

The
**PHYSICAL
ASPECT** 990,
of the
LIVING CELL

Edited by
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MIKLÓS KELLERMAYER
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Akadémiai Kiadó, Budapest

THE PHYSICAL ASPECT
OF THE LIVING CELL

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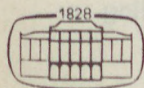
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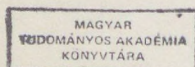
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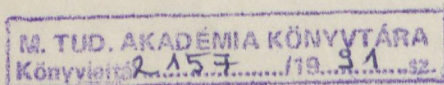


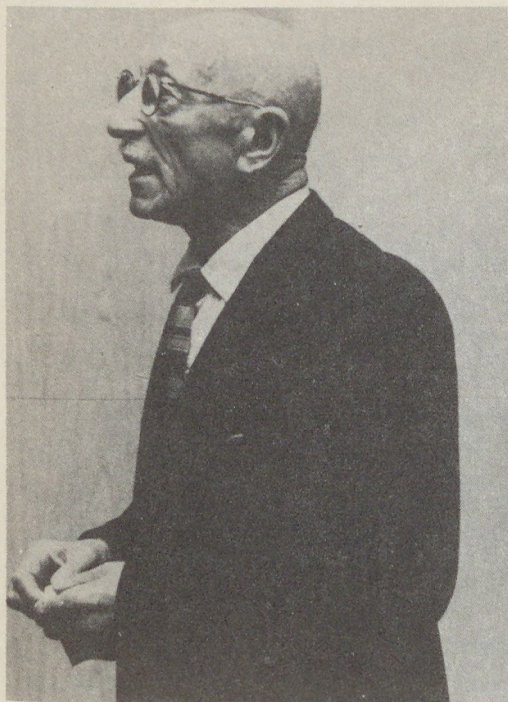
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"... Although the human being is able to perceive but small sections of his surroundings with the aid of his senses, his intellect immensely broadens out his capacity to get acquainted with his world..."

In: "Biology without Mysticism. A Biophysicist's Reflections"
by E. Ernst

Akadémiai Kiadó
Budapest, 1976

PREFACE

This book was inspired by a Symposium devoted to the memory of the late Eugene (Jenő) Ernst with a title of "The Physical Aspect of the Living Cell". The Symposium was organized as a satellite to the 2nd European Cell Biology Congress (Budapest) in Pécs (Hungary) on July 3-5, 1986.

The participants of the Symposium were either those who were acquainted with Eugene (Jenő) Ernst's pioneering work or those who, although they had not been acquainted much with Ernst's work, their recent findings were consistent with Ernst's concepts concerning the complexity of the protein-water-inorganic element interactions in the living cell.

A major concept embraced by the participants was that the mechanism(s) of life involves the exact three dimensional organization at the submolecular, the molecular, and the supramolecular levels. Life can be understood only by knowing simultaneously the relationships between those three levels of organization. We should be ever mindful as we intervene of the pitfalls that our respective technologies may impose on this delicate three dimensional organization.

The contributors were asked to discuss freely their views and opinions. In the course of the Symposium, a general theme seemed to emerge: That the search for truth is well served by open and frank discussions, regardless of each participant's viewpoint. This theme was recognized frequently at the Symposium and is evident in the manuscripts contained in this book. To paraphrase the theologian Paul Tillich, every

attempt was made to see that "the passion for truth was not silenced by answers which have the weight of undisputed authority". As a result, the views expressed in this book do not form an integrated overview of a single concept about the living cell, but rather different concepts which, for the most part, differ with conventional views of the cell.

Pécs, 1989

J. Tigyi

M. Kellermayer

C.F. Hazlewood

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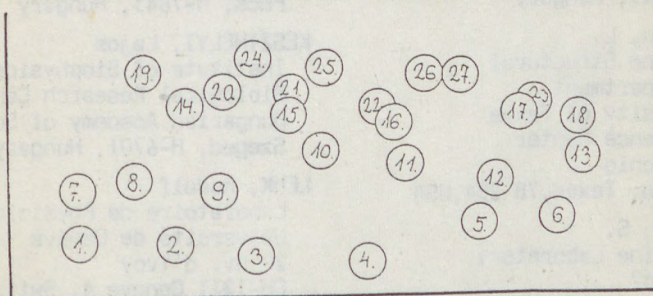
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OPENING - WELCOMING ADDRESSES

ALBERT SZENT-GYÖRGYI'S ADDRESS

Dr. Eugene Ernst was a very dear friend of mine and I am unable to accept in my mind really his death. His opinions and feelings were always so very vivid that it is difficult to believe that they will ever end. We often differed in our opinions which made the bond between us still stronger, the essential point of our opinions being always that science is a search for truth, and that truth is the only thing that matters. I was fortunate enough that I was brought in near contact with him repeatedly and even had an opportunity to contribute to his safety during the disastrous Nazi period of Hungary under German occupation. He was a wonderful friend with a very deep understanding for everyone's opinion, though his own beliefs were very often at variance with those of the majority. If science is a search of truth, then he was a real apostle of scientific knowledge.

BÉLA FLERKÓ'S ADDRESS

On behalf of the regional Center of the Hungarian Academy of Sciences I am pleased to welcome the participants of the Eugene Ernst Memorial Symposium.

In the words of W.L. Palmer "Institutions are the shadows of men - men who come and go, cast their shadows, and disappear into oblivion of the past". The shadow of Eugene Ernst is the University Medical School of Pécs in the first decade following the second World War. After having returned to Pécs from the labour service in the days of the liberation of the town by the Soviet troops, he started to reorganize the Medical Faculty of the University with enlightened brain and with political power in his hands. He initiated the recruitment of brilliant young scientists to vacant chairs and developed an excellent Medical Faculty in a few years. I have to add that in the most difficult years of our post-war history.

Eugene Ernst was Marxist and as a biologist he stood on the basis of Darwinism but, at the same time, he realized the truth of D. Morris' words: "Using a harsh evolutionary argument, it might be suggested that if two human groups clash and one exterminates the other, the winner is biologically more successful than the other. But if we view the species as a whole this argument no longer applies. It is a small view. The bigger view is that if we had contrived to live competitively but peacefully alongside one another, the species as a whole would be that much successful." He followed this line and firmly guided and protected the Faculty during the difficult years of the early fifties.

As a scientist, he agreed with Jacques Monod: "In science, self-satisfaction is death" and with Victor K. McElheny "Unquietness, anxiety, dissatisfaction, and torment, those are what nourish science. Without

fundamental anxiety, there is no fundamental research." He made great discoveries and developed excellent hypotheses having always in mind that a hypothesis can be thought of as a draft law, and the everyday scientific process consists essentially of examining the logical consequences of hypotheses to see if what follows from them or what they predict does in fact correspond to real life. In this process disproof is a logically stronger process than corroboration, which always falls short of completeness, so that no general hypothesis, however solidly established it may seem to be, is ever proved beyond the possibility of further critical examination or questioning. What unites us all in science is the characteristic that we are all in our several ways seekers for truth. In the words of Karl Popper: "It is not the possession of knowledge that makes the man of science but his persistent and relentlessly critical quest for truth". This made Eugene Ernst a great scientist.

SZILÁRD DONHOFFER'S ADDRESS

Professor Tigyi asked me to give you a glimpse at the characteristic features, the totality of which made up the unique human being called Jenő Ernst: an impossible job. My only conceivable qualification which could have motivated Tigyi's request is that I met Jenő 60 years ago and that our friendship covered more than 50 years lasting until his death.

Ernst had a very complex personality which, perhaps, could be portrayed by a great novelist, although the result would certainly bear only a very faint resemblance of Jenő's true self.

Volunteering in World War I, being decorated with the highest award, and ultimately spending 5 years as POW in Russian camps, he returned converted to communistic ideals and remained a staunch Hungarian patriot until the end.

With his war record and scientific achievements, he could have obtained easily a tenured appointment, had he changed over from the Jewish religion to any other denomination. This he consistently declined to do, despite completely rejecting all kinds of religion.

Jenő usually put up a gruff manner and, when displeased, burst out in intimidating language. This quite frequent event disturbed nobody acquainted with him more than quite fleetingly. Despite the often gruff manner, Ernst could be gentle and compassionate, and he helped many people both financially and, more important, in other ways. I myself certainly would not have obtained the tenured chair without his countering powerful influences opposing it.

Regarding theoretical problems, Ernst held rather dogmatic, radical leftist views; in practice, he was an understanding humanist camouflaged by uncompromising gruff language.

Ernst never accepted office at the University; he was, nevertheless, during the decade after the War, behind the scenes, the almost dictatorial leader. He used his, at that time immense, influence to build a faculty which, in those years, was generally acknowledged to be the best in this country. He accomplished this in a period in which, as in the pre-war decade, other criteria than scientific performance often prevailed.

Jenő being a formidable, intimidating, provocatively aggressive debater himself, enjoyed a fierce debate - no holds barred - immensely and few could stand up to him. I miss these debates very much indeed.

Despite his, in many ways ascetic, lifestyle Jenő enjoyed occasionally good food, a good drink and congenial company - he never suffered fools gladly. His strong physique permitted him to keep up cycling and shooting into his seventies, and he continued swimming and tending his orchard even during his last summer and autumn. So fate was ultimately kind to him, retaining his mental and physical capacities up to the very end, he was spared from the disablements of senescence and chronic invalidity.

If Jenő could read these lines he would shout at me: "How could a man whom I thought to be rather intelligent for 60 years write such sentimental nonsense" - while, in himself, he would probably not be utterly displeased, and consider that some important aspects have been missed or understated - and he would be right.

CARLTON F. HAZLEWOOD'S ADDRESS

I would like to just present some memories that I have of Professor Ernst. My interactions with him started in March of 1976, which was my first trip to Hungary. It became a common occurrence for me to meet with him for two to three hours in the morning. (At all times there was a bottle of plum palinka: Ernst's Palinka. It was from his own plums, which apparently he fermented himself and then carried to the national distillery, very officially.) After the morning meeting he would assign me to someone to take me to lunch or to do something until about two in the afternoon, when we would again have intensive discussions about ions and water. As he would say "those of us who are preoccupied with the boundedness of water and ions must be about our business". Then he would take me on tours in his laboratory to meet the younger faculty and I would be permitted to meet with them.

I would like to discuss one small matter which relates to his publication: "Facts and Texts". It took me a long time to understand what he meant by Texts - it meant words. He didn't pay much attention to or care much for words. He wanted facts. I remember one time he reprimanded me for having referred to Ernst's theory. He said Ernst has no theory. He only has facts. That's all he would publish, the facts, and would make very minimal interpretations of these facts. He would nevertheless, spend lengthy periods of time in discussion of the interpretation of these facts.

During my many visits to Pécs, I had some wonderful times. I remember on one occasion, when Zoltán Hummel was assigned to me. We needed to make some calculations about something to do with potassium. Zoltán was assigned as my body-guard and to take me to his room and to begin to have discussions and to make necessary calculations. But it was beautiful day

and I said "why should we stay inside, why don't we go to the Mecsek mountain and make the calculations there"? We went. But, of course I wanted to go jogging. Zoltán set me off for an excursion on a simple three kilometer track in the woods. After some two hours of jogging I became convinced that I was lost, since I could not read any of the signs, I decided to run to the top of the mountain because, once I got to the top, I knew then I could find a road down to the city. But in the meantime Zoltán had run off trying to find me. Finally, we found one another. We now had to face Professor Ernst. We were very late and when we arrived at the Biophysics Institute Professor Ernst was getting ready to close the laboratory. He wanted to know why we were late, and I tried to tell him, that I had become lost in the woods while jogging in such a way that Zoltán would not be in trouble - also, without getting myself in too much trouble. I remember he got very upset, and, as Professor Donhoffer has said, his temper could flare very rapidly and very intensely. And, for such a small man, he it's amazing how big seemed when he was on his toes, yelling at you. He began to scold Zoltán that he should not have let me go by myself. But I said how can I run in any other way in the woods? The anger subsided as quickly as it began - he said "it is over, you are bad boys". I remember him very fondly and I miss him. He could give wise council and his dedication to the search for truth was inspirational. Thank you.

INTRODUCTORY SPEECH

WHAT WAS NEW IN ERNST'S THOUGHTS ?

J. Tigyi

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I am really very honoured having the opportunity to give a survey about thoughts and ideas of Jenő Ernst because he was my scientific master and later paternal good friend. I spent with him in the laboratory and in the scientific life 36 years between 1945-1981.

I was very fortunate to enjoy this scientific symbiosis, a lot of difficult discussions and debates, his brilliant humor, his wise philosophy, his very strictly disciplined ascetic life-style and his sincere cordial attitude towards his coworkers.

Jenő Ernst was a unique intellectual giant of this century and his influence on the world biophysics and on the whole Hungarian biology was very significant.

Biographic data: Jenő Ernst was born in 1895 in the town of Baja, in a small agricultural community situated at the border of the great Hungarian plain, on the bank of the river Danube. He finished his secondary school education in the famous Roman catholic ecclesiastic gymnasium in 1913, in spite of the fact that he was Jewish. He was admitted to the Medical School of the University of Budapest. In 1915, as second year medical student volunteered to the army and was transported in a few months to the Hungarian-Russian front of the Ist World War. At the end of the year 1915 he was fallen into captivity and spent 5 years in Siberia as Russian war prisoner. Returning to Hungary, in 1920, he continued his university studies and graduated as MD in 1924.

After his graduation he joined to the Medical School of the Pécs University where he worked 54 years - except an involuntary interruption of three years during the second World War - until his death (1981) in Pécs.

The main events of his scientific carrier are as follows: (Tigyi, 160) 1928 privat docent, 1935 extraordinary professor, 1945 head of the department, 1947 full professor, 1946 member of the Hungarian Academy of Sciences, 1959 head of the Biological Section of the Academy, Kossuth Prize 1948 and 1956, Golden Medal of the Academy 1971. He got numerous orders and awards but he refused also a large number of medals and orders. He was exceptionally modest and he was living during his whole life in his laboratory like a monk devoting his whole life to the science. He was never married, his coworkers were considered by himself as his family. He was working day by day from 4.30 am to 10 pm, about 16 hours in the laboratory with an incredible rigorous discipline and fanaticism. His intellectual authority among Hungarian scientists was supremely outstanding.

During his scientific activity of 57 years he published 160 original papers, 3 monographs and 4 text books. (See references)

Without mentioning the very interesting details of his struggling life I should like to give you a picture about the main ideas of Jenő Ernst analysing his original papers. As Fig. 1. shows, his publications can be classified in 3 large and 3 small groups.

FIELD	Nr. OF PAPERS
1. MUSCLE	61
2. POTASSIUM	31
3. WATER	26
4. THERMOOSMOSIS + THERMODIFFUSION	10
5. RADIOACTIVITY	8
6. BIOL. SEMICONDUCTORS	9
7. MISCELLANEOUS	14
TOTAL	159

Fig 1. The classification of Ernst's publication

The main fields of his interest were: the muscle biophysics, problems of the potassium and the water. In reality, his main interest was the problem of the muscle contraction but during this study he met the problem of the K and water as very decisive factors of the muscle contraction, therefore

he focused his interest to that area so his K and water research was also oriented to the muscle problem. Situation is similar also with the 3 smaller fields of his research activity.

I. New concept on the muscle research

He started the muscle research in 1924 two years after Nobel award of D. Meyerhof and A.V. Hill were awarded by Nobel prize about the lactic acid theory. After studying the publications of Meyerhof the young Ernst met the famous Prof. Mansfeld and explained him that "no one word is correct in the lactic acid theory". That time it was absolutely a heretical idea but fortunately Prof. Mansfeld accepted Ernst's objections, and offered him an unpaid post in his laboratory giving him opportunity to verify his ideas.

The idea of Ernst was the following: the time relations of the muscle contraction and lactic acid formation are different. The lactic acid appears only in the late period of the tetanus therefore it cannot be a cause of the contraction. In order to solve the real problem one should study the quick phenomena of the muscle contraction which are mainly physical, not chemical phenomena. In this way Ernst come to the volume measurement during the muscle contraction.

He elaborated a completely new method as shown in Fig. 2 and Fig. 3

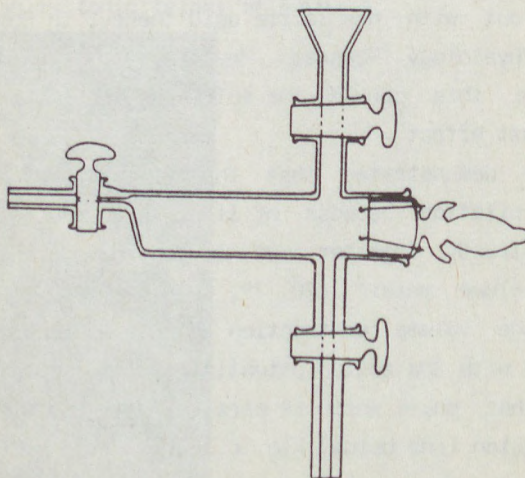


Fig 2. The glass volume-meter of Ernst

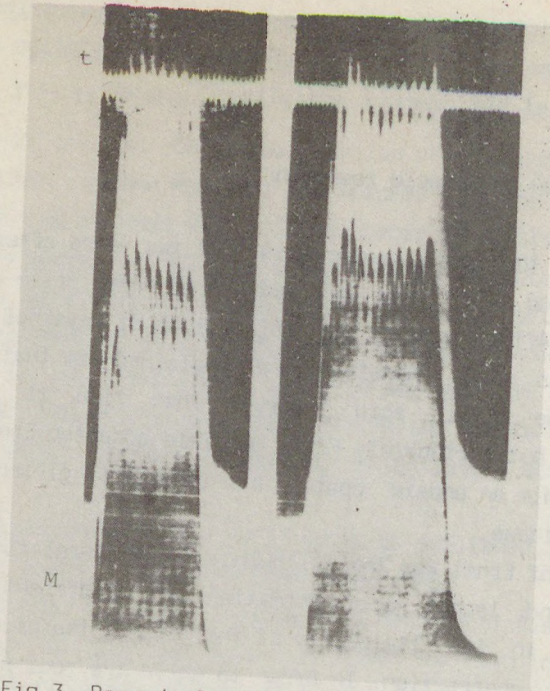


Fig 3. Record of volume changes of muscle.

which gave him a possibility for the exact study of muscle volume. Between 1925-1940 he published 7 papers (1, 4, 5, 23, 38, 45, 50) using and constantly improving this method which resulted that D. Meyerhof was totally knocked-out with his lactic acid theory. In Rome in 1933 at the International Physiology Congress Meyerhof himself accepted Ernst's objections. After this period the volume contraction was quoted in the textbooks as "Ernst effect".

In 1934 he demonstrated that the volume decrease seems to be a phenomena of excitation because of its time course, after 1948 when we were working already together and I had developed the so called 'piezoelectric volume meter' (70, 79, 80) (Fig. 4), we were able to discover that the volume contraction has two phases: 1. initial phase which is parallel with the action potential, latency relaxation etc. (Fig. 5) and 2. another phase which is parallel with the time course and the extent of the tension (see below) Fig. 6.

In the studies about volume changes, Ernst's interest was converted to the ionic changes during contraction. In his classical work in 1929 he was the first who verified - the K-Na exchange during the muscular

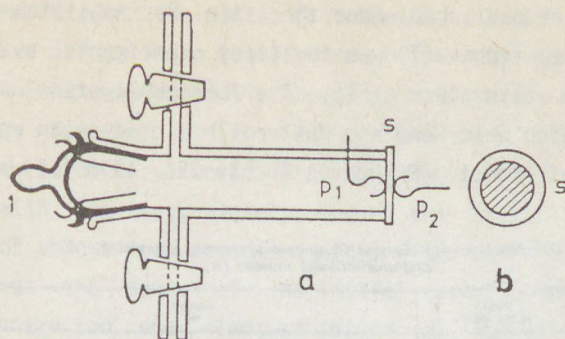


Fig 4. a./ Volume meter with piezoquartz transducer
b./ The quartz disc with gold foil

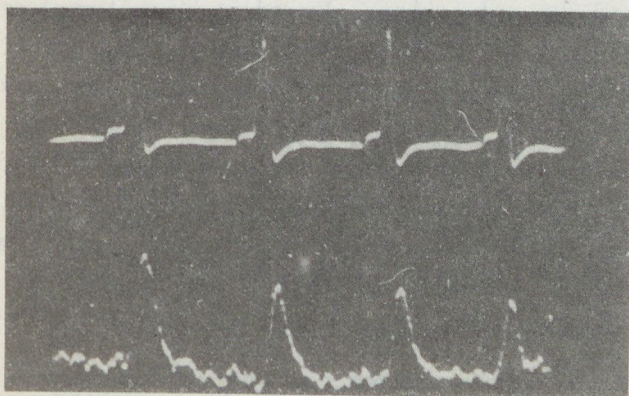


Fig 5. Synchronous record of action potential (above) and volume decrease (below) in an isotonic tetani of 50 Hz.

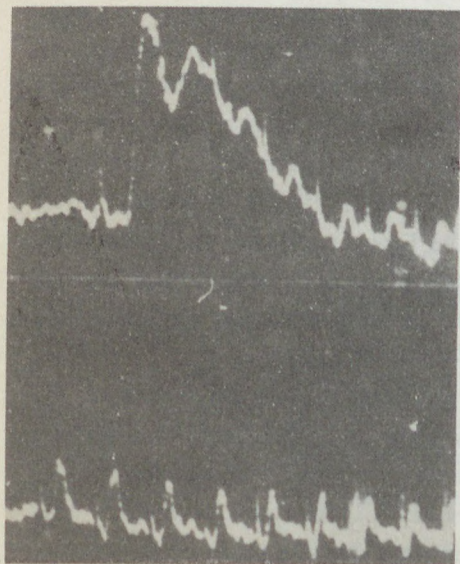


Fig 6. Record of volume curve in tetanus.
Above: isometric, below:
isotonic contraction.

activity. The paper published under the title: Permeabilitat und Tatigkeit in the Pflüger's Archiv (7) was the first experimental evidence of Na-K exchange in the literature (Fig. 7). This publication caused a famous priority discussion with Mond and Netter. This discussion ended with the total victory of Ernst (Pflüger's Archiv 226. 1930 (13) and 230. 1932) (19).

*Differences in K-Na and Cl content of directly stimulated (d)
and unstimulated muscles (u)*

No.	Weight of the muscle		Content mg					
	g	diff.	K	diff.	Na	diff.	Cl	diff.
1	d	1.98	1.88	— 2.30	3.70	1.66	3.56	
	u	1.86	4.18		2.04		1.70	1.86
2	d	1.36	0.64	— 2.38	2.20	0.65	2.95	
	u	1.20	3.02		1.55		1.41	1.54
3	d	1.20	0.69	— 1.74	2.95	1.00	2.91	
	u	1.11	2.43		1.95		1.97	0.94
4	d	1.32	2.10	— 1.10	2.55	0.80	1.80	
	u	1.51	3.20		1.75		1.31	0.49
5	d	1.52	2.35	— 1.08	2.73	0.78	2.18	
	u	1.78	3.43		1.95		2.03	0.15
6	d	1.66	3.05	— 0.94	2.50	0.30	2.10	
	u	1.90	3.99		2.20		2.29	— 0.19
7	d	1.40	1.13	— 1.79	3.08	1.13	3.45	
	u	1.35	2.92		1.95		2.10	1.35
Total		— 0.05		— 11.33		+ 6.32		+ 6.14

Fig 7. Results of perfusion experiments from (7)

The phenomenon of Na-K exchange served later as the basis of all kinds of excitatory events and many details of such events are still related to the phenomenon of Na-K exchange. So we might state that the concept of the Na-K exchange has been survived for more than 50 years. It is very interesting to notice that at the beginning R. Höber and other outstanding German scientists have cited Ernst regularly but later when W.O. Fenn took over from Höber's monographs and cited only Höber instead of Ernst, the American literature quoted the original paper of Ernst very seldom.

During the second World War Ernst lost his unpaid status at the University of Pécs because of his Jewish origin, but Szent-Györgyi was generous inviting him and so he was active as scientific consultant in muscle research in Szent-Györgyi's Biochemistry Laboratory in Szeged. This period when Szent-Györgyi changed his field from the field of biological

oxidations and started the muscle biochemistry studies. The experiences and advices of Ernst were significant in the development of the Szeged muscle school. Ernst was fortunate enough to survive the very cruel period of the Hitler fascism and in 1944 he was able to return to Pécs and continued his laboratory work. From September 1945 I was lucky to collaborate with him very closely. During that period we reinvestigated many aspects of volume measurements improving the methodology. The opinion of Ernst about the cause of the initial quick volume changes was the reversible ionization and electrostriction of the water because of the hydratation of new formed ions. The second slower phase of the volume changes was supposed to be originated by the conformation changes of the muscle proteins. This hypothesis was based mainly on three independent studies: 1. with polarizing microscopic methods we were able to demonstrate the increase of birefringence caused by mechanical stretch (89) 2. in magnetic volume-meter (Fig. 8) we were able to demonstrate in muscle and in myosin threads the volume decrease due to mechanical tension and 3. microcalorimeter studies in which we measured the parallelism between mechanical tension and heat production (63, 89, 103). In this way the polymer crystallization of muscle protein caused by external or internal mechanical tension has been verified. Between 1948 and

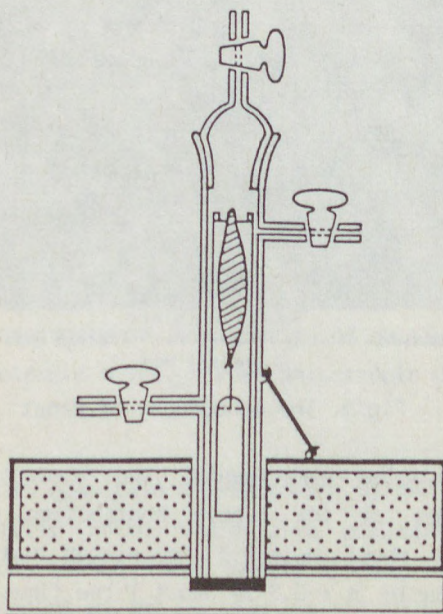


Fig 8. The magnetic volume-meter

1954 he published 8 papers about this field. According to my opinion this hypothesis remained a very considerable idea of muscle research till now.

There is no time to enumerate all the new ideas and suggestions of Ernst in the muscle research but his monograph which was published first in German: "Die Muskeltätigkeit" in 1958 (89), then later in English in 1963 (103) "Biophysics of the Striated Muscle" became the bible and thesaurus of muscle biophysicists for many decades (Fig.9).

However, I should like to mention two additional results from his ideas: 1. He initiated the work of Garamvölgyi to try the electrical stimulation of the single fibril of the honey bee thorax muscle. Garamvölgyi succeeded to get contraction in these single fibrils, giving an unsolved enigma of the membrane theory even until today, because in these experiments there was a complete absence of the membrane of the fibre (94, 103).

BIOPHYSICS OF THE STRIATED MUSCLE

By

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PROFESSOR OF BIOPHYSICS
PIAS UNIVERSITY

SECOND EDITION
WITH 100 FIGURES COMPRISING 311 ILLUSTRATIONS



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BUDAPEST 1963

Fig 9. The monography of Ernst

2. Ernst conducted experimental and theoretical studies about mechanical efficiency of the muscle contraction on contrary of the classical knowledge about $\eta = 0.3$ he stated that the real mechanical efficiency of the muscle $\eta = 0.9$ is about three times as large (60, 103). The basic reason of this difference is that the classical concept did not make difference between specific function and metabolic function of the muscle.

II. The Potassium

At the beginning of his muscle studies Ernst's attention was drawn to the very large molar concentration of K in muscle. In his paper in 1928 (7) he already emphasized the important role of that element. After elaborating a very fine analytic method he never ceased the work and theoretical studies about K. In his more than 30 papers about this problem he stated that the majority of K exists in bound, non-ionic state. According to his opinion this binding is not covalent but a labile adsorptive one. Many studies were conducted with many different methods which demonstrated the non uniform distribution of potassium in the muscle fibre and even in the A and I bands of the muscle fibre. Fig. 10 shows a picture made with K^{42} ELMI radioautography and Fig. 11 demonstrates the X-ray microanalyzer diagram in which identical volumes of A and I bands are compared with each other.



Fig 10. ELMI radioautographic localization of potassium in the muscle.

Above: isotropic discs, below: anisotropic discs

Ernst started to elaborate a methodology of the ultra-high frequency electrical conductivity which could give a possibility to explore the binding state of ions in the living muscle tissue. (31) These experiments proved also the concept of the bound potassium in muscle. Dr. Masszi his successor in that field will present more details about the problem.

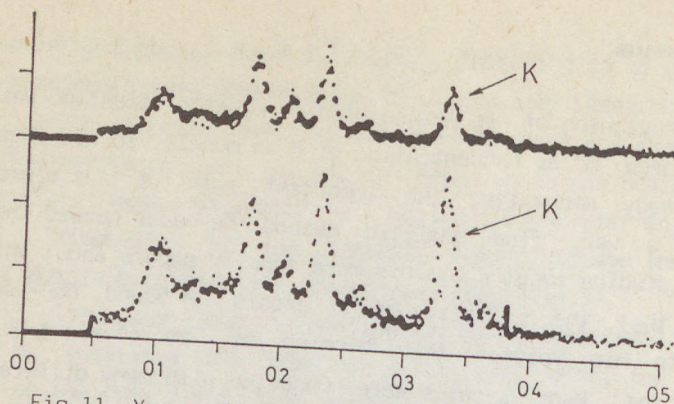


Fig 11. X-ray microanalyser record of potassium

The essence and basic consequences of these studies are the strong oppositions against classical membrane theory. In this case of bound potassium and non-uniform distribution it is difficult to imagine how to use correctly the Goldman equations (145).

Anyhow, the problem of the muscle contraction cannot be solved without exact knowledge about the largest inorganic component of the muscle (excluding water).

III. Water

The special role of water in biological systems was emphasized by Ernst very early when the official opinion was the validity of the classical concept of the osmosis in which all ions and water molecules are in a free diffusible state. Using the up to date terminology: all water in biological tissues is "bulk" water. In his publication in 1926 (2) he demonstrated that the mechanical tension influences very significantly the water content of the muscle. The title of the 8th paper was "Wasser-verschiebung als Grundlage der Zuckung" 1928 (8). He published very important papers about the water problem in 1938 in *Nature* (43) and in 1938 in *Kolloid Zeitschrift* (48), the title of the later one was: "Osmose und Quellung und ihre biologische Bedeutung".

After a break of 12 years he renewed the investigation of water problem. I was fortunate to collaborate with him together with Dr. Zahor-csek (64). We worked out 3 separate methods: 1. differential manometer (Fig. 12) and 2. microbalance vapour pressure equipment (Fig. 13). These

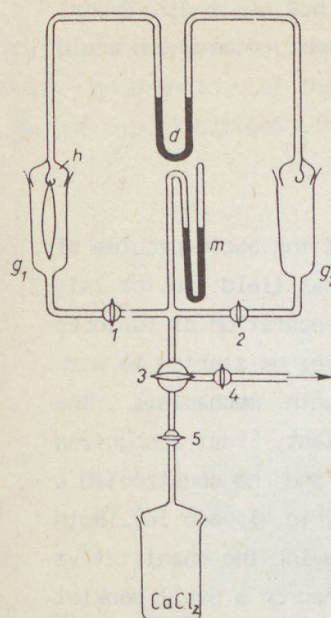


Fig 12. Differential manometer for measuring the vapour pressure of muscles during progressive drying

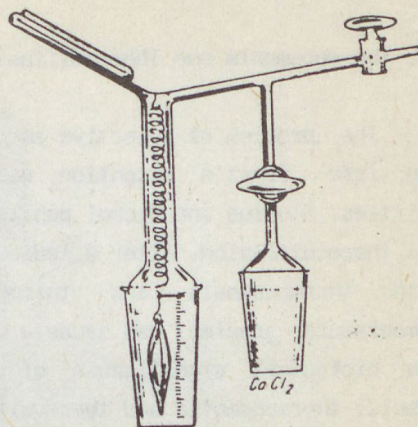


Fig 13. Microbalance for measuring the relation between water content and water vapour pressure

combined with a 3rd known method (the sulphuric acid-series method) gave us very convincing experimental data about the existence of bound water in muscle (Fig. 14). This paper which was published in 1950 became later one of the basic publication of the bound water problem. Dr. Pócsik is continuing the study of the water problem and achieved a lot of new experimental results. Ernst gave a summary of his thoughts in collaboration with C. Hazlewood in 1978 in the publication: "Inorganic

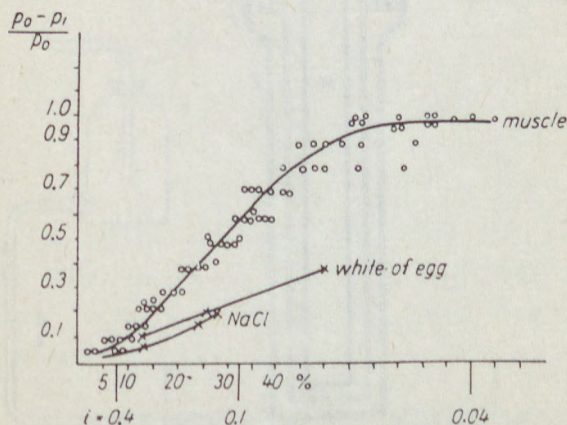


Fig 14. Relative vapour pressure lowering as a function of the water content of muscle

Perspectives in Biology and Medicine" (155, 156) but all at his papers about water problem are containing many very important ideas which could be useful for future investigations.

IV. Thermoosmosis and Thermodiffusions

The problem of selective accumulation is one of the basic problem of the life. Ernst's attention was also drawn by that field. In the late thirties, Clusius and Dickel published papers about separation of isotopes by thermodiffusion. After a tedious study of that area he started to work with thermoosmosis and thermodiffusion. In both mechanisms the temperature gradient can cause a concentration gradient. Ernst recognized the biological significance of those processes and he constructed a special thermoosmotic and thermodiffusion equipment (Fig. 15 and 16). Both apparatuses gave him ample opportunity for studying the quantitative aspects of the phenomena. The results were published in a small booklet Thermoosmosis and Thermodiffusion in 1941 (53). (Unfortunately it was not dispatched at all because of the Second World War).

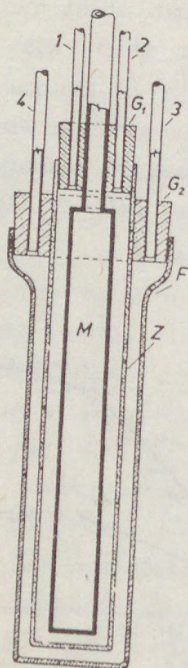


Fig 15. The thermoosmotic device of Ernst

As a continuation of this work, he developed a special thermodiffusion apparatus: the so called "capillary thermodiffusion" which is a very good analogy of the tubular system of the kidney (Fig. 17) (75, 95). He had 6 publications about this field.

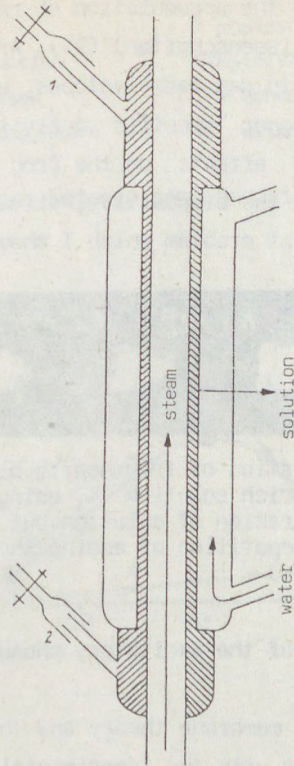


Fig 16. Thermodiffusion-device for separation in liquid

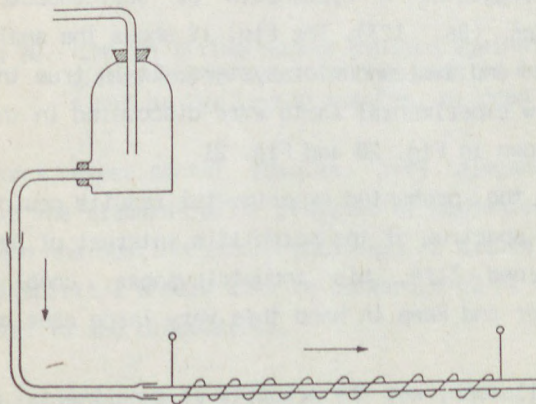


Fig 17. Capillary-thermodiffusion

V. Biological Role of Radioactivity

The old Zwardemaker problem about the biological role of natural isotope of K the K^{40} excited very much his inquisitiveness. The first paper in which he studied the accumulation of radioactive K in plants was published in 1934 (Naturwissenschaften) (24). After the second World War, having the opportunity to get radioisotopes, we were able to study this problem with K^{42} of higher specific activity. Fig. 18 shows very interesting radiopositive effects on the frog heart activity (90). This and other papers about the biopositive, stimulating effect of ionizing radiation led us to the last problem which I should like to demonstrate.

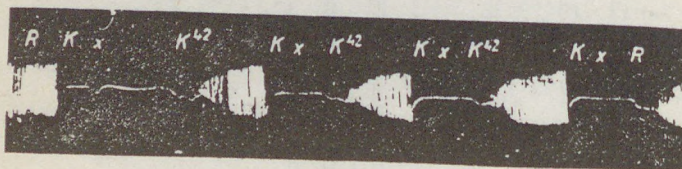


Fig 18. Reactivation of frog hearts blocked with K-rich solution (K) using the same concentration of solution but with K^{42} (K^{42}) repetition of adding the previous fluid (x)

VI. Semiconductor concept of the excitatory phenomena

The criticism of the membrane theory and the radiosensitivity of the excitable tissues together with the experimental data from the laboratory about the similarity of electrical conductivity and other electronic parameters Ernst suggested a hypothesis of semiconductor aspects of excitatory processes (86, 123). The Fig. 19 shows the analogies between biological systems and semiconductor systems. It is true that using that hypothesis many new experimental facts were discovered in the laboratory. Two examples are shown in Fig. 20 and Fig. 21.

I hope that the presented experimental results could convince you about the very wide spectrum of the scientific interest of Jenő Ernst. His rigorously disciplined life, his industriousness, combinative talent enabled him to cover and keep in hand this very large area of biology and biophysics.

His basic philosophy was the priority of experimental facts against speculations but he had the capability to create new theoretical concepts

Semiconductor:

Rectification of pn-junctions
Starting voltage of diodes
Photoelectric responses
Frequency modulation
Sensitivity to contamination
The conductivity increases
with increasing temperature

Living system:

Rectification of the axon membrane
Stimulus threshold
Photodynamic effects
 $f_{\text{postsyn}} \neq f_{\text{presynaptic}}$
Sensitivity to trace elements
The conductivity increases
with increasing temperature

Fig 19. Analogies between semiconductors and nerves

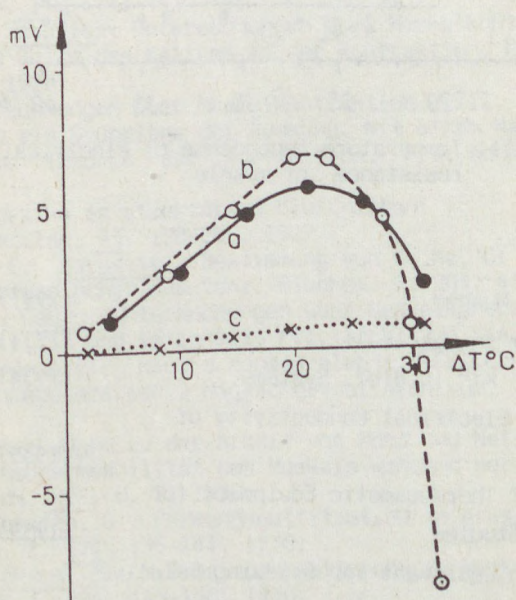


Fig 20. TEMF in living muscle plotted against temperature difference between the two ends of muscle
a and b: two living muscles, c: dead muscle

based on exact experimental results. Very elegant examples of that capability is the elaboration of problems of mechanical efficiency of the muscle work and the semiconductor hypothesis of excitations.

In this spirit I should like to summarize 12 of his most significant scientific results and discoveries.

1. Volume Contraction (Ernst Effect) (1925)
2. Na-K Exchange during Excitation (1929)

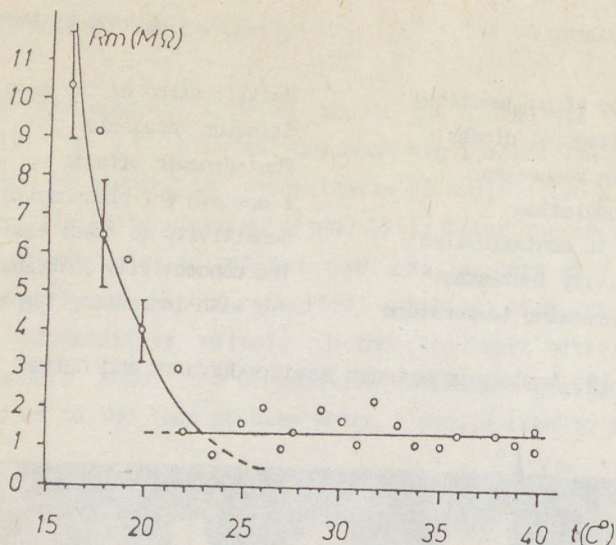


Fig 21. Temperature dependence of electrical resistance of muscle

3. Bound Water in Muscle (1931)
4. Bound Potassium in Muscle (1934)
5. Accumulation of K^{40} in Biol. Systems (1934)
6. High-Frequency Electrical Conductivity of Muscle (1935)
7. Construction of Thermoosmotic Equipment for Biological Studies (1939)
8. Thermodiffusion Equipment for Studying Relative Accumulation in Liquid Systems (1940)
9. Differential Micro-manometer (1949)
10. Capillary Thermodiffusion (1954)
11. Semiconductor Concept of Excitation (1955)
12. Ionizing Radiation Like Stimulus (1959)

I think no additional comment is necessary to be convinced about his admirable scientific achievements in biophysics which assures him an immortal position among the intellectual giants of the biology of the XXth century.

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STRUCTURE AND DYNAMIC ARCHITECTURE OF THE LIVING CELL

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THE ORGANIZED GROUND SUBSTANCE OF THE LIVING CELL

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Read by James S. Clegg

I wish to convey first of all Keith Porter's regrets that he couldn't be here. And that's truly a loss, because his image of the cell is an extraordinary one which, we should recognize, in controversial and by no means unvirally adopted by the cell biology community. However, he has mustered a large body of evidence in support of that particular image. Keith sent to me a letter and about eight slides in which he wished to convey to the group here his ideas and observations. So I am going to try to put myself in Keith Porter's skin and convey as much as possible of that. I must say that the slides that you will see here and my wonder don't really present the artistic or esthetic side of Porter's work, as well as it could be done by him. Well, let us take a look at the slides. The first one is a low-power high voltage image taken with the million volt electron microscope at Boulder, University of Colorado, which I think was the first one in the United States. We are looking through the whole cell here: these are very thin NRK cells spread out in tissue culture. The next slide will show a small region of the periphery of one of these cells. The micrograph shows the periphery of one of these cells with a rich supply of stress fibers in the cytoplasm. These are rich in actin as you doubtless know. Note that we are seeing a lot more organization than the disorganized representation that so many held to be the case for so long in cells; namely, a kind of disorganized aqueous phase in which there are all kinds of molecular running around. Slide number 3 is a higher power field which you can identify in figure number 2. It's a small region in which the individual filaments of the stress fibers can be seen, as well as vesicles of the endoplasmic reticulum. Pervading all the rest of the space and even in the region of the stress fibers, is this tremendous display of structures which Porter calls the microtrabecular lattice, or

MTL, the individual trabeculae of which are readily seen here. And I think it's fair to sum up Porter's general view of the microtrabecular lattice as encompassing, connecting virtually everything in the cytoplasm. His description is very important to us because, if correct, it says the cell is virtually organized down to the level of individual macromolecules. The volume in between all of this structure is, therefore, exceedingly dilute with respect to macromolecules. That doesn't mean that the water is the same as the kind one would find in a glass by no means. We are looking progressively at smaller and smaller parts of the cell as we go along. On slide 3., the two vesicles are derived from the endoplasmic reticulum, and adjacent to these are few microtubules. The vast majority of what you see here is of course the microtrabecular lattice. Some cell biologists believe the MTL is an artifact of the preparative method, in which "soluble" proteins are deposited on conventional cytoskeletal elements: intermediate filaments, microtubules, microfilaments. The issue is whether or not this MTL is an artifact of the preparation. Now I don't know whether we can resolve that or not, to everyone's satisfaction at least. I would like to suggest that a key element of this whole argument is the extent to which soluble proteins exist in cells. That's a fundamental issue. If most proteins are not diffusing in three dimensions, freely, if they are not soluble in cells, then the criticism applied to Porter's preparation doesn't hold. If there are large quantities of proteins freely diffusing in three dimensions, then it is a legitimate criticism.

The two vesicles you see are derived from the ER and besides these you can observe a few microtubules, stress fibers, and a number of polysomes suspended in the lattice. He is talking about ribosomes with presumably messengers attached that were, when "still alive", presumably making proteins. So even the protein synthesising apparatus, according to Porter, along with everything seen here, with the possible exception of mitochondria, are tied up in this network. The next slide is from Porter's Scientific American paper. We are still looking at NRK cells, and because of greater contrast, this slide provide a specially good image of the MTL, and its cross connections. The dense body is a lysosome, and there are pieces of the endoplasmic reticulum, several microtubules arranged in parallel, and some clathrin-coated pits. The next slide is just a magnified part of the previous slide. And it is interesting, that one does not resolve fine structure within these trabeculae. In other words, one does not see any hint of individual units, or any sort of semirepetitious

pattern. Porter's general view of things is expressed with some artistic licence, in the model that is shown on the next slide, which probably some of you have seen. I don't think actually that this slide does justice to this MTL. The previous slide gives you the impression of a lot more structure, a lot more crowding, smaller spaces or volumes in between all this structure than does the model. A support that the major purpose of this slide is to emphasise the vast interconnections of this MTL. It is almost as if you pull on one part of the cell then you can communicate that to everything else through this highly branched network. A most interesting possibility!

The last slide summarizes his current position on the MTL, along with some other evidence. Thus the cytomatrix is highly structured and appears as a three dimensional network of slender strands called microtrabeculae. In its structural features it resembles a gel.

We might recognize of course that many people over the years have referred to cells in this kind of context. The early cytologists certainly were predisposed to take that position. They commonly viewed cells that way. Put with the advent of cell fractionation we began to emphasize the randomness of the cytosol, for instance, which exists in cells.

- Available evidence supports the contention that the lattice is not an artifact of preparation technique.

I think Porter presented a convincing case for that in a paper written with John Woloszewick, who carried out much of that work in the late seventies.

- The MTL is continuous with centrioles and satellites of microtubule organizing centers (MTOC's), and together with microtubules, microfilaments and intermediate filaments, it comprises a unit structure.

It seems to be the only structure in the cytoplasm available to control the nonrandom or organized distribution of organelles and filamentous components. Its form can be distorted reversibly by abnormal conditions of temperature, osmolarity, Ca^{2+} , Mg^{2+} and cytochalasin. It seems to possess a preferred form to which it returns after distortion.

Porter and his students and associates have done some interesting work on this notion of deforming cells and watching things go back to where they were. In pigment cells they have followed the motion of granules in living cells and interpreted them in terms of the image seen with high voltage electron microscope. It is a dynamic structure and

probably contributes to the saltatory motion of lysosomes and the directed motion of secretory granules, pigment granules, and chromosomes. I am sure, if Keith Porter were here, he would show you some much more beautiful pictures, and I think you would probably at least appreciate their beauty even if you don't accept the evidence as final end.

Once again I'm really sorry that Professor Porter couldn't be here! I'm sure he would have enjoyed this meeting.

Thank you very much!

Note from the editors:

Although, Professor Porter was unable to attend the meeting and also to submit a manuscript for this book, his findings through Professor Clegg's presentation contributed much to the meeting and to this book. For pictures and details the reader's attention is called to a few major publications of K. Porter.

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DISCUSSION

Mátrai: You told us that cytochalasin distorts this structure, and it may support the findings about the mechanical properties of the leukocyte which is a rather elastic body. But if you treat it with cytochalasin it becomes a lot softer.

Clegg: Detailed study, as far as I know, hasn't been done on this issue. Certainly, you expect that anything that disrupts the structure should alter the mechanical properties of the cytoplasm. That's reasonably prediction.

Kellermayer: First, I'd like to call attention to two major mistakes that were made by mankind: One is to divide the potassium content with the volume of water and say that we have 200 mM KCl solution in the cell. The other one was to create the word "cytosol". I would like to ask whether the cell releases a part of its microtrabecular lattice, when it starts to "agonize". I am asking about the energy dependence of this structure. That is, if before you make these pictures you injure the cell, and let it start to die, - because death is not absolutely sudden, there is an "agony" - would the cell release these microtrabeculae?

Clegg: I do not think that Porter has published on that matter, certainly an interesting and worthwhile project. Some observations on transformed cells suggest that they exhibit a much more disorganized pattern. But I don't believe he has published those in detail. What he has pointed out, and a number of earlier cytologists also observed, is that you don't see much Brownian motion in cells until they do die, or begin to do so. In other words if the cell does die or goes through, as you referred to it, an "agony", then Brownian motion becomes apparent, suggesting the MTL has lost its integrity.

Cameron: I am not overly impressed by the amount of surface area because you are looking through a thickness that seems to me to be a micron or so. If you disassemble this some time in the cycle, then wouldn't you have a lot more surface rather than less surface after you assemble something like that, from subunits.

Clegg: Yes, but all surfaces are not equivalent, the surface chemists tell us. For example, long flat surfaces have much different effects on their environments than small globular ones. For instance an inorganic ion doesn't really influence water over the same distance than that of, let's

say, mica sheets.

Secondly, you are apparently not disagreeing with the surface area measurements which Porter has carried out with some of his colleagues. They show that for a cell 16 μ m in diameter, taken as a sphere and omitting the nucleus, that the cytoplasm alone has somewhere between a 100,000-150,000 μ m². That is indeed a very large surface to volume ratio, and it is that number which looms large in importance, when evaluating effects on the properties of the adjacent aqueous phase.

Cameron: Could you tell me how many layers of water would have to be put on that surface in order to have all the water organized.

Clegg: Don't you read my papers Ivan? If you put a monolayer on, you only use about 3-4% of cytoplasmic water as I recall. This is just a calculation, a very simple one, but should be moderately accurate. We assume absolutely smooth surfaces no rugosity, which exists and minimizes the estimates. So, if you put ten layers on, we use up about a third and so on. That's an instructive calculation, I think, because it says the surfaces are influencing large amounts of water.

Cameron: All I would point out is that if you make this microtrabecular lattice out of subunits, and then you disassemble the subunits you have increased the surface area even more.

Clegg: Yes this is true but you may not have increased the effective surface in terms of perturbing the adjacent solvent. That's another matter! On this issue of disassembly and reassembly, I don't think there is evidence that the lattice is assembled at any given point. In fact Porter has found that if you put tissue culture cells at 4°C, this lattice falls apart. You don't see the highly detailed network. If you then put those cells back at 37°C within 30 minutes the whole structure is there again. This has great implications, I think, for things like the transmission of structural information when cells divide. The ability to assemble and disassemble this structure presumably resides in the cell as a whole.

Edelmann: I have a question concerning the problem of fact or artifact. Are there specific differences between the trabecular network seen in cells and in protein solutions.

Clegg: I can't pretend I have a tremendous grasp of the literature on this, but I do remember a paper by John Condeelis in the late seventies in which he looked at actin gels and observed a lot of structure remarkably

similar to the MTL. And that's only actin, there is nothing else there.

Edelmann: I only want to mention a paper of Kondo in 1981. He had studied protein solutions and showed that they form the same trabecular network as seen in the cell. So the question is, do we have some more information about the specific differences between the trabecular networks seen in the solution outside the cell and inside?

Clegg: That's really astonishing. Do you mean if you prepare a protein, like B-lactoglobulin, or something else, that it would form such a structure?

Edelmann: Yes!

Ling: I very much enjoy this conference, because you brought people from all over the world. And as Professor Tigyí has pointed out this morning we also bring people who have departed from us. And I want in this spirit to remind us, that the story you are telling, and the questions following, of course have a "deja vu" experience. That one time there were fibrillar theories of cytoplasm and it was defeated, in fact completely forgotten as a result of protein solution fixation, and demonstration of similar fibrillar and alveolar structures. And I think that does not in anyway distract from the importance of Porter's finding. But it is important to remember history to address ourselves to the question: is a dramatic demonstration enough to say here this is, here that is. Then, since it happens here and this environment differs from that therefore that environment doesn't exist.

That does not follow.

Clegg: Just a brief comment. It seems to me that reductionism was a very valuable and important approach in the recent history of cell biology. That allowed us to really attempt to see what these structures did, so I would not argue against it, I think it was a good idea. We'd still be in the dark ages if we didn't burst cells open and study their parts. However, the time has come may be to address some of the questions that probably can't be answered by destroying cells but rather only by studying them when they're alive and working, or as close to that as possible. Surely the early cell biologists were right on the mark if one believes this, which incidentally I do.

Ling: But not in detail, in other words, you may see a distorted version of something existing. That possibility should not be forgotten.

Clegg: Absolutely, Gilbert! The idea to be considered is that Porter's

image, even if one believes, does not give us a truly reliable picture. I don't think anybody would believe that. The HVEM images give us a static and dead look at what things might roughly be like in living cells. In other words, I believe Porter's view in by for the best approximation we have at present.

Keszhelyi: My question is very simple. You mentioned that at 4°C the structure is dissolved and at 37°C restored. If you repeat it, do you get back the same structure?

Clegg: You mean if you cycle?

Keszhelyi: Yes.

Clegg: I don't think he has done that.

Keszhelyi: Because that would show whether it has some meaning. At least it should connect the same type of organelles.

Clegg: Absolutely. That's right, and a really good study to do. There are a lot of experiments to be done on this. It's unfortunate that Porter came across this so late in his career. There are lots of things to be done just along these lines. I think a good source of evidence for Porter's MTL is that if it were an artifact of protein deposition due to the supposed presence of lots of soluble proteins in the cell then why doesn't appear in cells at 4°C?

CYTOSKELETAL PROTEINS; THEIR ARRANGEMENT IN CELLS AND THEIR USES IN THE DIFFERENTIAL DIAGNOSIS OF HUMAN TUMORS

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INTRODUCTION

There are two ways to define the cytoskeleton. One is a functional way i.e. to reserve the term for those components of the cell which are involved in the maintenance and change of cell shape, in migration, and in the internal organization and movement of organelles. The second is an empirical one to use it to refer to those components of the cell that are left behind after treatment of a cell with a buffer containing a detergent such as NP40 or Triton X-100 which extracts many of the "soluble" protein components of the cell (9,50,64). The cytoskeleton includes the three major filament systems with the cytoplasm i.e. microfilaments, microtubules and intermediate filaments (97). Here I want to concentrate on the anatomy of these three systems - rather than on their physiology - about which we still understand relatively little.

ARRANGEMENT OF DIFFERENT CYTOSKELETAL ELEMENTS IN CELLS IN TISSUE CULTURE

It is important to realize that the cytoskeleton should be viewed as a dynamic three dimensional organization in which the individual components are constantly being built up and broken down. Unfortunately the microscopical methods used to study the cytoskeleton usually yield only static snapshots from which the dynamic behaviour must be inferred. Moreover the impression obtained is sometimes critically dependent on the method selected to study the cytoskeleton. Thus methods which give an overview over the whole cell, or a large part of the cell, such as immunofluorescence microscopy (48), scanning electron microscopy, high voltage electron microscopy (105) or quick freeze deep etch microscopy (35,37) are for certain purposes much more informative than methods such

as transmission electron microscopy where only a small section of the cell is viewed at high magnification.

This is particularly true when considering the overall arrangements of microfilaments, microtubule and intermediate filaments within a cell. Biochemical and protein chemical methods complement and extend the microscopical approach. The purified proteins - indeed if monoclonal antibody technology is used the proteins do not even have to be pure - can be further used to raise antibodies, and if the antibodies are subsequently used in immunofluorescence microscopy (or to a lesser extent in electron microscopy) an unambiguous location of a particular protein to a given structure is often possible (48). Thus it is possible using a combined biochemical or protein chemical approach to build up a biochemical or protein chemical anatomy of a particular cytoskeletal component (98). Moreover the antibodies can then be used to compare and contrast the arrangement of particular cytoskeletal structures in different cells of the same type, in different cell types, and in cells in situ in normal tissues and in pathological specimens.

Microfilaments Proteins located to the microfilament system by such an approach include not only actin (48) (Fig. 1a), but also myosin (98), α -actinin (Fig. 1b), tropomyosin filamin and fimbrin (for review see 99). Some actin associated proteins locate to different actin containing cellular structures e.g. α -actinin and fimbrin are characteristically found in the ruffle; myosin is not (34,94,98). Likewise in the brush border of epithelial cells actin and fimbrin are found in the microvilli while myosin is concentrated in the terminal web (Fig. 2). Alternatively careful immunofluorescence and immunoelectron microscopy show that different actin associated proteins can be found in different regions of the same structures i.e. in the microfilament bundles ("stress fibres") characteristic of many cell types in culture and in particular of endothelial cells in situ. Stress fibers appear able to contract in vitro (e.g. 41) and can be disrupted by a variety of treatments (Fig. 3). The actin containing systems of the cell are involved in cell movement and their display usually changes rapidly with time as do the surface movements of whole cells. However in fragments of cytoplasm isolated by cytochalasin treatment (cf Fig. 3a), disruption with a water jet, trypsinization and replating, each fragment repeats a single characteristic

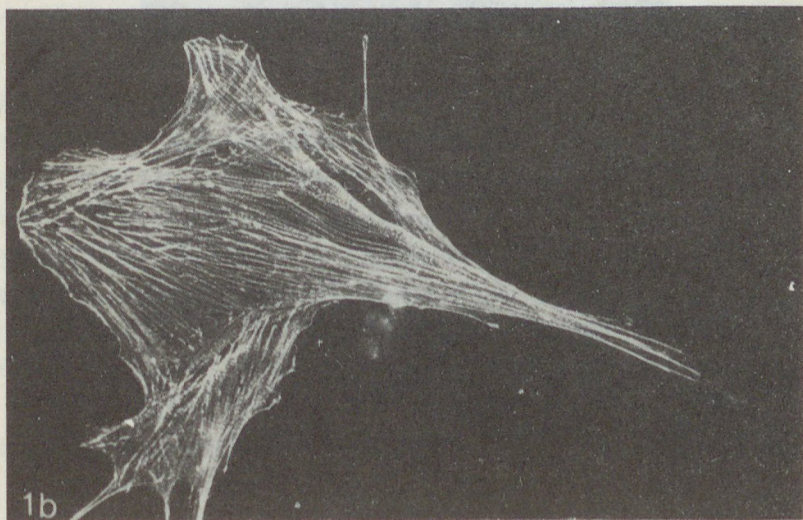
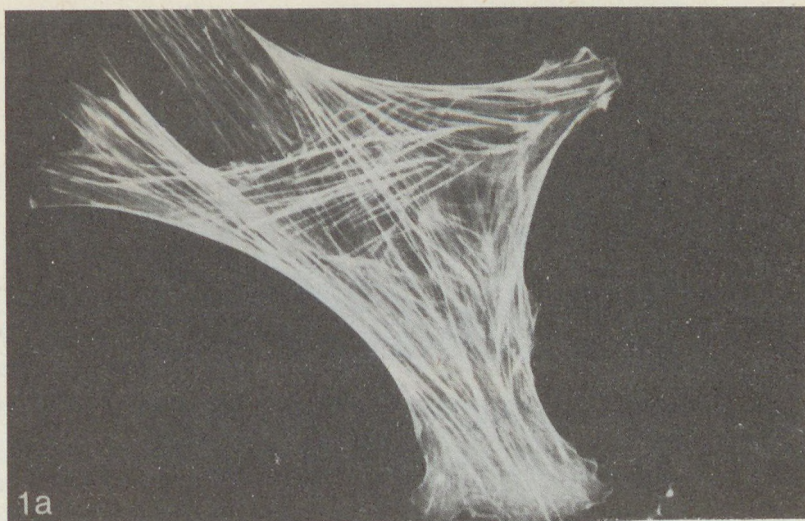


Fig 1. Rat RMCD cells labeled with an antibody to actin (a) and with an antibody to α actinin (b). Notice that both antibodies stain the stress fibers and that the actin antibody does so in a continuous manner, while the α -actinin staining is striated. (a) x600 (b) x380.



Fig 2. Localization of actin (a) and myosin (b) in mouse intestinal epithelial cells. Cells were lysed in phosphate buffered saline causing the microvilli to become more splayed out than in vivo, and were then treated directly for immunofluorescence microscopy. Note the strong staining of the microvilli by the monospecific actin antibody, and of the terminal web region by myosin antibody. (a) x600, (b) x750. Micrographs courtesy Dr. A. Bretscher (for further details see Bretscher, A., Weber, K. J. Cell Biol. 79, 839, 1978).

motion such as blebbing or ruffling time and time again for periods of at least 6 hours (2). Presumably the particular motion adopted by each fragment depends on the particular complement of proteins and/or other substances present in the fragment.

Microtubules The dominant proteins in microtubules are of course the α and β -tubulins with the subunit being an $\alpha\beta$ -heterodimer. In addition high molecular weight proteins - the so called MAPs (microtubule associated proteins) - and lower molecular weight Tau proteins may also be present. Whether different MAPs and Tau proteins define functionally different microtubules is still not clear. Microtubules are involved in intracellular transport (see below).

Intermediate Filaments Whereas F-actin filaments, and microtubules, are polar structures, intermediate filaments are not. Thus intermediate filaments are unlikely to be direction markers within the cell. Intermediate Filaments can be the most abundant (e.g. in nerves) or second most abundant protein (e.g. in many other cell types). In spite of this fact no unambiguous function for intermediate filaments in isolated cells other than a cytoskeletal one has so far emerged. This is particularly clearly emphasized by experiments in which antibodies specific for vimentin, or for an intermediate filament associated protein have been injected into cells, and where they appear not to inhibit easily measured cellular properties such as locomotion or cell division (23,44,51). Likewise at least three cell types have been described which lack all intermediate filament types (91). The arrangement of intermediate filaments in different cells in situ and the close connection of some IF types with membrane specialization (e.g. keratins with desmosomes) suggests that at least in these instances they probably subserve a cytoskeletal function. Intermediate filaments contain one or more than one type of IF protein (see below), and in addition several different intermediate filament associated proteins of differing molecular weights have been described (91).

Figures 4a-d show the distribution of stress fibers, microtubules and two intermediate filament types in an epithelial cell in culture as shown with antibodies to filamin, tubulin, keratin and vimentin, respectively. A variety of techniques - including stereo immunofluorescence microscopy (60) show that while the stress fibers underlie one or both membranes, the microtubules and the intermediate filaments fill the interior of the cell.

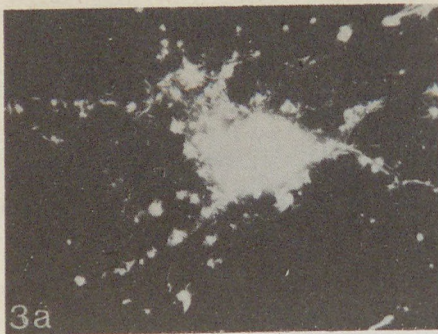


Fig. 3. Treatments affecting the actin arrangements in cultured cells with stress fibers. (a) Effect of cytochalasin B on 3T3 cells. 3T3 cells, treated with cytochalasin B ($2 \times 10^{-5} \text{M}$) for 5 minutes before being fixed and treated with antibody to actin. Stress fibers are no longer visible and instead the actin is present in arborized heaps. $\times 640$ (for further details see Weber, K., Rathke, P.C., Osborn, M., and Franke, W.W., Exp. Cell Res. 102, 817, 1976). (b) Effect of microinjection of the actin-specific drug phalloidin on 3T3 cells. The cells were microinjected with 0.2 mM phalloidin and after 3 hours fixed and stained with antibody to actin. Note the "islands" of aggregated actin, and also that the stress fibers are stabilized. $\times 670$. Compare with the effect of cytochalasin shown in (a). Micrograph courtesy Dr. J. Wehland. (For further details see Wehland, J. and Weber, K. Exp. Cell Res. 127, 397, 1980). (c) Induction of nuclear actin paracrystals by DMSO. PtK₂ cells were incubated with 15% DMSO for one hour at 37°C , then fixed and stained by antibody to actin in the nucleus. The arrowheads indicate the outer cell margin. $\times 900$. (For further details see Osborn, M. and Weber, K. Exp. Cell Res. 129, 103, 1980).

Keratin filaments often form thick intermediate filament cables or bundles with a characteristic arrangement in epithelial cells (Fig. 4c) (61).

Individual microtubules

run uninterrupted from the plasma membrane to the area around the nucleus where they often appear to connect to the centriolar region. Regrowth experiments in which the regrowth of microtubules was monitored after treatment with colcemid to depolymerize all existing microtubules showed a synchronized regrowth from the centriole with a rate of approximately 1-1.3 μ /min in mouse 3T3 cells (70,95). More recent experiments, in which microtubules synthesized in a particular time interval in the interphase cell are specifically labelled yield a value of 4 μ /min in monkey BSC-1 cells (79). Such experiments emphasize that microtubules within the cell are constantly being broken down and reformed, a process which has been termed "dynamic instability" (43). Microtubular profiles such as that shown in Fig. 4b where it was clear that single microtubules could be visualized in immunofluorescence microscopy (67), as well as drug experiments (e.g. 100), originally suggested that microbutules might be important as tracks along which particles could be moved. This was dramatically shown by the introduction of video intensification microscopy (3) in which movements along filamentous structures could be documented. Subsequently these filaments could be shown to be individual microtubules (3,77,86). In this system the development of a suitable in vitro assay has paved the way for biochemical attempts to purify the molecules (or "motor") responsible for movement of particles along microbutules (86).

A CONTINUUM BETWEEN PLASMA MEMBRANE AND NUCLEUS

That the cytoskeleton may form a continuum between the plasma membrane and the nucleus is emphasized by two recent findings. First the demonstration of a "membraneous cytoskeleton" underlying the plasma membrane. This is particularly clearly shown by experiments in which membranes from a very special cell type - the mammalian erythrocyte which lacks a nucleus - have been extracted very briefly with a non-ionic detergent and then viewed as whole mounts in electron microscopy (11). These pictures show a framework in which actin, spectrin, ankyrin and a transmembrane protein called band III are dominant components. Glycolytic enzymes may be anchored to band III, a transmembrane protein which

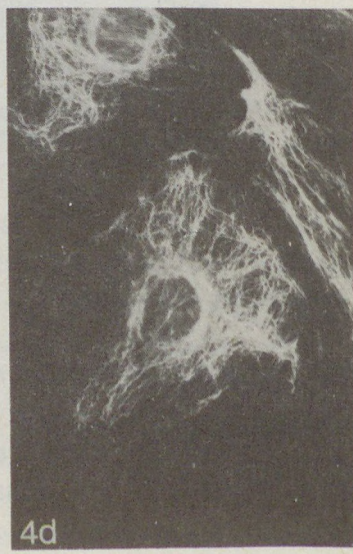
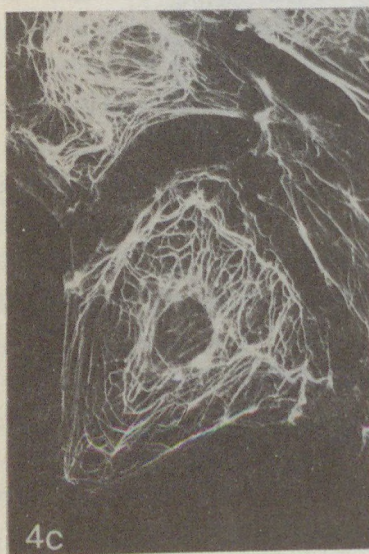
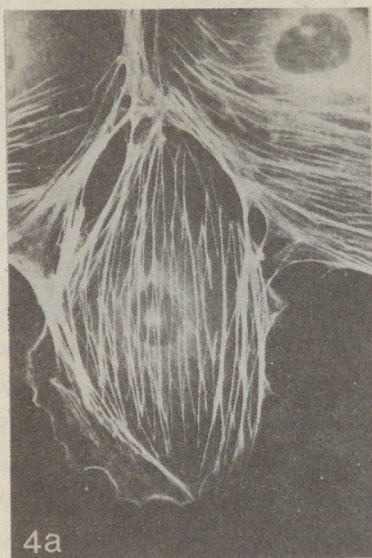


Fig. 4. Differing arrangements of the three filamentous cytoskeletal elements in a single cell type, rat kangaroo Ptk2 cells. (a,b) Double label immunofluorescence microscopy with (a) filamin antibody revealing the stress fibers and (b) tubulin antibody showing the microtubular array. (c,d) Double label immunofluorescence microscopy with (c) keratin antibody and (d) Vimentin antibody showing the arrangements of the two different intermediate filament types in Ptk2 cells. Epithelial cells are characterized by the presence of keratin filaments (see text). x640.

mediates the breakdown of glucose, and also acts as a channel allowing ion exchange across the membrane (7). In other cell types certain protein kinases which are expected to play a regulatory role in cell physiology may be present in this layer. This submembraneous net may also be influenced by signals received in the outer surface of the cells by receptors that bind messenger molecules. Sort actin filaments can be visualized at the intersection points of this net. The intermediate filament protein vimentin binds by its headpiece to ankyrin in inverted membrane vesicles from human erythrocytes (26). These results if they can be extended to other less specialized cell types suggest therefore that at least some of the "classic" cytoskeletal elements may bind directly to the submembraneous cytoskeleton underlying the plasma membrane. Note also that keratin filaments abut on to desmosomes in epithelial cells. Intermediate filaments may also anchor the nucleus (49,64). Recently, sequencing of the cDNAs for lamins A and C has revealed that these proteins, which together with lamin B are the major proteins of the nuclear envelope, also appear to be members of the intermediate filament family (19,55). Thus lamins A and C show extensive homologies to intermediate filaments and in particular also contain the rod motif which appears characteristic of all intermediate filament proteins (24,25,32,82 for review of IF structure see 24). All three lamins i.e. lamins A,B and C react in immunoblotting (66) with the IFA antibody (71) suggesting that when the lamin B sequence is known that it will also be IF related. Striking pictures showing en face views of the nuclear lamina in oocytes reveal it as a meshwork or net in which individual filaments have a diameter of approximately 10 nm (1). Whether cytoplasmic IFs anchor into this network from the cytoplasm is not yet clear. However such results raise interesting questions as to how the cell manages to sort the lamins and intermediate filament proteins into separate cellular compartments. Since both the nuclear lamina (10), and some IF proteins (in particular some epithelial keratins) (22,46) show a similar sequential timing of depolymerization-repolymerization during mitosis it may be that the cell uses similar control mechanisms to regulate the aggregation state of lamins and intermediate filaments. There is some experimental evidence for the involvement of phosphorylation-dephosphorylation events in both systems (10,85).

The cytoskeleton can thus be thought of as a three dimensional network linking the plasma membrane to the nuclear envelope. That

individual cytoskeletal elements are also in some instances linked laterally is clear. Such linkage may be between like elements or unlike elements. To draw attention to these lateral linkers Porter and his colleagues have used the terms microtrabeculae, ground substance or cytomatrix (76, 103, 104). Microtrabeculae have diameters between 4 and 10 nm and interconnect not only microtubules, microfilaments and intermediate filaments but also cell organelles such as ribosomes and internal membranes. The fibers are usually thinner in the center and thicker at the ends where they meet other cytoskeletal elements. Although the microtrabeculae concept has been valuable in emphasizing the three dimensional nature of the cytoskeleton it is not universally accepted for the following reasons:

- 1) The evidence in support of microtrabeculae relies in large part on high voltage electron microscopy. The discrepancies between Porter's group (76, 103, 104) and Ris (74) who also used the same technique are obvious from the literature, with Ris arguing that the microtrabeculae represent "a distorted image of the cytoplasmic filament network produced by critical point drying by traces of water or ethanol in the CO₂". Ris supports this point of view with micrographs of F-actin and cytoplasm by different fixation techniques. When other methods such as rapid freeze, deep etch are used microtrabeculae are not seen (35).
- 2) In parts of the cell cytoplasm where F-actin is very abundant very different images are obtained depending whether high voltage microscopy or a combination of fluorescence and/or electron microscopy is used. Two examples will suffice. First in vaccinia induced macrovilli in high voltage microtrabeculae are visualized (83). In fluorescence and whole mount electron microscopy however regular bundles of F-actin can be demonstrated (36) (Fig. 5). Second when structures at the leading edge of a tissue culture cell are compared in high voltage, and in electron micrographs of detergent fixed and negatively stained material the results are very different. Using the first method microtrabeculae are visualized (103, 105), while in the latter method impressive ordered arrays of F-actin filaments of uniform diameter are seen (38, 39, 81). In another study the compactness of the microtrabecular lattice has been shown to depend on the concentration of protein present in the model system (45).

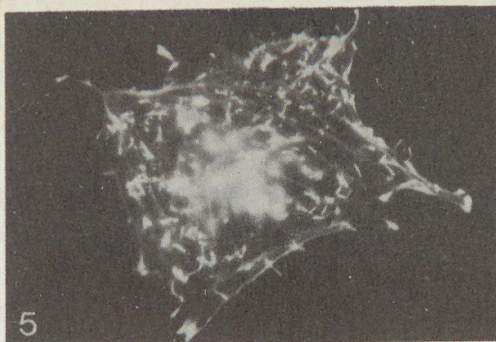


Fig. 5. At late stages after infection of chick embryo fibroblasts with vaccinia virus "macrovilli" are apparent on the cell surface, and are strongly stained by antibody to actin. This cell is 15 hours post infection. The "macrovilli" are 3x thicker in diameter than the normal microvilli found on the cell surface (see text). x950. Micrograph courtesy Dr. G. Hiller (36).

- 3) Two dimensional gels of proteins from a variety of cell lines (e.g. 76) do not reveal the existence of a novel protein present in sufficient amounts to be the building block of microtrabeculae in the same sense that actin is of microfilaments or tubulin of microtubules.

Thus it seems valid to raise the question whether the microtrabecular lattice can be derived from a mixture of the major cytoskeletal proteins, and/or from several proteins which may project from the three major filament systems in the cell, or be rod-like themselves. Examples of such proteins include MAP proteins, neurofilament and other intermediate filament tailpieces, α -actinin, plectin as well as proteins which even though they are not an integral point of any cytoskeletal element may become trapped at the moment of fixation because they are transiently bound to a cytoskeletal component (54).

CELL TYPE SPECIFICITY OF DIFFERENT CYTOSKELETAL PROTEINS

Many cytoskeletal proteins occur in the cell as multigene families i.e. they are coded for by more than one gene. The rules governing the differential expression of the different actins and the different intermediate filament proteins have been largely worked out, while for the different tubulins the results are more complex.

Actins Amino acid sequence studies have shown that there are six different vertebrate actins (87). The β and γ cytoplasmic actins are two homologous isoactin forms characteristic of non-muscle cells. γ smooth muscle actin is dominant in visceral tissues while the α form is the major actin in vascular smooth muscle cells. α -cardiac and α -skeletal muscle actins comprise the skeletal muscle forms named according to the tissue in which they predominate. Because these six actins are strikingly similar in sequence not all actins can be separated from each other on two dimensional gels, and although the occasional antibody has been described which seems to see one or the other actin preferentially (e.g. 28) a set of actin antibodies each member of which is specific for only one of the six actin types is not yet available. For many purposes however the specific actin isotypes present can be determined by actin typing, a protein chemical procedure requiring tissue specimens some 0.5-1gm in weight (88). It is not necessary to purify the actin, and the procedure exploits the documented differences in the highly charged amino terminal peptides which characterize the six different vertebrate actins. Clearly from their distribution in the body the actin isoforms are most useful for distinguishing different muscle types.

Tubulins Vertebrate tubulins are expressed in complex developmental patterns (14,92) "which all but rule out simple kinds of tissue specific expression of individual polypeptides." (14). Between 10-20 α tubulin sequences, and a similar number of β -tubulin sequences per haploid gene have been reported for human, rat and mouse. Many of these sequences are pseudogenes; for example in liver only 3 out of 12 such genes coding for the human β -tubulin family code for functional sequences. Tissue specific patterns of six α -tubulins mRNAs encoding five distinct isotypes showed a complicated expression pattern when tissues such as brain, heart, kidney, liver, leg muscle, spleen, stomach, testis and thymus were examined. Most notable was the apparent testis specific expression of two sister genes. High resolution two dimensional electrophoresis of vertebrate brain tubulin reveals some 7 α species and 10-14 β species. The expression of the different genes is modulated during neural development. In the rat all 7 tubulins are present during development but the relative amounts of each component varies. For the β species, 3 β -tubulins are found in the embryo whereas 14 are expressed in the adult. Although for a tissue as complex as brain it could be imagined that the complexity of tubulin expression is due to the fact that brain contains a variety of different cell types, 5-6 electrophoretically different tubulins have been documented within a

single neurone in culture (29). The complexity of tubulin expression, indicated by the results just discussed suggests that the different α and β -tubulins within a single cell, might subserve different functions. Posttranslational modification of tubulin and in particular the tyrosination of α -tubulin is well established. Although such modifications may be related to differing functional specificities or stabilities of different microtubules within the cell the evidence equating particular α or β -tubulin isotypes with particular functions is still weak (31,100,101).

Most immunological studies have been performed on microtubules in cells in culture. Microtubules can be visualized in tissue sections if special precautions are taken, and methods have been developed to determine microtubular profiles in developing embryos e.g. in the sea urchin (33) and in *Drosophila* (42,93). Although originally it was suggested that microtubules in transformed cells were "fragmented, disordered or absent" (8) it is now clear from both immunofluorescence and electron microscopical studies that microtubules are ubiquitous cytoplasmic components (95). Tubulin antibodies often show broad species specificity - for example a single antibody can reveal the microtubular arrangement in plant cells as well as in humans. Antibodies specific either for β -tubulin, for the tyrosylated form of α tubulin, or for the detyrosylated α -tubulin (31) have been described and provide useful probes to further investigate possible functionally significant differences between different tubulins. However because of technical difficulties in visualizing microtubules in tissues - and in particular in frozen cyrostat specimens - so far there is little data on tissue specific distribution of the different tubulins by immunohistological methods.

Intermediate filaments

The striking features of the cell type specific distribution of intermediate filaments is how closely it resembles the major cell divisions made in histological textbooks. The cell type specific distribution of IFs in normal human tissues and in tumors is summarized in Table 1 and in Figures 6,7 and 8 (for original references see 6,21 for reviews see 20,40,62). Thus keratins are characteristic not only for the classic keratinizing epithelia such as skin, but also for the so-called non-keratinizing internal epithelia (20,62,84). Vimentin is the IF protein characteristic of cells such as fibroblasts, endothelial cells and chondrocytes where it is the only IF type present. Desmin is the IF

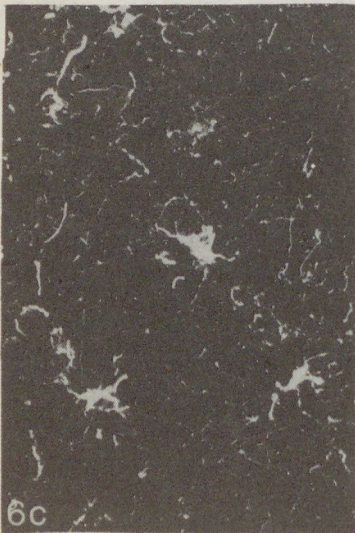


Fig. 6 Normal human tissues stained with antibodies specific for a single IF type (a) bladder epithelium showing positive staining of the epithelial cells with a broad specificity monoclonal keratin antibody. (b) muscle showing positive staining of the z discs with a desmin monoclonal antibody. (c) brain with a monoclonal antibody specific for glial acidic protein showing positive staining of the astrocytes. (d) brain showing positive staining of neuronal processes with a monoclonal antibody specific for NF-H the highest molecular weight neurofilament peptide. Notice that only the cell types mentioned stain and that other cell types present in the section do not. (a,b) x250 (c,d) x400.



Fig. 7 Use of antibodies to intermediate filaments in the differential diagnosis of human tumors. (a) breast carcinoma shows tumor cells positively stained with a monoclonal antibody to keratin. (b) infantile fibrosarcoma showing tumor cells positively stained with a monoclonal antibody to vimentin. (c) rhabdomyosarcoma showing tumor cells positively stained with a monoclonal antibody to desmin. (d) neuroblastoma showing tumor cells positively stained with a monoclonal antibody to glial fibrillary acidic protein. Note that IF typing gives histogenetic information about tumor cell origin. For instance the tumor cells in (a) are stained only by keratin antibodies not by antibodies against other IF types (a,c) x160 (b,d) x250.

protein typical of skeletal, visceral and many vascular smooth muscle cells. GFAP expression is found in astrocytes and in Bergmann glial while neurofilaments are found in cells of the peripheral and central nervous systems. A few cell types appear to lack IFs.

IF distribution can be assayed by the use of antibodies specific for one IF type, and in many instances the immunocytochemical results can be supported by biochemical or protein chemical studies on the purified IF polypeptides. The use of well characterized antibodies sometimes allows individual cell types to be picked out by positive staining against a background of negative cells. Thus the Langerhans cells of the skin and melanocytes are positively stained with the vimentin antibody, while epidermal cells do not stain with vimentin, but are stained by the keratin antibody (52). Likewise different cell types can be distinguished in brain by use of antibodies specific for different intermediate filament types (78,80).

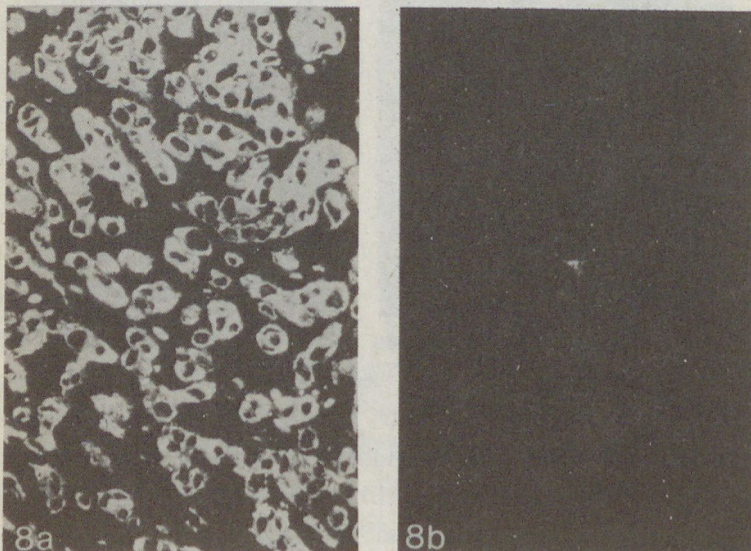


Fig 8. Use of a monoclonal antibody specific for keratin 7 (and which does not recognize other keratins) to differentially diagnose pancreatic carcinoma. This antibody stains tumor cells of ductal carcinoma of the pancreas positively in a liver metastasis (a) but does not stain tumor cells of a stomach carcinoma (b). x250.

USE OF CYTOSKELETAL PROTEINS TO ESTABLISH A DIFFERENTIAL DIAGNOSIS ON PATHOLOGICAL MATERIAL

In general two questions have to be answered by the pathologist or cytologist confronted with a histological or cytological specimen. First whether the specimen is benign or malignant. Second if it is malignant a differential diagnosis has to be provided where possible, since such a diagnosis is of utmost clinical importance and has therapeutic and prognostic consequences. By far the majority of specimens (90-95% in histology, somewhat less in cytology) can be classified by a careful analysis of morphologic features displayed by the tumor cell at the light microscopic level. In these cases examination of specimens stained with routine stains such as hematoxylin-eosin is sufficient to separate the major human tumor groups. However in the remaining cases (5-10% in histology, somewhat more in cytology), although it is relatively easy to recognize that the cells are malignant, no specific diagnosis of tumor type can be made. In such instances which are often reflected by use of terms such as small cell, anaplastic, undifferentiated, or where the possible differential diagnoses are modified by terms such as "might", "could", "is consistent with" etc. other methods have to be used to look for distinguishing features which may help to further differentially diagnose a particular tumor. Chief among these are immunocytochemical and/or ultrastructural characteristics.

Intermediate filaments That human tumors can be classified according to their IF content has been established by a variety of studies in different laboratories using sets of antibodies specific for a single IF protein type on tumors classified in parallel by conventional histological techniques (57,59,63,73,89). In general the primary as well as the metastatic tumor displays the same IF types as does the parent cell. Thus carcinomas retain the IF type typical of keratinizing and non-keratinizing epithelia and are keratin positive. Non-muscle sarcomas, lymphomas and melanomas express vimentin as the only IF type. Muscle sarcomas i.e. leiomyosarcomas and rhabdomyosarcomas retain the muscle specific IF type - desmin (5). Certain tumors with a glial origin retain GFAP. And finally tumors originating from the sympathetic nervous system such as neuroblastoma, ganglioneuroblastoma, and pheochromocytoma retain the IF type typical of neuronal cells i.e. neurofilaments. Thus IF typing distinguishes the major human tumor groups. More quantitative data listing

the number of tumors in each group can be found in the references cited above. Further support for the subdivisions shown is provided in some instances by IF typing of tumors induced in animal model systems (e.g. 5). Additionally and more importantly biochemical support for the presence of particular IF types in different tumor types can be provided by one or two dimensional gel electrophoresis (15,58,72). Particular IF proteins can then be identified either by their gel coordinates or by transfer to nitrocellulose and immunoblotting.

Conversely in a situation where a differential diagnosis is difficult because of lack of detail at the light microscope level, IF typing can be used to aid the differential diagnosis. IF typing would seem to be of particular use for distinguishing the small round cell tumors of children since rhabdomyosarcoma is desmin positive, neuroblastoma neurofilament positive, Ewing's sarcoma and malignant lymphoma vimentin positive while blastema cells in nephroblastoma can be vimentin positive or vimentin and keratin positive. Among tumors of adults IF typing can distinguish anaplastic carcinoma which is keratin positive versus lymphoma which is vimentin positive. Or carcinoma versus melanoma since the latter tumor is vimentin positive. Or carcinoma versus non-muscle sarcoma where the sarcoma is vimentin positive. Tumors where only very few tumor cells are present, or where they have aberrant morphologies, can also be easily visualized if the appropriate IF antibody is selected. One example in this category is signet ring carcinoma of the stomach identified with a keratin antibody. A second is the identification of only a few carcinoma cells in a lymph node with a keratin antibody.

Other tumors appear to be characterized by the coexpression of two (or very rarely more) IF types (56). For example, coexpression of keratin and vimentin is very often detected in renal cell carcinoma and in certain thyroid carcinomas. Coexpression of keratin and neurofilaments has been noted in many but not all Merkel cell carcinomas, while vimentin, keratin and neurofilaments may be present in medullary thyroid carcinoma. Other situations where IF typing clearly has a useful role to play include the classification of tumor cells in bone marrow smears, and in cytological specimens where the positional information inherent in the histological specimen has usually been lost (17,18). Thus for example a recent study of aspirates difficult to classify by conventional techniques showed that the results of IF typing could in a significant fraction of the specimens

refine the diagnosis i.e. make it more specific and in a few instances even alter it (17).

Keratin The human contains at least 19 keratins which can be distinguished by their molecular weights and isoelectric points on two dimensional gels, excluding a further 8 hair keratins (15,58,106). Different epithelia contain different but sometimes overlapping keratin subsets. The number of keratins in a particular cell type can vary from two (e.g. in hepatocytes) to more than ten (e.g. in skin) (for a complete listing see 15,58,72). An alternative approach to the characterization of keratins has been to isolate monoclonal antibodies specific for only a single keratin polypeptide (15,16,47). Such antibodies can thus be used to define the keratin content of normal and tumor cells in a manner similar to that discussed above for IF typing. Antibodies specific for single keratins include those with specificities directed against keratin 1,3,4,7,8,10,13,14,18 or 19. Thus far the results obtained by keratin typing correlates well with those obtained by biochemical methods using 2D gel analysis. Keratin typing can be used to try to obtain information about the cell of origin of a particular lesion (12,13,15) or to subdivide apparently homogeneous tumor groups such as breast carcinomas according to their keratin content (4). Keratin antibodies with predetermined specificities can make diagnostically useful distinctions. Thus for example a keratin 7 antibody can separate pancreatic carcinoma which is keratin 7 positive from colon carcinoma which is keratin 7 negative (68) (Fig. 8a,b). Finally in some instances determination of keratin content can be useful in determining whether a carcinoma of unknown origin is for example an adenocarcinoma as typified by being keratin 18 positive, or is a squamous cell carcinoma the vast majority of which are keratin 18 negative (16).

Tissue polypeptide antigen, thought to be a cellular tumor or proliferation marker, and present in the circulation corresponds to the soluble rod fragments of keratins 8,18,19 (96), a finding recently confirmed by DNA sequencing for keratin 8 (53). This raises the possibility that assays detecting intermediate filament derivatives in serum might be of diagnostic value.

Other proteins characteristic of particular differentiated cell types
In this section I list only a few examples of proteins which have been first characterized by cell biological or biochemical studies in

differentiated tissues and then later used to test whether the expression is preserved in tumors.

1. Desmosomal components. The biochemical anatomy of the desmosome, a junction typical of epithelial cells has been largely elucidated (for review see 57). Antibodies to protein components of this structure such as the high molecular weight proteins desmoplakins I and II can be used to positively identify epithelial cells and carcinomas (Fig. 9a,b) (57,65). Thus desmoplakin staining, which is visualized as an interrupted staining of the cell membrane can provide an additional check of epithelial cell origin (although note that there are a very few non-epithelial cell types that also have desmosomes).
2. Villin. This 96 Kd protein is expressed by a restricted set of epithelial cells i.e. those which have a brush border (94). Examples include intestinal epithelial cells and kidney proximal tubular cells. Villin expression is also preserved in most but not all colon carcinomas and renal cell carcinomas with the least differentiated tumors being least likely to express villin (30,75).
3. Actin. Antibodies specific for skeletal muscle actins might be able to specifically identify tumors of skeletal muscle origin i.e. rhabdomyosarcomas.
4. Titin. A further marker of interest is titin a high molecular weight protein (10⁶Kd!) important in skeletal muscle organization (90). Titin antibodies stained skeletal but not visceral or vascular smooth muscle cells. Tumors of skeletal muscle i.e. rhabdomyosarcoma are positively stained while tumors of smooth muscle i.e. leiomyosarcoma are not. Thus titin may be a useful additional marker with which to positively identify rhabdomyosarcoma, although a lower percentage of tumor cells are positive for titin than for desmin (Fig. 9c,d) (69).
5. Synaptophysin is a membrane glycoprotein with an apparent molecular weight of 38 Kd which is a component of presynaptic vesicles (102). Synaptophysin expression can be used to positively identify certain tumors of neuroendocrine origin (27).

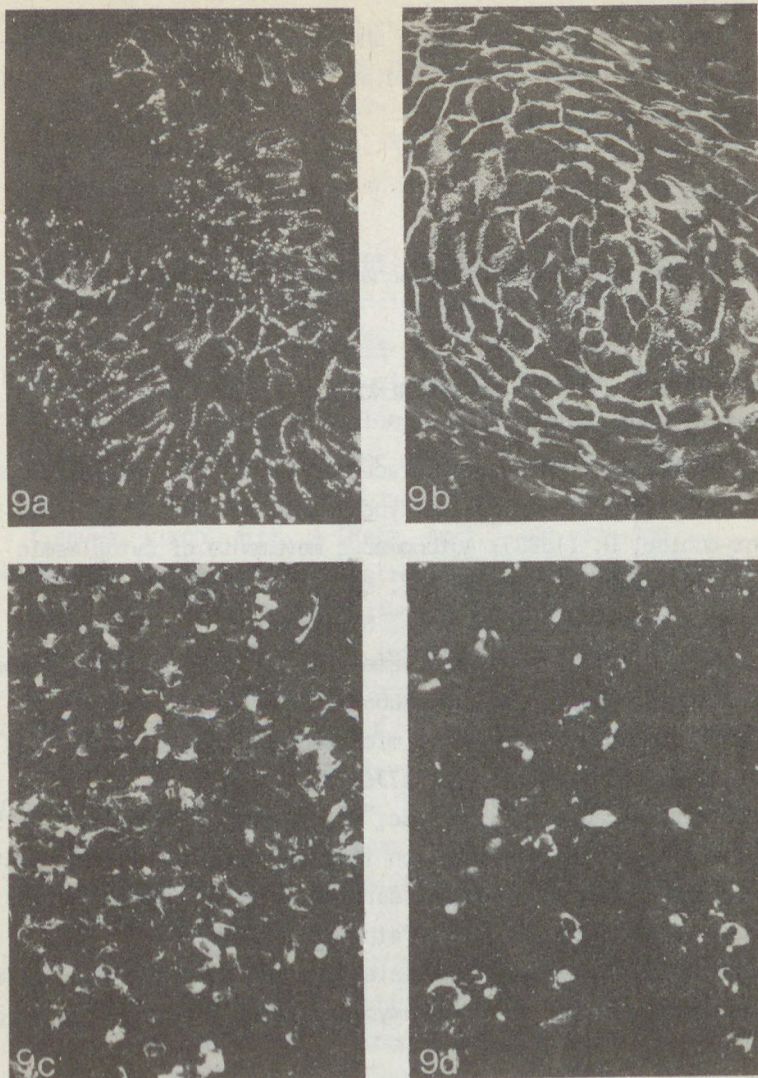


Fig 9. Examples of the use of antibodies to other cytoskeletal proteins in differential diagnosis of human tumors: (a) normal uterine epithelium and (b) a squamous cell carcinoma stained with a monoclonal antibody to desmoplakin I (65). The dots at the membrane represent for the most part single desmosomes and desmosomes are in general characteristic of epithelial cells and of carcinomas. (c,d) a section of the same human rhabdomyosarcoma stained in (c) with a desmin monoclonal antibody is in (d) with a monoclonal antibody against titin. Note that fewer tumor cells are stained with the titin antibody than with the desmin antibody presumably because titin is expressed later in development than desmin. (a) x400 (b-d) x250.

The results summarized here show that certain proteins first identified in cell biological studies may also have a part to play in routine pathological diagnosis.

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DISCUSSION

Tigyi: The antibody staining and the scanning electron microscope are both very powerful methods. But you must not forget that polarizing microscope is a very good tool for the study of native cells, and I'd like to mention that about 20 years ago in our lab we made a lot of polarizing microscope-studies and we were able to see microtrabeculae between the fibrils in the muscle fibers. This is published in the monograph of Ernst.

Osborn: The talks this morning have shown several microscopical methods for visualizing the cytoskeleton, e.g. immunofluorescence microscopy, high voltage electron microscopy, electron microscopy and quick freeze deep etch microscopy and now you add polarization microscopy. Sometimes the impression given by one technique aids the interpretation of pictures by another technique. One good example is microtubules, where the first immunofluorescence pictures of microtubules in the interphase cell, taken by Weber and Birbing ten years ago contrasted sharply with the images of short, apparently discontinuous pieces of microtubules known from standard electron microscopy and in retrospect of course it is clear that with the latter technique it is hard to get an overview, since only a section of the cell is usually examined.

Whether any of the techniques we've talked about give you the absolute truth as to what is going on in a living cell is open to debate. However in the case of immunofluorescence microscopy one way in which it has been tested, is by injecting fluorescently labelled proteins. When one views such experiments done over the last four to five years they give the same impression as what you see when you open the cell, fix it and use antibody to the microinjected protein. So, I think, to a large extent what you see with immuno-, fluorescence is all right.

To return briefly to connections between the different cytoskeletal elements. Certainly they have over the years been visualized by a variety of different techniques. The current differences in opinion center more on their morphology and on their constituent proteins rather than on their existence.

Ling: I really have a small comment and a question. The comment is about the natural tendency for us to put something inside a cell and let that something tell us about what's in the cell normally. And I would just submit that one has to be very very careful whatever one puts in that it

does not disturb the original condition. I point for instance to the intracellular electrode, which has been used extensively to measure the physical state of ions in the cell.

Although this has been carried on for nearly twenty years, the bottom line as we can draw it now is that it disturbed the cytoplasm and most of the results reflected that disturbed condition. Now, the question I would like to ask relates to a subject of considerable interest to this meeting, that is the state of water. And it was the concern about the state of water that led to the recognition that there may be differences in cancer cells and normal cells. And of course when you deal with cancer cells, the problem is very complex. You have various states of development of cancer. And most of our studies were made on cells, that were called maximally deviated cancer cells. That is on cells that have been passaged in vitro many many times. And under these conditions, as far as we could detect, fifteen strains of maximally deviated cancer cells protein-wise were almost identical. That is, the one dimensional gel tracings could be superimposed on each other. This is in contrast to what you are demonstrating which indicates that they still maintain the original protein pattern.

Osborn: I agree with the comment although as just mentioned fluorescently labeled cytoskeletal proteins can be introduced into cells, and do usually show the same locations as the intrinsic proteins. To the question you raise as to how similar the protein profiles are of normal and tumor cells let me make it clear that I did not say that all the proteins in the normal and transformed cells remain the same. I don't know that. There are people who have spent a lot of time trying to look for specific differences by two dimensional gel electrophoresis of paired normal and transformed cells. What I did say is that in general the intermediate filament complement of the normal cell is retained in the tumor cell. I don't want to say it's without exception that they keep the major intermediate filament type(s), but it's a good working rule. In considering why they seem such good markers, one should remember that they appear to be early markers of a cell's commitment to develop along a particular developmental pathway. For example in skeletal muscle development, desmin is expressed relatively early, while specialized skeletal muscle proteins such as titin and myoglobin are expressed relatively late. In rhabdomyosarcomas more than 95% of the tumor cells

express desmin while a much lower percentage express titin or myoglobin. Thus this tumor faithfully expresses an early protein - desmin - but expresses only in some cells protein markers such as titin and myoglobin.

Ling: I think, I have given the wrong impression. I was very impressed by what you presented including the possibility of using these markers in clinical diagnosis. However the Erlich ascites cells that have been passaged for thirty, forty or fifty years are probably not typical of cancer cells dividing inside a patient, a situation which more likely reflects a transitional state. And that's a practical problem. But on the theoretical side as to what ultimately cancer cells become, that is a different problem and has nothing to do fundamentally with clinical diagnosis, the question is: does it really revert back to an early embryonic state? The estimates made by one dimensional or two dimensional gel are both rough estimates. At best one can see a trend and the thing that was remarkable to us is that all the water seems to behave in a certain common way and so do the protein profiles in terms of molecular weight and abundance.

Osborn: Let me not comment on water, because one of the reasons I'm here is to learn about water in the cell. Let me make one comment about tissue culture. Tissue culture is terrific. A lot of us couldn't do experiments if we didn't have the cells growing in the lab. But tissue cells in culture are sort of an approximation. For example when we started looking at the intermediate filament content of cells growing in culture we found that the set of cell lines that you have in cell culture collections is not very representative of those in the body. They are highly biased towards epithelial lines and fibroblast lines. That is true even now, but ten years ago all the epithelial cell lines we had available had both keratin and vimentin. In spite of screening a large number of cell lines for additional IF types we had one cell line only that expressed desmin, one cell line only that expressed GFAP and one cell line that expressed neurofilaments. We started studying tumors because of a totally wrong idea coming from the cell culture work. We thought originally that since epithelial cells in situ had only keratin that tumors would then add vimentin to the IF complement in situ and that vimentin would thus be a marker of proliferation or of malignancy. In retrospect, it is now clear that indeed there are more recently isolated cell lines in culture which only express keratin, such as MCF 7 or HT 29 cells. But what really made

the difference for us in understanding the rules that govern IF expression was going from the cell culture system and looking at IF expression in sections of normal tissues. Thus I think cell culture is a useful but imperfect system and that you need to constantly check the results obtained with it against in vivo or in situ results. I don't want to exclude the possibility of reversion back to an early embryonic state e.g. in teratoma, but I do think there are enough examples where one can guess at the cell of origin in the adult tissue or tumor by looking at the IF complement of the normal cell and of the tumor.

Kellermayer: As you can see in the program, we wanted to have Poyton here to talk about spatial memory. I don't know whether this is the appropriate time to speak about spatial memory, but at any rate the spatial memory must be "located" somewhere in the supramolecular organization of proteins. So, there are two types of protein structures which can be taken into consideration: One is the cytoskeleton, the other is the proteins which associate either in a lattice or in some other way to the cytoskeleton. The supramolecular organization of these proteins inside the living cell means that water and ions associate to them. If we go to the spatial memory, it must be "buried" and expressed somehow in the dynamic associations of proteins and ions. Could you comment on this?

Osborn: If by spatial memory you mean morphology each cell has a group of structural proteins, which, depending on the protein complement, the ionic environment and other circumstances in the cell, adopts one of a certain restricted number of macromolecular organizations. For example, the morphology of a cell - whether it is round or flat - correlates well with the absence or presence of stress fibers. In normal cells actin appears in stress fibers, while in the transformed cell such bundles are not present. To explain the structure one has to understand not only what one sees within the cell, i.e. the biochemical anatomy of the stress fiber but also the biochemistry of the isolated proteins in the test tube. To explain dynamic changes the whole environment surrounding the structures within the cell including ion concentrations, ATP levels etc. is likely to be of importance.

Németh: I think that the immunofluorescent morphological demonstration shows that in the last ten years we have had a very fast development in morphology and in pathomorphology. But we have to remember, that the classical analytical histochemical methods could demonstrate the same

structures without biochemical background. My question is, could you explain the functional significance of the orientation of the cytoskeleton. If we see these pictures, sometimes our impression is that the cytoskeleton forms a random network while in other pictures the cytoskeleton is orientated. Thus, if you compare tissue culture cells and tissue cells highly organized in different organs, you can find a different organization. Cytoskeletal elements are much more fixed in cells in situ: in contrast you can observe dynamic changes in this orientation if you analyze the tissue culture cells. Is there any significance to this orientation; I would also like to recall Prof. Tigyi's comment that if you use polarization microscopical methods, and compare it to the immunofluorescent pictures, you can observe this different level of orientation on these filamentous elements inside the cell.

Osborn: Let me reply with one specific example, the intestinal brush border cell which has several thousand microvilli projecting from its surface. Here the distributions of keratin filaments within the cells in situ and within the cytoskeleton of that cell after scraping those cells off the intestinal mucosa and extracting it with detergent, are almost identical. The keratin filaments outline the intestinal cell below the brush border, and form a true "cytoskeleton". Above the brush border one sees microvilli. These contain bundles F-actin filaments and three other major proteins, villin, fimbrin and a 110Kd protein. Using the purified proteins in vitro one can duplicate some of the same effects seen in vivo. Thus there is a system where one is quite close to being able to correlate what happens in the test tube with proteins such as F actin, fimbrin and villin, to what one sees ultrastructurally in the electronmicroscope. Additionally there is a human intestinal cell line HT 29, studied by Zwiebaum and Louvard and their collaborators. Under certain conditions the cell will differentiate and express and assemble microvilli specific proteins, so that the cell is polarized in vivo. Again in culture things are not quite so good as in situ in the three dimensional tissue and to me that's no surprise. I think, every pathologist or anybody who has looked at tissue sections, is aware of the point that you make, which is how organized, how regular, how directed the cytoskeletal organization is in tissues in comparison to the situation in tissue culture where the cells are often not even connected to each other.

In situ the contacts between neighbouring epithelial cells i.e. the

desmosomes as well as contacts between the cells and the basement membrane i.e. hemidesmosomes clearly play an important role. And, so, again what you are saying is that growth in a Petri dish in two dimensions is not quite the same for the cells as being part of a three dimensional tissue.

ON THE MECHANISM OF CONTRACTION

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In his long career, Professor Ernst, to whom this symposium is dedicated, rarely hesitated to express his point of view forcefully and lucidly, even if it differed radically from what was accepted at the time. Until his dying day, he held that the sliding filament theory was incorrect, despite widely accepted views to the contrary.

Professor Ernst was convinced that thick filaments shortened during contraction; he felt this phenomenon was connected intimately with the underlying contractile mechanism. Thus, protein contraction rather than filament sliding appeared to underlie active shortening. Further, Professor Ernst and his collaborators long maintained that the myofibril was not merely the sum of thick and thin myofilaments; that a network of longitudinal and transverse structures held the myofibril together.

Although these views are not widely held today, it is quite possible that they are all correct. In this presentation, we will show that all of these propositions are not only likely, but that they probably play an important role in the contractile process. We begin with a phenomenon studied intensively in our laboratory, stepwise shortening, and then investigate how the views described by Professor Ernst help to elucidate the underlying mechanism.

Stepwise Shortening

More than a decade ago we stumbled upon an unexpected result. The advent of lasers and high-resolution optical sensors made it possible to track the time course of sarcomere shortening with high precision. To our surprise, we found that sarcomere shortening did not occur smoothly; it was punctuated by periods of pause, during which there was little or

no length change. This conferred a staircase-like character on the shortening waveform. Examples of early results are shown in Figure 1.

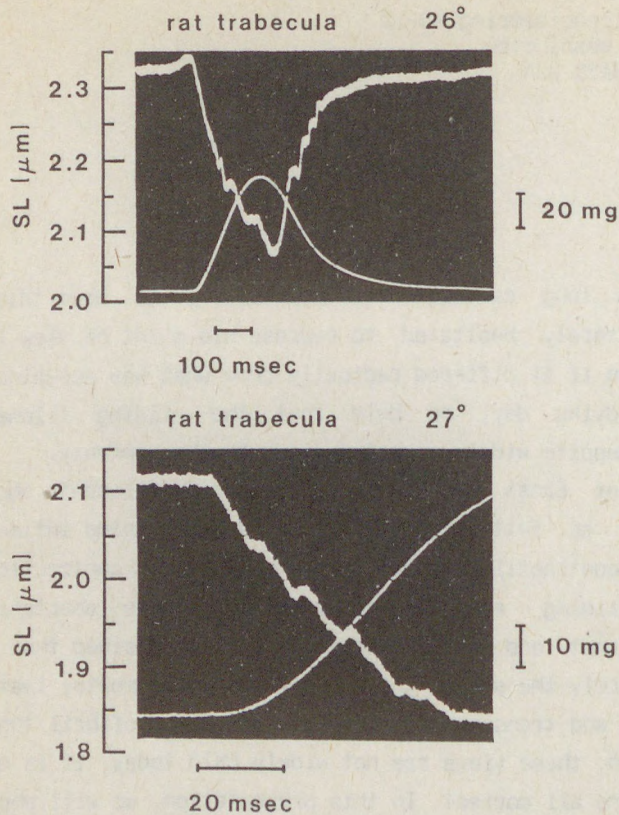


Fig. 1. Stepwise sarcomere shortening in cardiac muscle, as measured by the optical diffraction technique. Specimens were mounted between fixed clamps and stimulated. The central, viable region shortened at the expense of end compliance. Sarcomere length changes are interspersed with pauses during shortening and relengthening. Tension trace shown below on each panel.

The pauses intrigued us. The optical diffraction method that was used reflects the collective behavior of a sizeable fraction of sarcomeres ranging over the field illuminated by the laser. A pause in this ensemble implies that this fraction pauses synchronously.

The difficulty with this conclusion is the implication that molecular elements, whether inside or outside the contractile apparatus, act in concert; otherwise pauses would not be detectable in so large an ensemble. A typical region sampled by the laser beam may contain 100 million filaments. Could an ensemble so large - or even a fraction of it - act synchronously?

This question is the essential root of the controversy that has developed. According to presently accepted views, there is no provision to synchronize the action of cross-bridges. The bridges are thought to act randomly. Thus, the theory predicts smooth shortening, not stepwise shortening.

Several papers have challenged the phenomenon of stepwise shortening, claiming it to be an artifact of the methodology. Perhaps the best summary of results and of criticisms may be found in the back to back articles to appear in *Circulation Research* just at the time of this symposium. The first, prepared by the senior author of this paper (Pollack, 1986), reviews the four methods used to detect stepwise shortening. The second, prepared by Sir Andrew Huxley (1986), offers criticism of each of the methods.

Meanwhile, since the criticisms have been largely directed toward methods based on the muscle's striations, our efforts have been redirected toward methods that were independent of the optical properties of the muscle. A method was devised to track the length of a fiber segment, demarcated by a pair of thin (ca. $10\mu\text{m}$) hairs taken from the ear of a black cat. Segment length was tracked optically.

Results obtained with the segment length method confirmed all of the optical methods. Stepwise shortening was seen regularly during both active shortening and unstimulated releases. Shortening patterns bore close resemblance to those obtained with microscopic methods. In fact, when segment length and diffraction methods were employed on the same fiber during the same contraction, the records were generally superimposable (Granzier et al., submitted for publication). Superimposability speaks strongly for the reliability of both segment length and diffraction methods.

Stepwise segment shortening has now been confirmed in two independent laboratories (Housmans, 1984; Tameyasu et al., 1985). Both used preparations of cardiac muscle, and different types of markers were employed in each.

In summary, methods that do not depend on the muscle's optical properties have confirmed the presence of pauses and steps in the shortening pattern. Careful controls have been executed to check for possible artifact; none could be found. The steps have been confirmed elsewhere. Thus, the phenomenon appears to be genuine.

Mechanism of Stepwise Shortening

Two approaches may be taken to this question. In the first, one may assume that the presently held theory is correct, and press on from that vantage point. In the second, one may be tempted to ignore the theory and begin afresh. In this latter approach, the steps themselves may be potentially useful in ferreting out the underlying contractile mechanism.

Many, along with Professor Ernst, have questioned the validity of the theory. Some of the more fundamental discrepancies between theory and observation are described in detail in a comprehensive review (Pollack, 1983). Thus, we begin with the notion that the sliding-filament swinging cross-bridge theory is not necessarily adequate. How, then might the steps arise?

Two clues help narrow the mechanism (Ganzier and Pollack, 1985):

(1) Activation is not required. Relaxed fibers that are stretched or released with a servomotor show stepwise shortening. This implies that actomyosin interaction is unlikely to be required to generate the steps.

(2) Steps remain in evidence when fibers extended beyond thick-thin filament overlap are forcibly stretched or released. This confirms that actomyosin interaction is not required.

These clues lead directly to a proposal for step generation in unstimulated fibers. When the fiber is stretched beyond overlap, continuity between Z-lines is not lost. As Professor Ernst knew long before the rest of us, the Z-lines remain interconnected. This is now well-established not only in insect flight muscle, but in vertebrate muscle as well: Each end of the thick filament is connected to the nearest Z-line by a "connecting filament". When the fiber is stretched, these connecting filaments are likewise stretched. Probably they support much of the resting tension (Magid et al., 1984).

An example in which connecting filaments can be seen particularly

well is provided in Figure 2. This specimen, taken from honeybee flight muscle, was placed in rigor, then stretched. As shown previously (White, 1967; Trombitás, Tigyí-Sebes, 1977) this stretch tears the thin filaments from their anchor points on the Z-line. Thin filaments remain bound to the bridges, while connecting filaments are extended by the stretch.

Since thick filament length remains constant in unactivated fibers, an inescapable consequence of the filament organization is that imposed length changes must be fully conferred on the connecting filament. If sarcomere length changes occur stepwise, the connecting filament must change length in steps. The stepping mechanism, therefore, at least in unactivated fibers, is likely to reside in the connecting filaments. No other element of the myofibril changes length.

While Occam's razor might favor the proposition that steps arise from a single source, connecting filaments cannot hold the full answer. Stepwise shortening can be detected in contractions at short sarcomere lengths (Vassallo and Pollack, 1982), well below those at which resting tension is present. Since connecting filaments are presumably slack at these lengths, another element must be responsible for these "active" steps.

A possible mechanism lies in stepwise shortening of one of the sarcomere's other filaments. The thin filament is evidently unlikely, for many studies have confirmed the constancy of thin filament length under a broad spectrum of conditions. This leaves the thick filament. Is this reasonable?

Figure 3 shows the sarcomeric structure seen by freeze-fracture. Structures revealed by this method are based on their physical presence, not on a pattern of chemical staining. Thus, the thick filament does not simply terminate; it interconnects with a (connecting) filament of slightly smaller diameter, which, together with the thin filaments, forms a congested I-band. The main point of this Figure (and also Figure 2) is that apart from the thin filaments, the sarcomere may be thought of as having one continuous filament running from Z-line to Z-line, the central region of which is thicker and more robust than flanking elements.

From such a perspective, the proposition that stepwise shortening of activated fibers arises from thick filament shortening is not at all preposterous - provided the data support such a contention. The division of labor would then be simple: connecting filaments in unactivated myo-

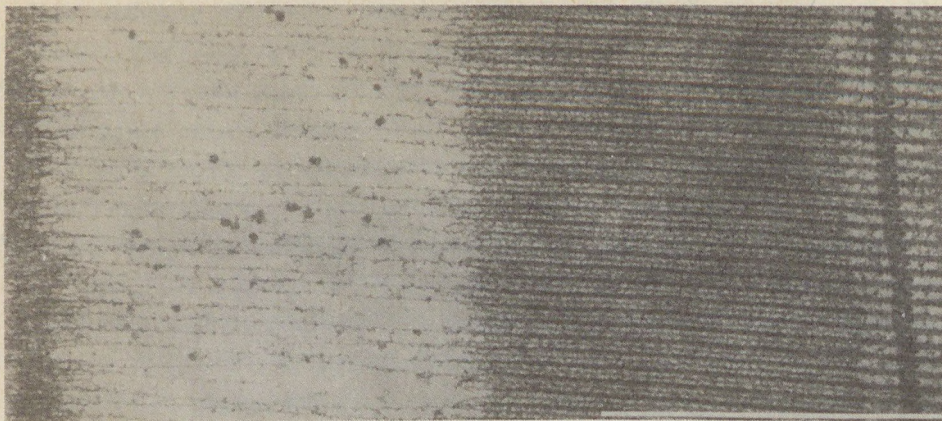


Fig. 2. Honeybee flight muscle stretched in rigor. Stretch tore thin filaments from their anchor points on the U-line; the length change was therefore conferred only on the connecting filaments. The latter are clearly seen on the left side of the micrograph, interconnecting the thick filaments (right) with the Z-line (far left). Scale bar - 1 μ m.



Fig. 3. Freeze-fracture image of relaxed frog semitendinosus muscle, stretched beyond thick/thin filament overlap (from Suzuki and Pollack, 1986). The gap between the tips of thin filaments and ends of thick filaments is spanned by connecting filaments, which run from thick filaments to Z-lines. Scale bar - 1 μ m.

fibrils; and thick filaments in activated myofibrils. Two regions of a single compound filament. Thus, Occam's razor is not seriously blunted.

Do Thick Filaments Really Shorten?

As Professor Ernst was keenly aware, the classical observations of H.E. Huxley and Hanson (1954) and A.F. Huxley and Niedergerke (1954) have not been broadly confirmed. On the contrary, a surprising large number of papers published since the mid-fifties have reported A-band shortening or thick filament shortening of substantial magnitude. In the above-mentioned review (Pollack, 1983), more than 40 papers can be counted, though not all are of equal quality. This does not include the vast collection of papers published during the first half of this century, in which A-band shortening had been confirmed time and again.

A number of studies were launched in this laboratory to pursue the question of A-band shortening. We have used both optical microscopy and electron microscopy. The preliminary results reported below appear to confirm the possibility of large-scale thick-filament shortening.

In the first study, single fibers of frog tibialis anterior muscle were stimulated to contract tetanically. During tetanus, sarcomeres were allowed to shorten slowly - typically by $1\ \mu\text{m}$ in 2 to 3 seconds. At this low velocity, the tension remained high during shortening, so the muscle did considerable work.

Figure 4 shows a representative result. Since polarization microscopy was used, A-bands are light, I-bands dark. We found that both A-bands and I-bands shortened during active contraction (Figure 4a). A-bands shortened by as much as half their initial length. As a control, we measured the changes of band pattern occurring during comparable shortening of the unstimulated fiber. Figure 4b shows that the length changes are largely restricted to the I-bands. Thus, there is no confusion between A- and I-bands.

A-band shortening has also been detected in the electron microscope, though a systematic series of studies has not yet been completed. Figure 5, for example, shows a specimen of dog cardiac muscle during an hypoxic episode. A region just outside this field has shortened extremely and irreversibly. Sarcomeres in the field are on the borderline between damaged and surviving regions. Note that these sarcomeres are of variable

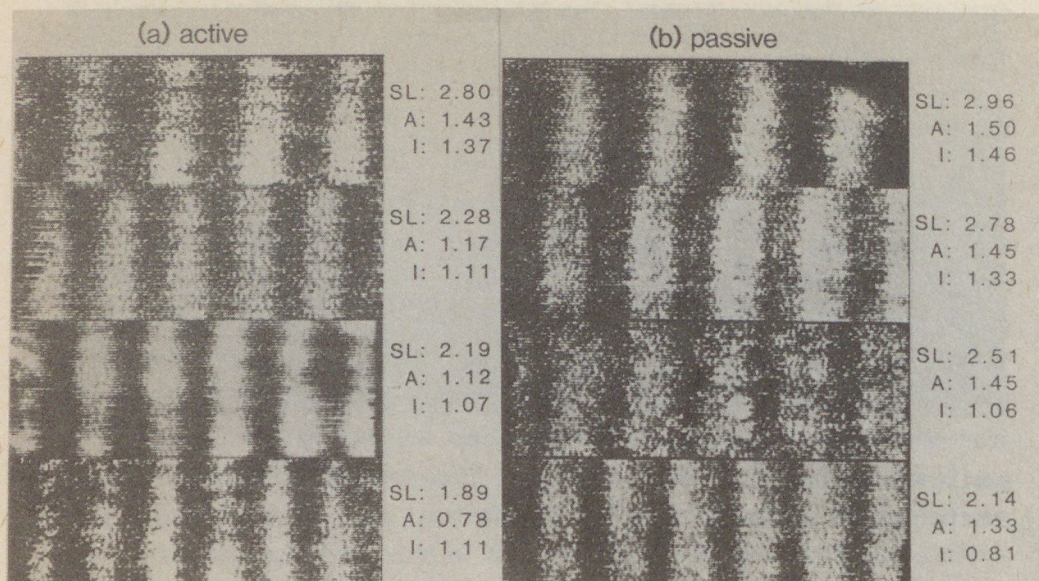


Fig. 4. Polarization microscopic images of frog tibialis anterior muscle. A-bands light; I-bands dark. Fibers were pre-stretched, then shortened with a servomotor. (a): In stimulated fibers, sarcomere shortening was associated with both A- and I-band shortening; (b): In unstimulated fibers, most shortening was confined to the I-band. Panels show representative images, each taken from a different fiber.

length, some near normal, others short. The shortened sarcomeres contain correspondingly short thick filaments. Although the sarcomeres under observation cannot be any stretch of the imagination be considered "physiological," the micrograph clearly demonstrates that thick filaments possess the capacity to shorten.

Figure 6 shows an example of thick filament shortening in frog skeletal muscle. Fibers were activated by brief glycerination at low temperature, which caused immediate local activation and shortening. Again, sarcomeres of normal length contained thick filaments of normal length, while shortened sarcomeres contained shortened thick filaments. In the example shown, thick filaments shortened from $1.6 \mu\text{m}$ to $1.1 \mu\text{m}$, and where A-band periodicity could be detected, it had diminished from 43 nm to 25-30 nm.

Another example is shown in Figure 7. Here, in honeybee flight muscle in rigor, two images taken from the same specimen are shown. In sarcomeres

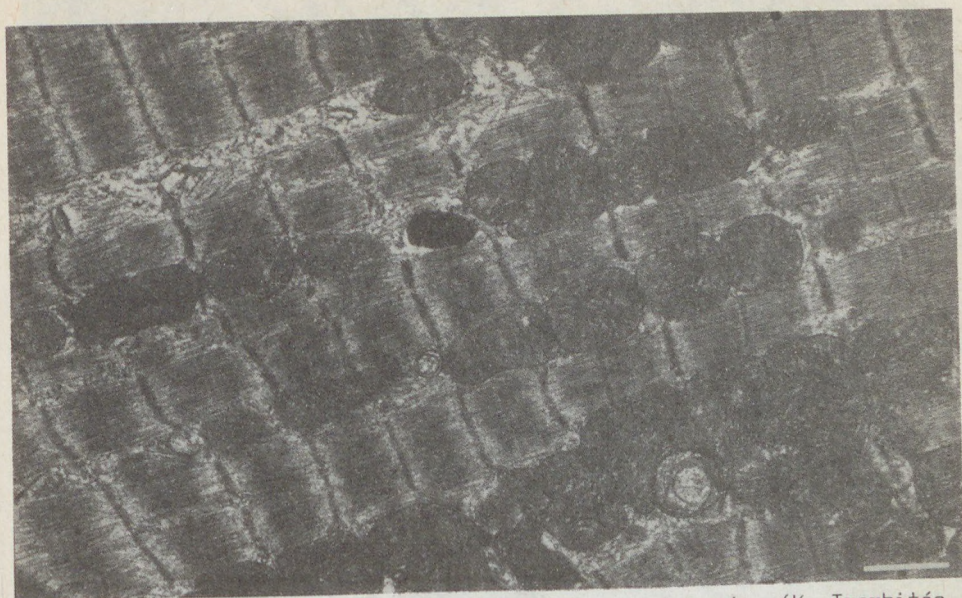


Fig. 5. Dog cardiac muscle subjected to acute hypoxia. (K. Trombitás, E. Róth, and B. Török, unpublished). Both normal and shortened sarcomeres are observed in the field. In shortened sarcomeres, thick filaments are correspondingly shorter. Scale bar - 1 μ m.

that had retained their initial length, thick filament length remained normal. In regions that had shortened, thick filaments shortened as well. In these latter filaments, cross-bridges were spaced more closely than in the unshortened filaments. Since all images came from the same section, many potential artifacts can be ruled out.

The decrease of inter-bridge spacing in the shortened filaments implies that some molecular rearrangement drives thick filament shortening. A possibility to consider is that thick filaments shorten in localized steps; that in activated muscle, stepwise shortening of the thick filament mediates stepwise shortening of the sarcomere. The mode of shortening would be segment by segment, a kind of gathering up of the thick filament. This mechanism would be fully parallel to the one in connecting filaments - the one that we surmised might apply in unstimulated muscle.

A preliminary description of this mechanism has been published (Pollack, 1984). A more detailed synthesis will appear in a forthcoming book (Pollack, in preparation), in which the evidence underlying stepwise

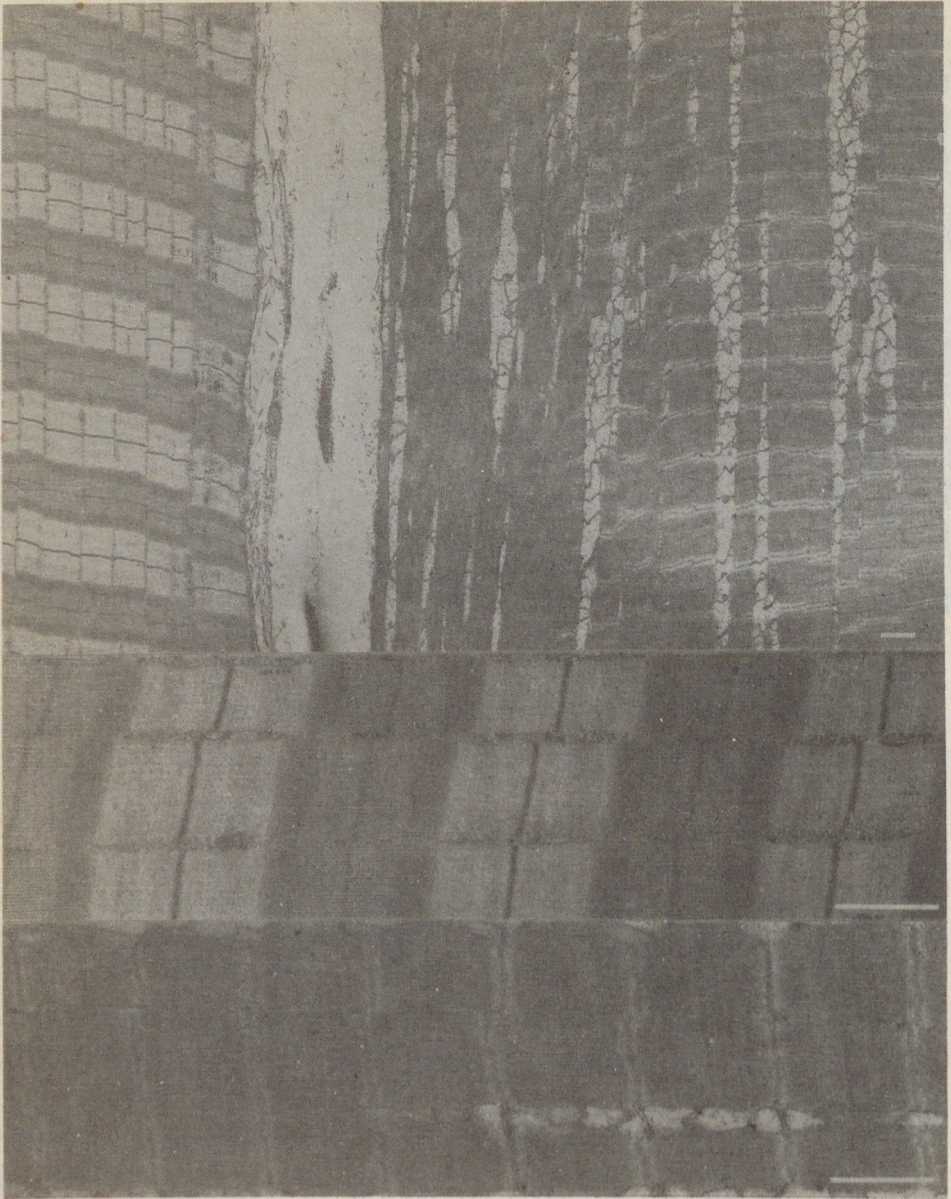


Fig. 6. Unloaded frog sartorius muscle activated by brief immersion in cold glycerol solution, then stretched. Top panel shows unshortened and shortened sarcomeres in neighboring fibers, implying that activation was localized. Higher magnification of unshortened and shortened regions (below) shows substantial thick filament shortening in the shortened sarcomeres. Magnification of two lower panels is identical. Scale bars - 1 μm .

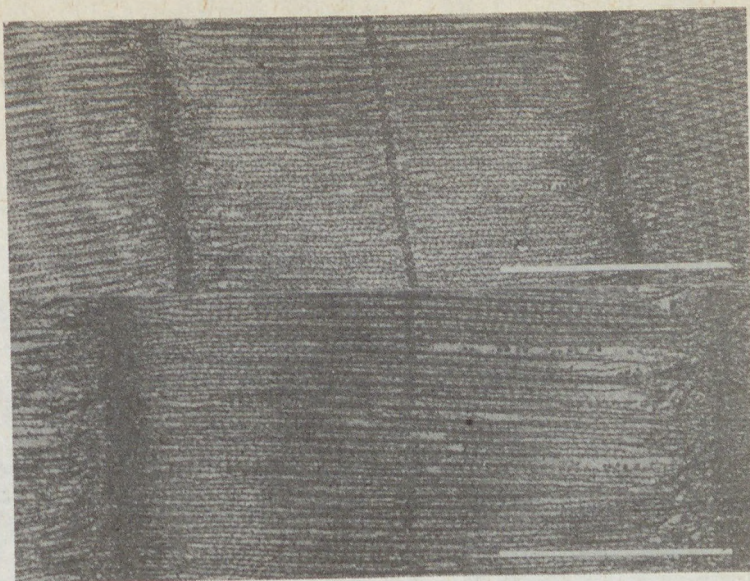


Fig. 7. Honeybee flight muscle in rigor. This specimen tore during the induction of rigor, allowing shortening in some regions. Sarcomeres of various length were found. Representative examples are shown. Sarcomeres that shortened during the induction of rigor had shorter thick filaments, as is apparent. Scale bar - 1 μ m.

shortening of thick filaments and connecting filaments is considered in depth.

One final aspect of stepwise shortening that has not been touched on so far is the issue of synchrony. How may one account for the vast synchrony implied by this phenomenon?

Synchronization of Steps and Pauses

The chief element underlying synchrony may be the transverse structural elements mentioned by Professor Ernst. Some years ago, we took note of transverse structures that appeared to interconnect adjacent thick filaments. Although M-connections had been well known, many electron micrographs showed rung-like interconnections also along flanking regions of the thick filaments, implying that filaments might be interconnected by the cross-bridges themselves (for review, cf. Pollack, 1983).

If thick filaments were really interconnected along their length, this would constitute a starting point for synchrony: One segment of a thick filament could not shorten unless corresponding segments in parallel filaments shortened as well. Segment shortening would be an all or none phenomenon throughout the myofibrillar cross-section. And given the known interconnections between myofibrils, all-or-none behavior would extend throughout the entire fiber. Thus, the basis for synchrony.

For this reason, a detailed study of cross-bridge structure has been initiated. The possibility that the interconnections were artifacts of chemical fixation were considered first. To check this possibility, we employed the freeze-fracture method, where chemical fixatives are not required. The results (Suzuki and Pollack, 1986) showed clear evidence of interconnections in relaxed, contracting, and rigor states. The interconnections seemed to be formed by two cross-bridge heads binding at their tips.

Additional studies are in progress with diverse methods. Some examples of thick-thick interconnections are shown below:

Figure 8 shows a cross-section from the non-overlap zone of relaxed frog semitendinosus muscle (one of a serial set). Interconnections between thick filaments can be seen. These interconnections are less robust and somewhat less regular than those seen in the M-region. Nevertheless, they are abundant and clear.

Figure 9 shows a freeze-fracture image prepared from honeybee flight muscle in rigor. Interconnections in the non-overlap zone can be clearly seen. No fixatives were used during preparation. Interconnections cannot be a fixation or staining artifact.

Figure 10 shows an "A-segment" prepared from rabbit psoas muscle and negatively stained. The A-segment contains thick filaments alone. In

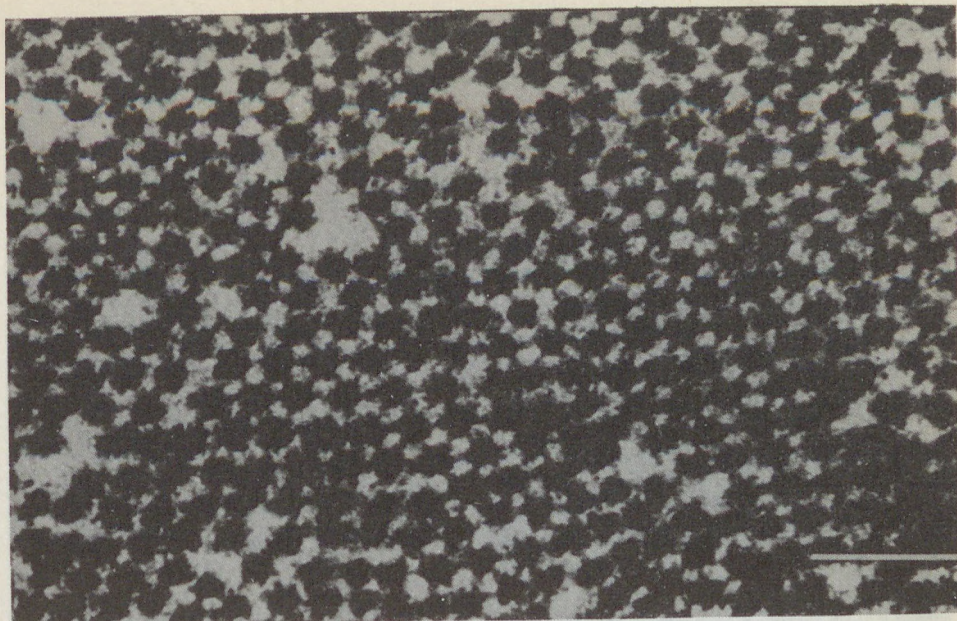


Fig. 8. Cross-section of relaxed, stretched frog semitendinosus muscle in the non-overlap zone. Abundant interconnections between thick filaments can be seen. Scale bar - 0.1 μ m.

instances such as the one in Figure 10, where the ends of the segment are frayed, structures interconnecting the filaments can be observed.

Figures 8, 9, and 10 show consistently that the thick filaments are interconnected along their length. The figures represent samples of three different methodologies; yet the results are similar. This implies that the interconnections are not likely to be preparation artifacts but genuine structures. Thus, the transverse elements implied by Professor Ernst's studies are confirmed.

These transverse elements probably underlie synchrony. Because the thick filaments are interconnected by rung-like interconnections along their entire length, shortening of myofilaments must be synchronized over the full myofibrillar cross-section; one filament cannot shorten unless all parallel filaments do so at the same time. Obviously, there is more to the issue of synchrony, and this is treated in detail in the forthcoming book mentioned above.

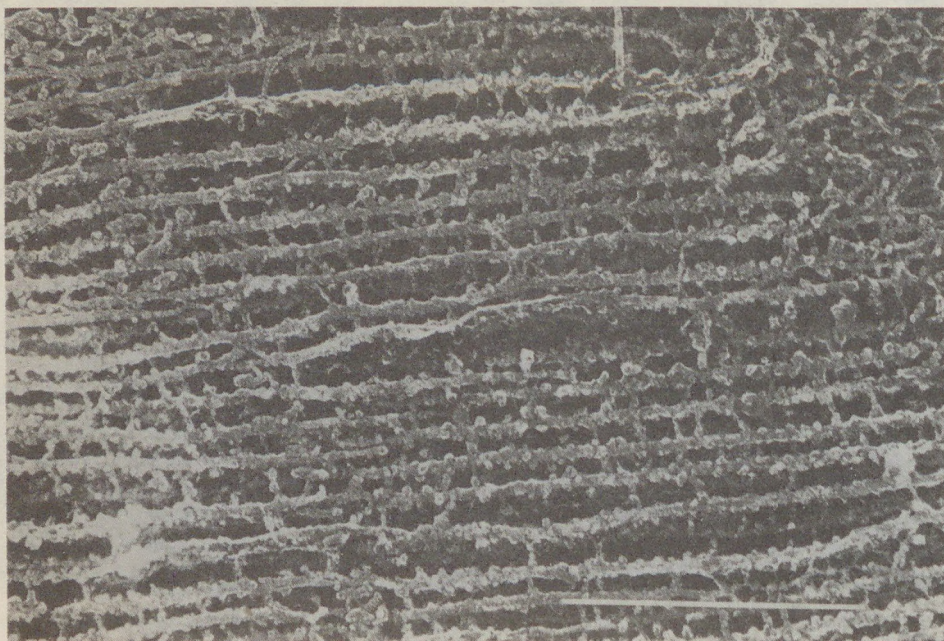


Fig. 9. Honeybee flight muscle, prestretched to withdraw thin filaments from the A-band. The specimen was placed in rigor, released, and prepared by the freeze-fracture method. The A-band lattice is shown. Note that adjacent thick filaments are interconnected by bridge-like structures. Scale bar - $0.5\mu\text{m}$.

Conclusion

Professor Ernst was a visionary. Despite popular views to the contrary, he remained convinced that sarcomere shortening involved thick filament shortening. His studies also convinced him of the existence and importance of structural elements within the sarcomere - both longitudinal and transverse elements.

If the conclusions drawn here are valid, Professor Ernst was correct in each of his assertions. The phenomenon of stepwise shortening requires all three of these elements for its interpretation. The steps appear to be generated by thick filament shortening in the activated myofibril and by connecting filament shortening in the unactivated myofibril. And the

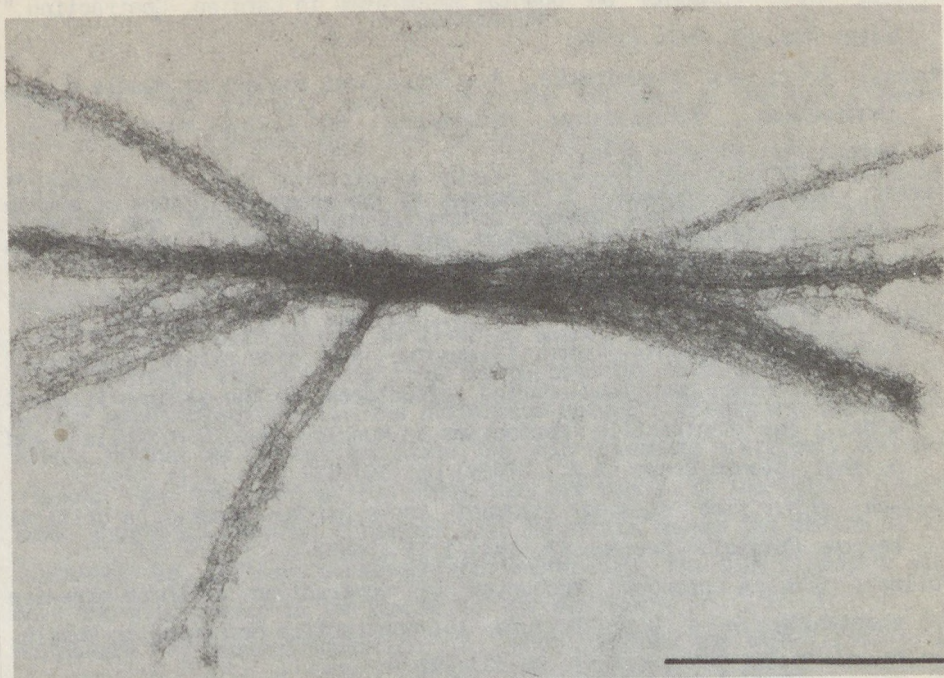


Fig. 10. A-segment prepared from glycerinated rabbit psoas muscle. Interconnections between adjacent thick filaments can be seen. Scale bar - 0.5 μ m.

transverse elements, which now appear to reside in the cross-bridges, seem to underlie synchrony.

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DISCUSSION

Hazlewood: How do you account for the movement of the thin filament through all of the structure that you have shown?

Pollack: When you activate a fiber, obviously there is an interaction between the thin and thick filaments. Through the action of calcium with troponin, the thin filament is allowed to bind to the cross-bridges. Many people think that the cross-bridges bind to the thin filaments, but I think that the cross-bridges may be fixed in space, and it's the thin filament that, through its mobility, has the opportunity to bind to sites on the myosin interconnections. So the difference between relaxed and contracting states is that in the relaxed state the thin filaments are freely movable, unbound; in the contracting state they are bound. If the thick filament then shortens, the thin filaments will penetrate toward the centre of the sarcomere. So, for each sarcomere shortening step, the thin filaments will move one step closer to the center of the sarcomere.

Hazlewood: My second question is, what suspends the thin filaments in their ordered array when they are stretched beyond the A-band?

Pollack: Interconnections. I haven't shown it, but we have evidence that the same sort of interconnections that exist in the A-band exist also in the I-band.

They can be seen on many old electron micrographs, and on newer ones. In carefully prepared micrographs, you can see them very clearly. If you look at the structure from a cross-sectional view, there is a kind of "cage" surrounding each thin filament, a cage that interconnects the connecting filaments. The thin filament rides through it. I think that is what keeps the thin filament, in its relatively ordered array. But it's by no means perfectly ordered.

Hazlewood: I'm reminded of extraocular muscle studies back in 1963, or so. Of course these were conducted in an intact organism, and of course you have the neural components included, but the eye seems to move in saccadic movement with a 250 ms time-dely. During the 250 ms, everything's cut off. After this time period the eye begins to track the object again by another saccadic movement. I noticed that your saccadic-like jumps are an order of magnitude less than that time. Do you see any relation between these?

Pollack? I'm not sure if the phenomenon you are talking about has a neural origin or muscular origin. Certainly, it may be related to the steps. I'm

reminded of a study conducted in the late 1940's by Gordon and Holburn. They put a sound transducer on the eyelid muscles and noted discrete sounds - similar to the sounds that we have observed in isolated sartorius muscles. They seem to correspond with the saccadic kinds of movements. As for the 250 msec interval, we need to bear in mind that the duration of the pauses, and therefore the separation between the steps, is a strong function of the load. If the load is small then the duration is short. If the load is large, the pauses last longer. Apparently, some sort of energy has to be built up before the next step. So, in the case of the eye, conceivably the load is large, the pauses last longer, and therefore the frequency is a little bit lower.

Tigy: You demonstrated a lot of convincing experimental data, and I'm sure that Professor Ernst would have been very happy listening to these. And I'm sure that the two Huxley's are very sorry because of these experiments. I want to make two comments. One: we started to study laser diffraction experiments but, according to our opinion, the fiber is too big, because the fiber has ten thousand fibrils. It would be convincing to have such laser diffraction studies with myofibrils.

The second: I don't remember whether the experiments of Sten-Knudsen, the Danish fellow who had a lot of nice laser diffraction studies, showed such a step-wise action.

Pollack: Sten-Knudsen's work mostly related to latency relaxation. He measured very small changes of sarcomere length that took place early in contraction. So perhaps in his experiments he didn't have enough shortening to permit the observation. But some half dozen other laboratories have observed it. It's a common observation: the question has been whether it's real, not so much whether it is observable.

Tigy: My third comment is that Huxley's method with the hair is not original. Because it originates from Bethe the old German. I can show it to you in my lab.

Pollack: That's interesting. To respond about the myofibrils, have a myofibril preparation setup, and we are considering such an experiment. The problem is that the amount of diffracting mass is very small, and so it is difficult to get a noise-free diffraction pattern from it. But with regard to the myofibril, the Hungarian, Garamvölgyi, took two microelectrodes, passed a current through a single myofibril, and noted that the myofibril, itself, shortened. This was without any membrane. This

result is consistent with the idea that contraction is due to a helix-coil transition. As Tanaka from MIT, has shown in polyacrylamide gels, which also undergoes such a transition, the transition can be precipitated by the passage of current. So Garamvölgyi's experiment is interesting and relevant.

Keszthelyi: I have a very trivial explanation of this quantal behaviour. And I would like to hear your refusal. It is simply that there are biochemical processes underlying muscle contraction. And we know from other experiments that chemical reactions can be oscillating in time. Such oscillations have been shown in cells, for example in the case of glycolysis. What is your comment?

Pollack: I think it is close to the truth. I haven't talked about chemistry; I've talked about structure. Obviously, underlying these structural changes must be some chemical changes, and they may well be oscillatory. For example, one way to synchronize the steps and pauses is if you have a step occurring that triggers the next step, which then triggers the next step, etc. That is a kind of "oscillatory" process, though the oscillation doesn't always occur in the same - it jumps from molecule to molecule. So I don't think your proposal is trivial; it may be an interesting way of looking at the process.

Keszthelyi: It is trivial because in this case it keeps the former theory, the sliding filament hypothesis.

Pollack: I don't think this evidence favors the sliding hypothesis.

Keszthelyi: Because the sliding mechanism is driven by an oscillatory reaction.

Pollack: If the thick filaments remain constant in length, your idea, I think, could be entertained as possible. I think the evidence favours the fact that the thick filaments shorten, even though the textbooks say otherwise.

Clegg: You've got to tell us, at least briefly what Huxley says about this.

Pollack: A.F. Huxley (Circulation Research 59:9-14, 1986) says that there are problems with each of the methods. His response can be divided into three points: First, all the methods are probably fraught with artifact; second, if they are not artifact, then they are probably not important - not important with regard to contraction. Third, if the observations are both real and important, he mentions that he proposed a mechanism to explain them ten years before we have observed the phenomenon.

INSIGHT INTO THE ORGANIZATION OF WATER IN LIVING CELLS

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INTRODUCTION

Water constitutes 60-90% of the weight of most biological tissues; however, its physical state within the cell has not yet been quantitatively characterized. Furthermore, any theory that is developed to explain the unequal distribution of ions between the inside and the outside of cells, the dynamic aspects of solute and water movement into and out of cells, and the bioelectric phenomena of cells requires fundamental assumptions concerning the physical state of cellular water. Thus, our ultimate understanding of cellular function will require detailed knowledge of the physical state of water.

Perhaps the most frequent assumptions made by biologists are: (1) that the aqueous cytoplasm of a living cell may be equated to a dilute solution and (2) that each cell is surrounded by a semipermeable membrane which separates the internal and external solutions (1-5). The popular description of bioelectric phenomena of cells comes from equations evolving from dilute solution theory. Osmotic theory and the ideas associated with cell volume regulation are entwined also in the theory of dilute solutions (4-6). This view can be better appreciated when one reviews its historical evolution.

By the end of the 19th century it was well known that inorganic matter (ions and water) played an essential role in cellular function. In fact, the normal living state could be characterized by its inorganic composition. In addition, cells were known to have an electrical potential across their surface and that this potential was related to the ionic composition inside and outside the cells. By utilizing the theory of dilute solutions, which had been developed by Van't Hoff and Arrhenius (1), and adding the assumption that the cell was surrounded by

a semipermeable membrane, a unified theory of the living cell emerged which could explain the selective accumulation of ions in cells and the cellular potential (2,3). Other investigators, utilizing the fundamental premises of dilute solution theory and assuming the presence of a semipermeable membrane, derived equations that described volume regulation of cells (4,5). Although these theories have been modified somewhat through the years, the more popular views which are being used today to describe cellular potentials, selective solute accumulation, and cell volume are essentially extensions of these earlier views. The assumption that most of the water and solutes within cells behave as dilute solutions remains fundamental to these conceptualizations.^x

A scientific formalization differing considerably from conventional wisdom was presented in 1960. This new proposal offered explanations for cellular potentials, and the selective accumulation of potassium over sodium (7). This theoretical development evolved to what is now known as the association-induction hypothesis (AIH) (7-9). In general, this hypothesis proposes that potassium (in preference to sodium) is selectively adsorbed to β and γ carboxyl groups of the amino acids contained in cellular proteins (10). In 1965, an explicit role for water in cellular function was formulated and introduced into the general equations of the AIH (11). In the context of this hypothesis, cellular water is considered to exist in a physical-chemical state different from that of ordinary water or water in dilute electrolyte solutions. Thus, it is proposed that the solvent properties are changed such that solutes (e.g. sodium) are excluded to some extent from the cell. A general prediction of this hypothesis is that the physical properties of cellular water can be altered by changes in the physiological state of an organism or tissue (12,13). It should also be noted that several other theories have been proposed which argue for selective adsorption

^xIt should be noted that Professor Ernst provided, throughout his early career, considerable scientific evidence which was inconsistent with the fundamental postulations of this view (See Chapter by Professor Tigyí). Nevertheless, the popularity of using dilute or ideal solution theory as the foundation for cellular theory continued in spite of his efforts. The lack of attention to his work served as one stimulus which led to his publication Biophysics of the Striated Muscle (Akadémiai Kiadó, Budapest) in 1963. It was through this publication that I first learned of Professor Ernst's work.

of potassium and a role for cellular water other than as a passive medium in which solutes were dissolved (14-18)^x.

In general, the AIH proposes that the equilibrium distribution of any solute within a cell is comprised of a minimum of two fractions; one fraction dissolved in the cytoplasmic water and another fraction adsorbed to cellular macromolecules (primarily proteins). Simply stated this means

$$|S|_{in} = \alpha |S|_{cw} + |S|_{ad} \quad (1)$$

where $|S|_{in}$ is the internal concentration of solute S (which could be Na^+ , K^+ , Mg^+ , non-charged solutes, etc.), α is the concentration of cellular water, $|S|_{cw}$ is the concentration of solute in the cytoplasmic water, and $|S|_{ad}$ is the concentration of the solute adsorbed to intracellular macromolecules. $|S|_{cw}$ will depend on the physical state of the intracellular water and may be described by the following relationship:

$$q_s = \frac{|S|_{cw}}{|S|_{ex}} \quad (2)$$

where q_s is the equilibrium distribution coefficient for solute S, and $|S|_{ex}$ is the extracellular concentration of solute S. The value for q_s can be equal to or less than one. If it is less than one, it means that the solute is partially excluded from the cytoplasmic water. That is, because of the physical state of the water, the solute cannot be dissolved equally in the cytoplasmic water as compared to the extracellular water.

Combining equations (1) and (2) we have

$$|S|_{in} = \alpha q_s |S|_{ex} + |S|_{ad} \quad (3)$$

^xDuring my second or third meeting with Professor Ernst, he called to my attention that I had, in past publications, referred to Ernst's theory. He said that this was incorrect because "Ernst has no theory - only facts". The very important "facts of Ernst" on this subject may be found in the Chapter of Professor Tigyí, and in Ernst's book Biophysics of the Striated Muscle.

Again, changes in q_s represent changes in the solvent properties of the cellular water which will result in changes in the concentration of solute S in the aqueous phase of the cell. Likewise, changes in the physical properties of cellular macromolecules could change the value of $|S|_{ad}$ (9,19). In theory, q_s and $|S|_{ad}$ may change independently.

These divergent views of cellular function arise fundamentally from different assumptions concerning the physical state of water and ions, and a resolution of these fundamental differences is needed.

This report focuses on the physical properties of water evolving from nuclear magnetic resonance (NMR) spectroscopy and from quasielastic neutron scattering studies of water protons. The diffusive motion of water in biological systems is particularly emphasized as a physical property that relates fundamentally to the divergent views of the cell reported above.

NUCLEAR MAGNETIC RESONANCE

Models for NMR parameters in biological systems.

The relaxation times of water protons in biological systems are substantially shorter than those for bulk water or water in dilute electrolyte solutions (20-25). Two general types of interpretations have been proposed to explain the reduction of the relaxation times: (1) "bound-free" models (26-29), and (2) the "ordered" water concepts (12, 30-33). These different interpretations have been in the literature in various forms for over 15 years and have not yet been satisfactorily resolved.

Within the context of the "bound-free" models, equations have been developed which allow some explicit testing of the models. In this general set of models the relaxation times are space averages over a wide range of proton environments. Therefore, the presence of a small, rapidly relaxing fraction of protons in rapid exchange (even with another much larger "free" fraction) will demonstrate the average relaxation time observed. This can be readily seen in the proportionality for the relaxation rate $T_1^{-1} \propto \sum f_i \tau_{ci}^{-1}$, where f_i is the fractional population of the water protons with a correlation time, τ_{ci} . That is, long correlation times will dominate. The diffusion coefficient, on the other hand, is dominated by the fractions of water containing free water. For

example, $D \propto \sum_i \tau_{ci}^{-1}$ and, therefore, dominated by short τ_{ci} . If the majority of the water is free (i.e., equivalent in its motional freedom to bulk water), then the diffusion coefficient for water in cells should be unchanged, or any observed reduction in its values should be explainable by compartmentalization or by obstruction effects.

In the case of the "ordered" water models, the polarized multilayer concept is the most specific but, at present, no formalized equations have been developed to describe the relaxation times. The polarized multilayer hypothesis, however, evolves from the fundamental assumptions that both the translational and rotational motion of water molecules are reduced. Therefore, in this view, the D of a substantial fraction of the water should be reduced, and the reduction must be greater than can be accounted for by compartmentalization or obstruction effects. This distinction concerning the diffusion coefficient between the two general interpretations provides an opportunity to experimentally evaluate the models.

Diffusion coefficient

The diffusion coefficient (D) for water in biological systems is moderately reduced when compared to bulk water, whereas the relaxation times are reduced by an order of magnitude or more. The reduction in D (in many biological systems) is by a factor of the order of 2-6. Explanations proposed to account for this reduction generally fall into one of the following categories: (1) membrane compartmentalization of cellular water; (2) macromolecular structures within cellular compartments serving as local obstructions to diffusion; and (3) the induction of intrinsic changes in the water-water interactions in a substantial fraction of the intracellular water due to water-macromolecule interactions. Clearly distinguishing between these alternatives is difficult. Much of the difficulty arises from the heterogeneous character of the cell as well as to the fact that a single NMR measurement is carried out over a period of time, t , of the order of a few milliseconds or more. During this measuring time, the water molecules are diffusing within the cell, and their NMR parameters are determined by averages over distances $\sqrt{2Dt} \approx 1$ micron, where D is the diffusion coefficient of bulk water and t is time over which the measurements are made. Interpretation of the results, therefore,

requires detailed modeling of the heterogeneous environment of the cell.

If the large protein structures, including membranes, within cells do form impermeable compartment, D , measured by NMR techniques, would not only be reduced, but also would be a strong function of the measuring time (34). This possibility has been investigated and the results of the study suggested that it is unlikely that compartmentalization is the cause (35). More recently measurements of D by way of pulsed field gradient techniques in which the measuring times can be varied have been used to evaluate this problem and it was found that cellular macromolecules serve as obstructions and can account for some, but not all, of the reduction of D in living cells (36).

The macroscopic scale of the measurement of D by NMR methods allows for the development of models based on the hydrodynamic interaction of water molecules with macromolecules. One of the earliest applications of the hydrodynamic method in biology was that of Wang (37). In this model the reduction of D for water in a dilute protein solution is due to interaction with the proteins (or macromolecules in general), which are impermeable for diffusion. This interaction is termed the obstruction effect and may be separated into two contributions: (1) the cross-section for diffusion is reduced due to the volume occupied by obstructions; and, (2), the path length for diffusion is lengthened.

An alternative calculation termed the cellular method has been developed for two geometries likely to be found in various cells: (1) an ordered array of cylinders; and, (2) cubic array of spheres. The development of the model, and its use in experimental analysis may be found elsewhere (38-41). In summary, the cellular method provides approximate solutions which are relatively simple and which can be experimentally tested. The following equations present the approximate solutions for an hexagonal array of cylinders (equation 4) and a cubic array of spheres (equation 5)

$$\frac{D'}{D} = \frac{1}{1 + 0.8 \phi} \quad (4)$$

$$\frac{D'}{D} = \frac{1}{1 + 0.63 \phi} \quad (5)$$

where D is the diffusion coefficient of bulk water, D' is the diffusion

coefficient of water in the biological system, and Φ is the volume fraction occupied by the obstructions (i.e., cylinders or spheres).

The solutions to this hydrodynamic model provide a means to test for obstructions in cellular systems. Equations (4) and (5) have been applied to skeletal muscle (39) to evaluate D as function of fiber orientation relative to the magnetic field (i.e., radial and longitudinal diffusion), to cysts of the brine shrimp to evaluate D as a function of hydration (40), and to barnacle muscle and macromolecular solutions to evaluate the overall effects of obstructions as a cause of the reduction of D (41). In these studies as well as those of Tanner (36), it is concluded that macromolecular obstructions cannot fully account for the reduction of the diffusion coefficient in the biological systems studied. Therefore, these model-dependent analyses have led to the conclusion that the translational diffusion coefficient of most of the cellular water is reduced (20,41).

QUASIELASTIC NEUTRON SCATTERING (QNS)

The QNS method provides information on the properties of scattering atoms which depend directly on the microscopic environment of the atoms. The correlation function for the atomic motion is evaluated over a space domain of a few angströms and during a time interval of approximately 10^{-12} second. In the QNS method, the positional correlation of the atoms determines the scattering whereas, in the case of NMR measurements, the relevant correlation function is that of the orientation of the protonic moments.

The method and results of our QNS studies are given in more detail elsewhere (42). This study was undertaken in an attempt to determine the diffusive motion of water protons in a space domain very short compared to intermacromolecular distances within biological systems (i.e., its measurement of D made before the water molecules have moved more than a few angströms). The study has thus far been restricted to the cysts of the brine shrimp (Artemia) because of the long time required to acquire the data needed for analysis (i.e., approximately 5 days). The Artemia cysts are well suited for this study, since the viability can be easily determined (43). The viability was $90 \pm 2\%$ in our studies and was unchanged by neutron irradiation.

In Table 1 the data from the QNS study on Artemia are compared to the

data obtained on pure water.

A summary of the results of this study is given in Table 1.

Table 1. Parameters for diffusion required to fit scattering law to QNS data

Sample	D/D_0	τ/τ_0	D_r (10^9sec^{-1})
Pure water	1	1	x
Agarose gel (4g $\text{H}_2\text{O/g}$)	0.8	1.4	-
Artemia Cysts (1.2 g $\text{H}_2\text{O/g}$)	0.29	3.9	6

- Notes: (1) x The rotational diffusion coefficient (D_r) for bulk water is $8.1 \times 10^{10} \text{sec}^{-1}$ as determined by dielectric relaxation
- (2) The diffusion coefficient of water (D_0) is $2.5 \times 10^{-5} \text{cm}^2/\text{sec}$.
- (3) The correlation time for water (τ_0) is $1.2 \times 10^{-12} \text{sec}$.

The conclusion from this study is that water in the Artemia cysts has strongly reduced translational and rotational diffusion coefficients that are not due to obstructions, compartments or exchange with minor phases but are instead an intrinsic feature of the cellular water. Hence, these findings are inconsistent with the assumptions that the cytoplasmic water may be considered equivalent to that of bulk water or water in dilute aqueous solutions.

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DISCUSSION

Hideg: You mentioned only measurements on tumor cells and dividing cells. Can you list data on other functional states of cells, like muscle contraction? What can you see with your technique? And what can you see in pathological conditions, like inflammation?

Hazlewood: Inflammation usually results in a change in the relaxation times, depending on the length and severeness of it. The presence of a tumor in the body of an animal can affect organs, that are spacially displaced from the tumor site in the animal. Also the serum seems to be effected by the presence of certain tumors. We call this the "systemic effect" of tumors. If you isolate cells and then begin to have drug interactions, with those cells you can observe changes in the relaxation times.

Hideg: And what's the case for muscle contraction.

Hazlewood: I don't know yet. There is one report from 1965, which shows the relaxation times to be increasing with contraction. I have made one study with chemical contracture with caffeine, and what happens is that muscle loses a lot of water. So we know water is heavily involved in the contractile process. And my general view is that for contraction to occur a change in the water structure is required.

Clegg: You said, Carlton, that the reduction in the diffusion coefficient which you have observed is consistent with polarized multilayers. I would like to ask what does the polarized multilayer model actually predict about the diffusion coefficient. I would assume that it would be a lot slower.

Hazlewood: I took only the fundamental assumption from which the polarized multilayer theory is built. Implicit in equation (2) is the assumption that the rotational and translational motion is reduced. And that's all that I tested. Equation (2) can be related to free energy which may, in turn, be related to entropy (see references 9, 11, 19 and 33).

Clegg: You know when I think of these highly polarized layers of water, I sort of intuitively imagine that water molecules would experience much difficulty in diffusing along such a structure.

Hazlewood: The problem with drawing a picture of polarized multilayers is that it makes it look like it's fixed in concrete. The polarized multilayers constitute a dynamic structure which will be a function of of

polymer type, hydration, temperature etc.

Ling: I wish to respond to Jim's question which is a very good one. Intuitively one often makes the wrong assumption. Here we are talking about a dynamic structure. Suppose you can take photographs of the assembly of water molecules. Let's take ice. You take one photograph, and you know all the water molecules to be specified at that moment. This is the extreme case. You then take liquid water, and no matter how many pictures you take, it's still random. Now, I'm saying the following: if you take one picture of the polarized water, you will see no structure; however if you keep on taking more and more pictures, then you begin to see more and more structure. This is the foundation this is the structure. You see, what is needed is an element that we cannot present on our slide, the time element. And if you want a still better picture, consider the bottom of the ocean where the giant algae swing slowly with the current. Again, at any one picture taken, it's almost random. You cannot predict from the position of one plant where the tip or the roots are going to be. But if you take an infinite number of pictures, then you begin to see their dynamic structure.

Now, what kind of parameters are we looking for? One of the most outstanding features of this problem leads to the question: how does the water of a given system handle solutes? This is the central issue between the membrane pump view and the association-induction hypothesis. The former view argues from dilute solution theory (i.e. normal water) whereas the latter argues for restricted motional freedom. The latter argument continues: if water suffers such dynamic motional restriction, then it will restrict the motion of large molecules and ions like sodium. Much as a butterfly that is free to fly in free space, becomes no longer so, if caught in a spider's web. But the butterfly is not fixed like a crystal. It can still fly, only much more restricted. And that's all you need, to capture the butterfly. And in the living cell, that's all you need to reduce the sodium concentration to approximately what we see in the cell. A relatively small reduction (of the order of 2-10 times) in the motional freedom of water is adequate to bring about the exclusion of sodium.

Tigy: I would like to have three comments. First, about contraction. According to my knowledge, no satisfactory experiments were carried out contraction. But contracture, the chemical contraction is different. There are significant changes. I think we have to improve the methodology.

My second comment concerns the importance of the NMR relaxation times. Consider, for example, our experiments about cataract formation. There is significant difference between the normal lens and the lens with cataract. It is very important for the practice of medicine. My third comment is that I feel that in the beginning of the NMR imaging technique, the theory was in advance of the practice. Now, according to my impression, the NMR imaging technique can offer you so much unexplained data. You can get many kinds of contrast in an NMR image, and it is very difficult to explain what is the real reason for it, so the technology is now more advanced than our theory.

Clegg: I don't think Dr. Ling answered my question which was: does the polarized multilayer model predict this kind of reduction in diffusion coefficient?

Ling: The polarized multilayer theory says that the motional restriction of water restricts the motional freedom of solutes and, hence, reduces their solvency. Now the exact formulation requires an exact knowledge of liquid water. We don't have that. One can still, however, design a theory that is semiquantitative, and therefore does not give an exact number, but it gives you the order of magnitude which my previous answer attempted to give. It is not crystalline nor is it a liquid, so it's somewhere in between. The difference in correlation times between liquid water and crystal ice is six orders of magnitude apart. And what we are expecting is close to one order of magnitude, which was the case in Hazlewoods' observation. So there is general agreement, but as long as we do not have an exact solution to liquid water, there is no way to unite an exact equation of the modification of liquid water.

THE WATER IN SWOLLEN SYSTEMS, AS SEEN BY DIELECTRIC SPECTROSCOPY

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A number of chemicals may be called 'biological substances' independently of their synthetic or natural origin (e.g. insulin, proteins, DNA, vitamins, and many others).

It is questionable what sort of physical systems can be considered biological systems, but many of the biological systems are swollen systems. (J.R. Katz, 1924; Ernst et al. 1951; Ernst, 1974).

I think that it is impossible to systematize the various swollen systems the time being, but I should like to underline a point that was considered very important both by J.R. Katz and the Nobel Prize laureate H.S. Staudinger: structural periodicity (crystallization) is an important feature of these systems (Katz, 1924; Staudinger, 1947).

Appearance of highly symmetric patterns in nature refers to a crystalline structure (Fig. 1., snow crystals). It is interesting to see that projection of human intelligence results in a similar appearance of highly symmetric patterns, too (Fig. 2. Hungarian folk embroidery). It is worthwhile to speculate if there is a crystalline structure behind this phenomenon.

As model substances we used solutions of mono-, oligo-, and poly(ethyleneglycol)s, which have periodically repeated groups, $(O-CH_2-CH_2-)_n$ with $n = 1$ to 450 (Masszi, et al., 1976; 1986). These polymers are called according to the Gilbert N. Ling terminology (negative-neutral) NO-NO type of polymers (Ling, 1984).

Fig. 3 shows the ϵ_0 "static dielectric constants" of the solutions, which were extrapolated from the microwave data to zero frequency. (Fig. 3). As it can be seen, the poly(ethyleneglycol)s have dielectric decrement if the volume rate of solute increases. But as it can be seen, too, these decrements are greater if n is greater, and the dielectric

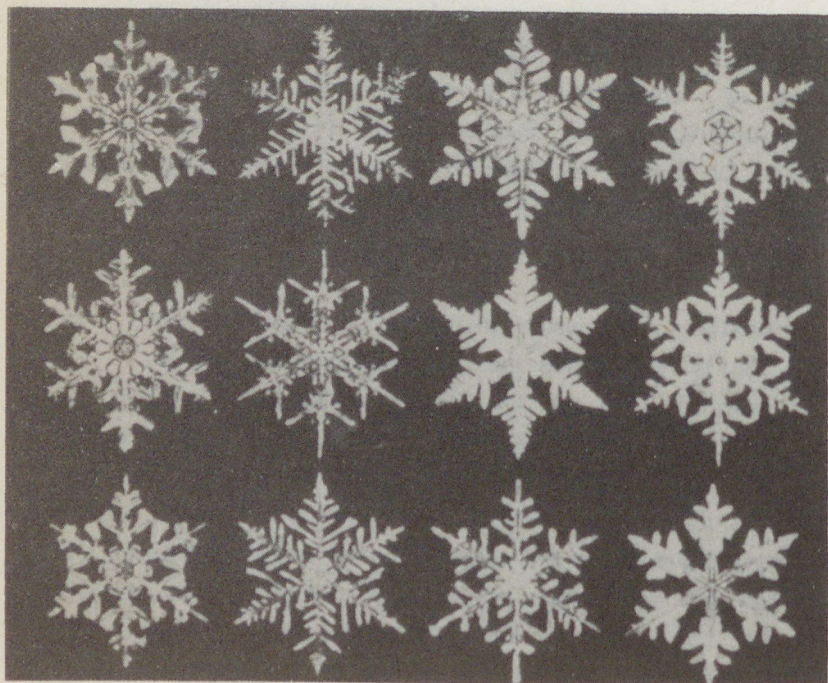


Fig. 1. Snow crystals.

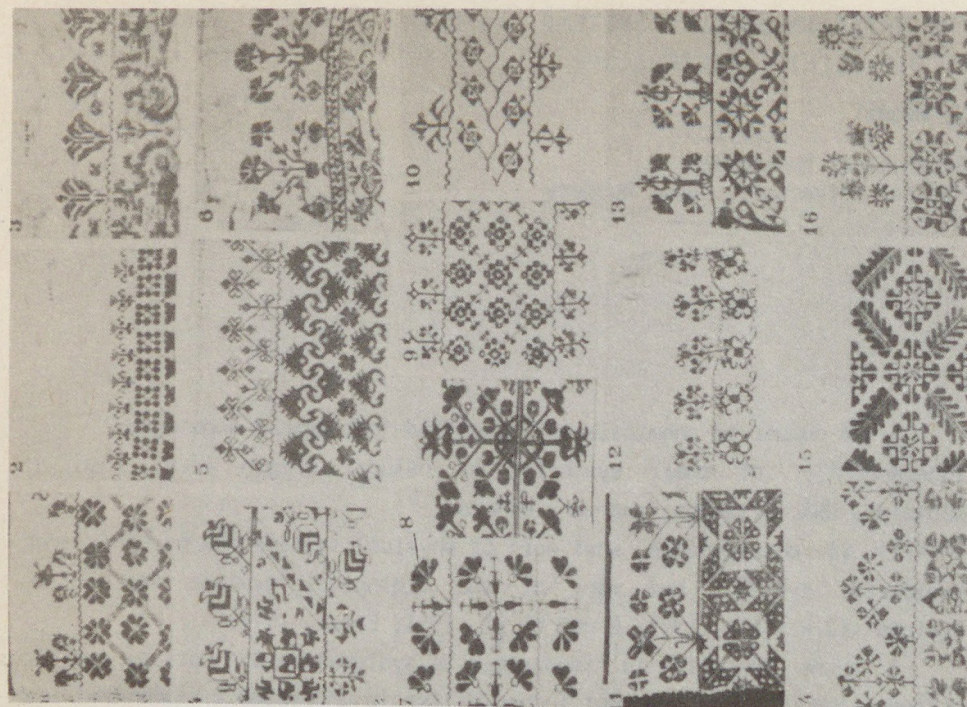


Fig. 2. Some projections of human intelligence.
(Hungarian folk needle-work).

decrements remain constant if n is greater than 22 and it is equal to the dielectric decrement of the dioxan solution.

This can be seen in Fig. 4., when the dielectric decrements of the solutions are plotted against $\ln(n)$.

From Figs. 3 and 4 it can be concluded that if n is larger than 22 the (polyethyleneglycol)s cannot rotate in the microwave field and their effect on the structure of water are similar to that of the dioxan solution.

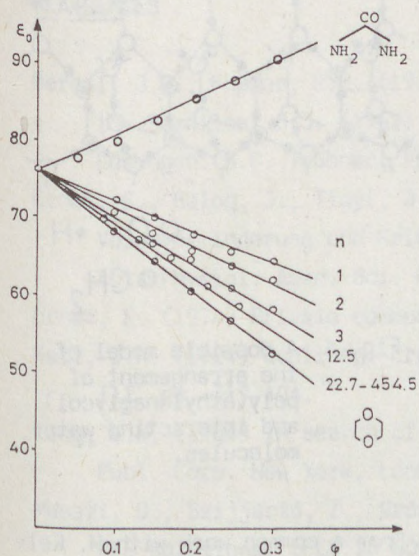


Fig. 3. The dependence of ϵ_0 on volume fraction ϕ in mono-, oligo-, poly- (ethyleneglycol) and urea solutions.

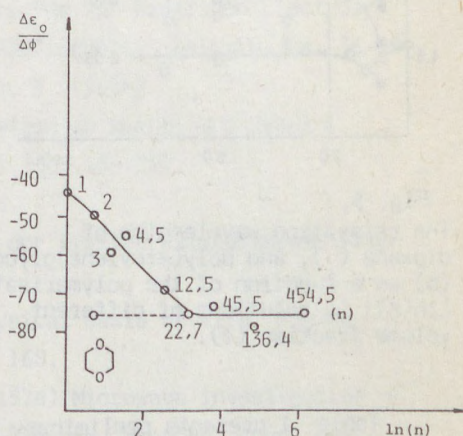


Fig. 4. The slope on the straight lines in Fig. 3 in dioxane and poly- (ethyleneglycol) solutions.

Fig. 5 shows the relaxation wavelength of the poly(ethyleneglycol) solutions.

As it can be seen, the relaxation wavelength is in contrast with the data of the dielectric decrement if n is so great that the poly(ethyleneglycol) cannot rotate in the microwave field; the resultant relaxation wavelength will not approach the relaxation wavelength of water or dioxan solution but it has a constant and greater length.

We think that the short lifetime crystal-structure of water around the periodically arranged hydrophilic groups becomes harder as Fig. 6 shows it according to the data of Staudinger and Bernal and Fowler. (Bernal and Fowler, 1933; Staudinger, 1947).

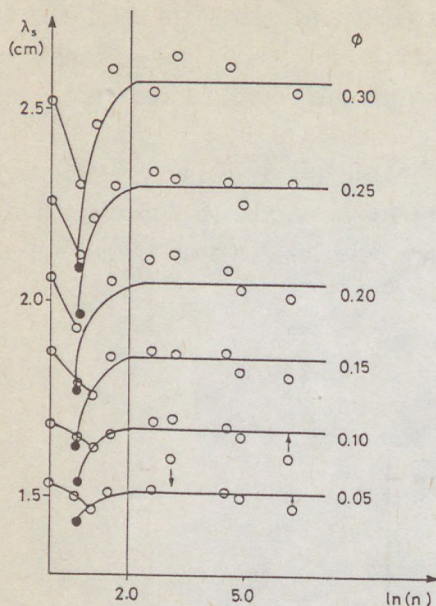


Fig. 5.

The relaxation wavelengths of dioxane (·), and poly(ethylene)glycol(s) (o) as a function of the polymerisation $|\ln(n)|$ in solutions of different volume fraction (ϕ).

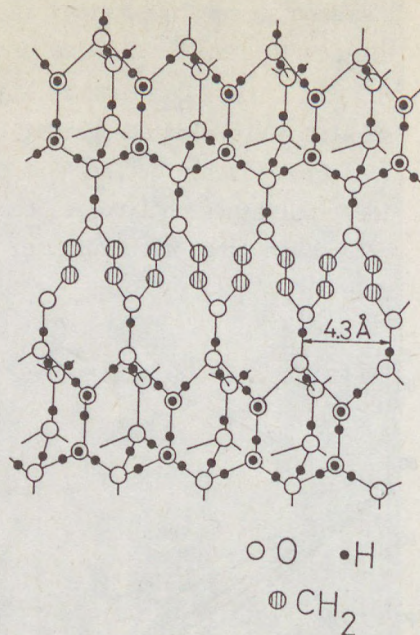


Fig. 6. A possible model of the arrangement of poly(ethyleneglycol) and interacting water molecules.

Table 1 presents preliminary results from a common work with M. Kellermayer and A. Ludány.

Table 1

Dielectric parameters of normal and treated human sera. (ϵ_0 static dielectric constant, extrapolated from the microwave data to zero frequency, λ_s relaxation wavelength of water). $T = 30^\circ\text{C}$. Preliminary result.

Substance	Dry weight %	ϵ_0	λ_s (cm)
Normal serum	10.4	68.0	1.47
Filtered serum (Filtrate)	0.88	74.1	1.38
Rest serum (Concentrate)	14.7	63.9	1.31
Water + 0.1 M KCl	-	75.4	1.36

The data shows that in normal sera the relaxation wavelength of the water is the longest. (I note that long relaxation wavelength of water is characteristic for living cells.) If we filtered the serum at 50,000 m.w. cut off value, the relaxation wavelength of water changed. The relaxation wavelength of water was shorter in case of filtrate and concentrate as well than it was observed for normal serum. We think that in normal serum the proteins have a certain "structuring effect" on water molecules.

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DISCUSSION

Clegg: What is rest sera?

Masszi: The sera were filtered, and rest sera were the ones that remained over the filter. They contained proteins over 50.000 D size. The interesting thing is that in spite of the large dry material content, the relaxational wavelength is shorter, certainly it is not greater than that of normal serum.

Ling: I will say only a very short comment. I enjoyed very much and I'am very glad to see other scientists across the world with similar interest. That's all.

THE ORGANIZATION OF AQUEOUS COMPARTMENTS IN CULTURED ANIMAL CELLS

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INTRODUCTION

Reductionism has been the hallmark of research on the structure and function of cells for decades: homogenize, isolate the structures of interest, and describe what they are and how they work in vitro. That approach has been exceptionally successful, and much of what we know about cell function has been obtained that way. However, I believe many cell biologists are nagged by the uncertainty that we may not be able to reconstruct the cell from a study of its isolated parts. Although any cell part could be chosen, let us take mitochondria as an example, the object of intense study in hundreds, may be thousands of laboratories. These efforts have led to an appreciation of the basic processes that occur in this important organelle. Had we been denied the delights of cell fractionation we would still be in the dark ages of understanding mitochondrial function. But given this success, what is the next step? Do isolated mitochondria behave the same way when they are in cells? How do we test that possibility? Certainly, the overall participation of mitochondria in cellular activities can be describe in vitro with reasonable confidence. But what about the fine-tuning? To what extent are mitochondria influenced by other parts of the cell, including the enveloping aqueous phase? Conversely, how do mitochondria "feed-back" upon other parts, so that the whole becomes integrated in activity? Can studies in vitro provide that information?

These are not unique questions, of course, they have been posed by many others over the years, not the least of which was Professor Eugene Ernst, who was guided by the conviction that "holism" was required for a reliable description of cells. While there are some ways to evaluate how different cell parts interact by reductionist tactics - the import of

cytoplasmic proteins into mitochondria, for example - these are limited, and in my opinion are not likely to be definitive, for any intracellular structure. Be that as it may, there is one "structure" in cells whose study in vitro is apparently not possible. I refer to the "aqueous intracellular compartments" which I should next define and justify referring to them as cell "structures".

II. THE AQUEOUS COMPARTMENTS OF ANIMAL CELLS, BRIEFLY DEFINED

I will use Figure 1 to develop my point. All will recognize Figure 1A as a photomicrograph of a thin section from transmission electron microscopy (TS-TEM). It happens to be a mouse fibroblast (L-929) but that detail is unimportant to us. I believe our thinking about cells, and experimental design, have been profoundly influenced by TEM images. A point of major importance is that about 70% of the cell volume is not observable by TEM; namely, "the aqueous compartments". To simplify, we can categorize these as the nucleoplasm the interiors of membrane bounded cytoplasmic organelles, and the aqueous cytoplasm ("cytosol"). All make up the aqueous phase(s) that we know must be there, in spite of their "removal" during the preparatory phases of TS-TEM. What are these compartments actually like in the intact cell? Although contemporary cell biology continues to pay little attention, I believe that question must be answered before any biologically meaningful account of cell structure and function can be advanced. During the rest of this article I will offer some of my own guesses on the matter. Before launching forth on that effort I wish to make one more point about how I think TEM and cell fractionation have influenced current conceptions of intracellular organization.

III. INTRACELLULAR LEVELS OF ORGANIZATION

Let us look again at an L cell as revealed by TS-TEM (Fig. 1A). We may construct some levels of organization from it, as I believe the historical development of cell biology has led us to do. The whole can be viewed to be composed of increasingly smaller structures, until the lower limits of resolution come into play. These images, I suspect, lead commonly to the impression that a sharp cut off exists in cells: the empty

spaces in Figure 1A are, thus, considered to be "structureless solutions" which surround the formed elements we may visualize with TS-TEM.

That impression has been reinforced strongly by cell fractionation: the disruption of cells is considered to release the contents of the aqueous cytoplasm, the "cytosol" (or the nucleoplasm, if we choose to isolate nuclei and disrupt them) and so on. I hasten to recognize that practically everyone appreciates the uncertainties of cell fractionation. My point is that this appreciation does not often play a significant role in the interpretation of such experiments, or in metabolic model construction: it is not translated into what we may or may not understand about cellular organization. Thus, I suggest that cell fractionation not only deludes us into thinking we are actually studying the aqueous cytoplasm when using the in vitro cytosol, but that it also reinforces our image of cells described by TS-TEM. It generates what appears to be a sharp boundary between the "aqueous compartments" and the "ultrastructure".

It seems to me that we have no good reason to believe that such a sharp interface actually exists in intact cells. On the contrary, it would seem equally appropriate to view the cell as a complete continuum in which relatively few of its parts are not functionally and structurally integrated into the whole.

To sum up this section I would like to suggest that if the idea of "levels of organization" is applied to the intact cell at all, that is because some choose to look at cells that way, and not necessarily because they actually exist. It is interesting that many of the earlier students of cell biology were very receptive to the "continuum-notion", and that this formed the basis for much of their thinking and experimentation (1). To avoid misunderstanding I should stress that I believe "reductionist" and "holistic" approaches are both useful means by which to study cells, each having its pros and cons. But I think there is one clear cut and unambiguous exception and that concerns the aqueous compartments: these are properties of the cell as a whole, and cannot be isolated in any form that has biological meaning. That alone is instructive, because it illustrates the continuum that exists between the aqueous phase(s) of cells and the "formed structures" that electron microscopy reveals. Our next task, then, is to inquire into what we know about these relationships.

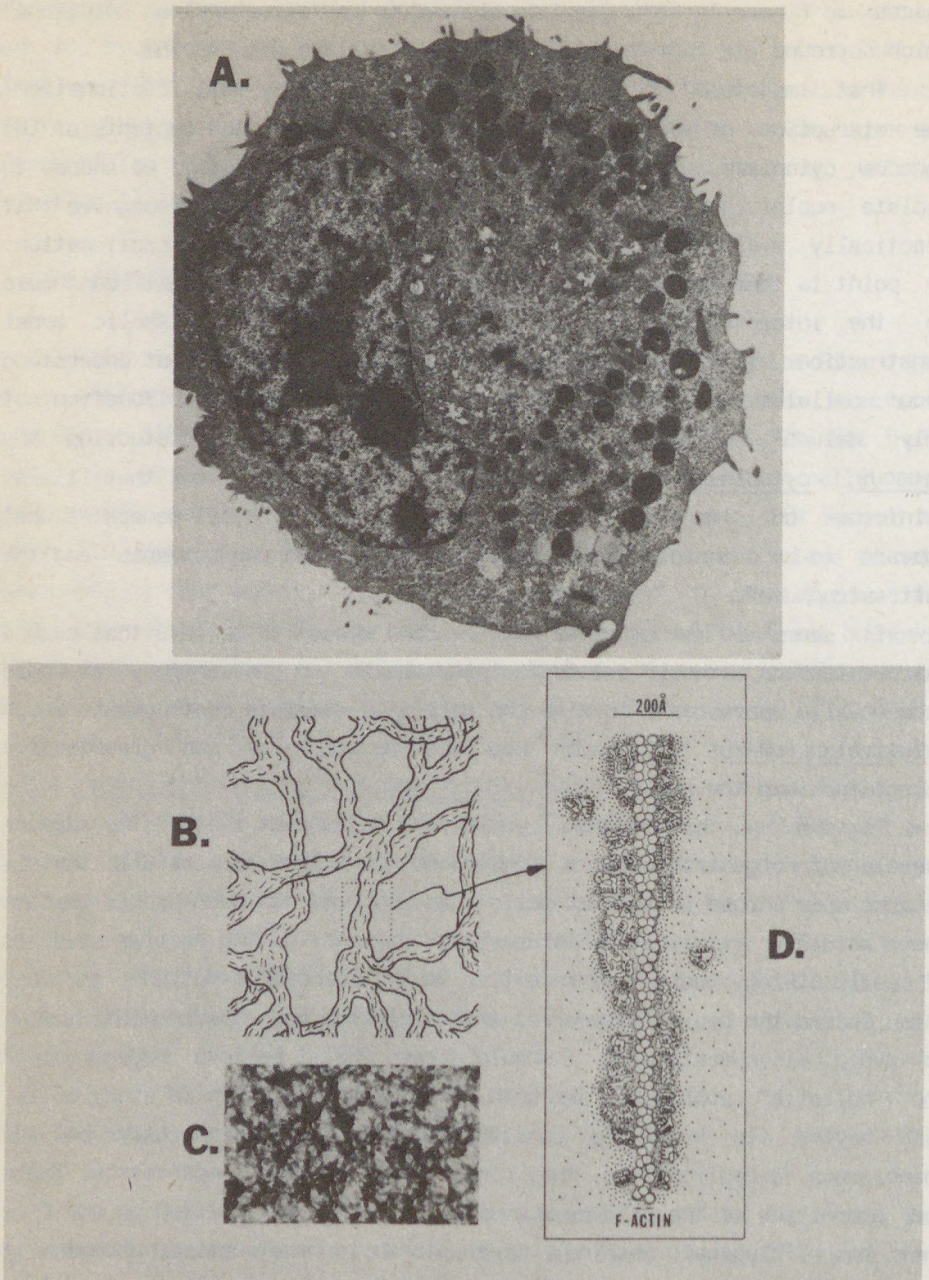


Fig. 1. Transmission electron photomicrograph of a mouse L-929 cell (A). Diagram of a small region of cytoplasm illustrating the microtrabecular lattice (B) which is shown below by high voltage electron microscopy (C). Hypothetical composition of a trabeculum (D, see text). Fig. 1C was provided by K.R. Porter.

IV. ARE THE AQUEOUS COMPARTMENTS CROWDED SOLUTIONS OF FREELY DIFFUSING MACROMOLECULES?

For reasons to be developed as we go along, I believe that question is a vital one to answer. If they are, then we can proceed, with confidence, to use the methods that have led us to the present; but if they are not, then we must develop new tactics if we are ever to understand them. What is more significant, the latter outcome will tell us that the extent of intracellular organization is not revealed by TS-TEM, but is hidden, and our perception of the continuum of organization halted at that level.

Cell fractionation, if accepted without question, indicates that the aqueous compartments are very crowded with macromolecules, containing as much as 50% of the total cell protein in solution (2). Examination of the contemporary literature shows that most appear to adopt this position; moreover, it is the general case that no assumptions are stated on this point. It seems to me that reference to a "cytosolic" enzyme is legitimate only if one restricts interpretation of its study to events in a test tube; it is unacceptable if by "cytosolic" is meant, or inferred, that its intracellular location and activity have been described by this method of study. I haven't cited specific papers in this brief account because there are thousands involved with cell fractionation, each as good (or as poor) as the rest.

However, there are some studies on intact cells that do provide evidence for the presence of macromolecules which diffuse throughout the cell (3). I believe the work of Paine and colleagues (4) also provides reliable information on this matter. Using an ingenious "reference phase technique", which involves the diffusive behavior of solutes between an injected gel (gelatin) reference and intracellular aqueous phases (5), Paine found that a variety of proteins are able to diffuse; however, the relative amount of total cellular protein exhibiting diffusive freedom was quite small compared to the total (see Fulton 1982^x). These elegant studies tell us that diffusing proteins exist, and can enter the reference

^x Fulton, A.B. How crowded is the cytoplasm?
Cell 30, 345-347, 1982

phase, but they do not show that this occurs by random thermal motion (diffusion) in three dimensions. It is possible that two dimensional diffusion of proteins along intracellular surfaces contributes significantly to the translocation observed. For the moment I simply note that results from the reference phase method provide an upper limit to the fraction of macromolecules that undergo free diffusion in cells and, therefore, exist in solution. We may suggest that, while the aqueous compartments may contain some freely diffusing macromolecules at a given time, they do not represent crowded solutions.

It is surprising to me how the paradigm of the crowded aqueous compartments continues to be adopted in the face of so much evidence against it, some of it being directly contradictory. Space constraints prevent an account of all that evidence here, but it is readily available in recent reviews and books (6-8). The evidence comes from the study of many cell types and the use of a wide variety of techniques including (a) fluorescence recovery after photobleaching to measure intracellular protein diffusion (9), (b) electron spin resonance spectroscopy of spin label probes to evaluate microviscosity (10), (c) high voltage electron microscopy (HVEM) (3,11) sometimes coupled to detergent treatment and protein electrophoresis (12), (d) use of the reference phase technique to evaluate intracellular protein diffusion (4), (e) analysis of the rate of protein (and enzyme) efflux from cells treated with different detergents (13), (f) combined use of immunofluorescence of tagged enzymes, cytomatrix disrupting agents, and microscopy on intact cells (14), (g) research on the association between F-actin, and other cytoskeletal proteins with glycolytic enzymes (15) and (h) ultrastructural and metabolic studies on osmotically perturbed and permeabilized cells (6, 16, 17). Indeed, this recent evidence is supernumerary to a significant body of data on the subject collected by earlier workers, some of it going back to the 1930s (6, 18).

However abundant this evidence, and how often reviewed, inspection of current literature reveals that the crowded solution paradigm remains firmly entrenched. Thus, it appears necessary to continue to collect data that tests further the matter of intracellular organization. I offer a few additional experiments in that regard.

V. STUDIES ON PERMEABILIZED L-929 CELLS

Recently we have been exploring the metabolic activity of L-929 cells permeabilized with dextran sulfate (6) using the method of Kucera and Paulus (19) which has the advantage of providing reversible conditions: the cells are reported to re-seal and resume growth and division. We have characterised the metabolism of glucose in these cells, showing that it requires appropriate levels of added NAD^+ and ATP, and a suitable ionic environment.

Figure 2 is a diagrammatic representation of two extremes in cytoplasmic and metabolic organization, A. being the crowded solution paradigm, and B. the organized one.

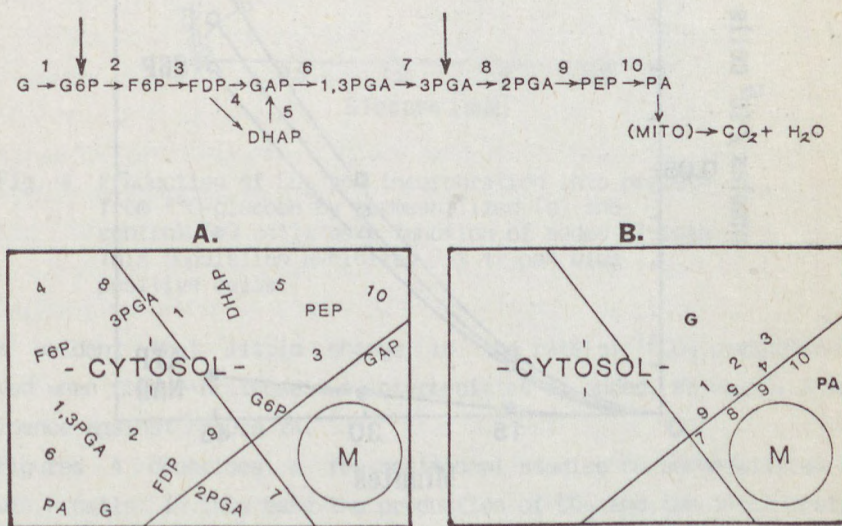


Fig. 2. Extremes in cytoplasmic metabolic organization:
A. represents the random disposition of all enzymes (numbers) and intermediates for the glycolytic pathway (top of figure);
B. represents these enzymes as a complex in association with the cytomatrix (lines). Mitochondria are represented by M.

The glycolytic pathway (a classical soluble pathway) is represented here, but any metabolic pathway considered to be "soluble" on the basis of cell fractionation could be considered similarly. The top of Figure 2 illustrates one experimental approach to test these alternatives in permeabilized cells: present ^{14}C -glucose and trap $^{14}\text{CO}_2$ in the presence and absence of added ^{12}C -intermediates (I). The prediction of the outcome, if Figure 2A is correct, is that the rate of $^{14}\text{CO}_2$ production should be markedly reduced by addition of ^{12}C -I. If Figure 2B is correct then no decrease in $^{14}\text{CO}_2$ production should be observed. Figure 3 shows the outcome for studies using two non-labelled intermediates (the arrows in Fig. 2).

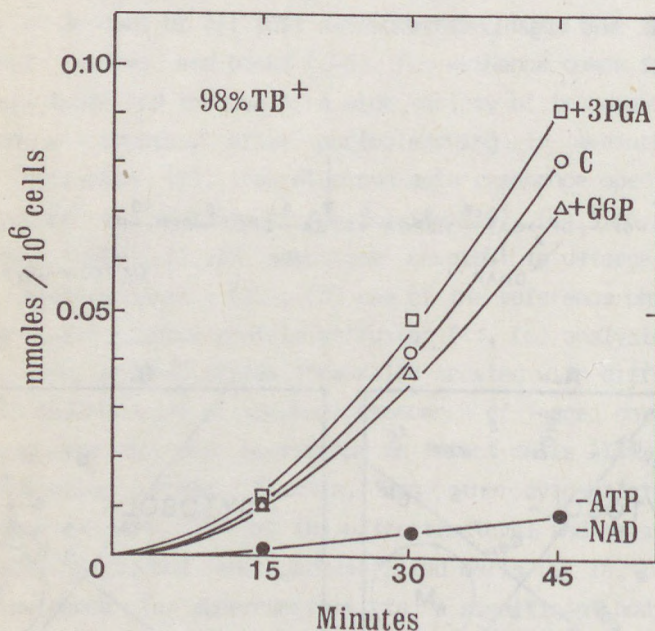


Fig. 3. Conversion of ^{14}C -glucose (nanomoles) to CO_2 in the absence (C) and presence of ^{12}C -glucose-6-phosphate (G6P) and ^{12}C -3-phosphoglyceric acid (3PGA), all being present at 1mM. ATP and NAD^+ are present at 2mM and 1mM respectively except for the filled circles where both were not added. TB = trypan blue (1%).

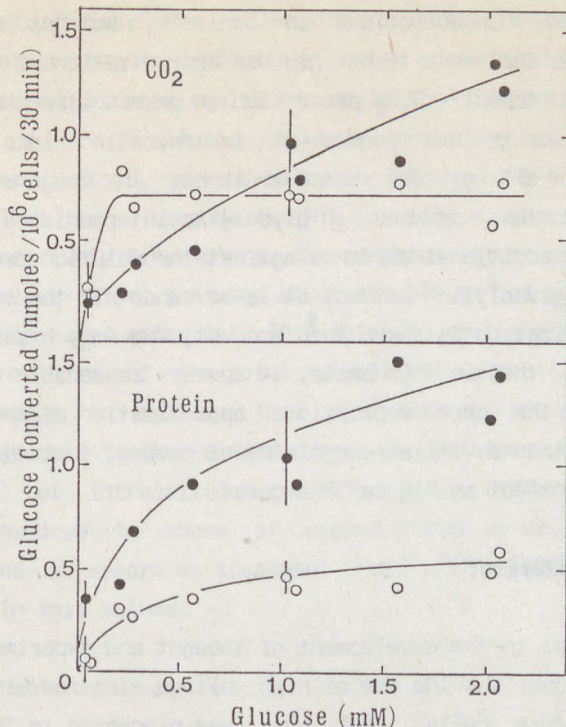


Fig. 4. Production of CO_2 and incorporation into protein from ^{14}C -glucose by permeabilized (o) and control (●) cells as a function of added glucose. This population exhibited 93% trypan blue positive cells.

It is evident that little change in the rate of $^{14}\text{CO}_2$ production is observed when either or these two intermediates is added. We may view this as evidence against Figure 2A.

Figure 4 describes a few additional studies on permeabilized and control L cells. In this case the production of CO_2 and the incorporation of ^{14}C from labelled glucose into protein (via free amino acids we presume) are assayed as a function of external glucose concentration. A major conclusion to be drawn from these studies is that a suitable "control" does not exist by which to evaluate the relative metabolic rates of permeabilized cells; thus, at low glucose concentrations the latter are "better" than controls in converting glucose into CO_2 . We may surmise that result is due to the contribution of glucose transport limitations at low

external glucose concentrations in controls, but not in permeabilized cells. Also note that an external glucose concentration of about 0.2 mM is saturating with respect to CO_2 production in permeabilized cells, and that permeabilized cells are poorer at incorporation into protein than controls, at all glucose concentrations. In conjunction with the previously described studies on glycolytic intermediates (Fig. 3) these results provide additional evidence against the solution description (Fig. 2A) for the glycolytic pathway at least. When all the evidence on this issue of organization is taken into account, and only brief attention has been given to that in this paper, it appears reasonable to suggest that Figure 2B is the more appropriate approximation of the intracellular aqueous compartments. It is recognized, of course, that the "coupling" is probably not as tight as Figure 2B suggests.

VI. CONCLUDING COMMENTS

A key event in the development of thought and experimentation on the topic of this paper was the use of high voltage electron microscopy (HVEM) on tissue culture cells. That work was pioneered in the mid-1970s by Porter, and his students and associates, who observed the existence of an elaborate network of filament-like structures ramifying throughout the cytoplasm which they called the "microtrabecular lattice" or MTL (11). The MTL is illustrated in Figure 2B which represents in diagram form an actual HVEM image provided by Porter (Fig. 2C). Figure 2D represents the possible composition of one part of a trabeculum, being a complex of F-actin associated with what has been referred to here as "soluble enzymes". The evidence for this model has been summarized elsewhere (6) but it should be noted that the key experiments are those of Schliwa et al. (12) and Masters and associates (15). Thus, this image of the cell leads us to the conclusion that an incredible degree of intracellular organization exists, perhaps down to the level of metabolic intermediates being passed from one enzyme to the next. Critics of the MTL (6) consider it to result from the artifactual deposition of "soluble" proteins on the conventional cytoskeletal elements. That criticism is not important if few soluble proteins actually exist in intact cells, which I believe the evidence tells us is correct.

A final comment concerns the aqueous volume enveloping the MTL and

other cell structures: the aqueous compartments. It appears to contain very little other than some nicotinamide and adenine nucleotides, a small amount of dissolved macromolecules and metabolites, and inorganic ions. In other words, a dilute solution. However, that does not mean the aqueous compartments are ordinary aqueous solutions of the kind one generates in a test tube. That is so because the MTL presents an enormous surface area within a comparatively tiny volume: the cytoplasm alone of a cell of 15 μm diameter (nucleus=10 μm) contains about 115,000 μm^2 of cytomatrix surface area, and a layer of water only 30 Å thick on that surface would consume well over one-third of the total cytoplasmic water (6). Since there is very good reason to believe that the effects of surfaces on water structure adjacent to them extend over such distances we may expect most (perhaps all) of intracellular water to exhibit differences in physical properties compared to those of ordinary bulk water. But that complex issue requires a separate treatment, and will be the topic of several other papers in this volume.

VII. Acknowledgements

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DISCUSSION

Damjanovich: Of course you know, but some people might be misled that you try to make an identification between the rotational relaxation and rotational diffusional constant. They are not the same. If you measure rotational relaxation, you measure a decay process, and it could be wobbling or a real spinning. And even if the rotation is really highly restricted, you still will measure a decay.

Tigyi: Your spin probe experiments are very sympathetic for me, because the difference between the rotational and translational movement is absolutely positive. But I'd like to ask you what was the chemical nature of these spin probes, because it is important to know. The second, did you not try to cool down this system because cooling will increase the viscosity and may give you some information.

Clegg: With respect to the kind of probe, the experiments of Mastro and colleagues that I described were done with tempol but they've used several probes as I recall. As any event, tempol is reported to sample the aqueous phase.

Tigyi: Was there a difference between different probes?

Clegg: Sure! If you use spin probes that have high hydrophobicity, then you have a lot of interaction with lipids and membranes. With regard to cooling down, that would be hard to interpret, because, if Porter's right, then the microtrabecular lattice dissolves, i.e. structure which is influenced by temperature, as well as the motion of the probe itself. Any result we have would be confounded by significant temperature change. Now, it might be possible to do it over a range of temperatures, but not to the point where the cell undergoes reversible change in its morphology. There is a paper just out in June issue of the Journal of Cell Biology, in which people use fluorescently labelled dextrans of increasing molecular weight. And they measure their motion in cells. This group, from Carnegie-Mellon University in Pittsburgh, measured the "pore size" this way. They interpreted their data to mean that the "pore size" would be about 400 Å. That is very surprising because it is much-much smaller than that Porter's measurement would suggest.

Osborn: Cooling down affects the microtrabeculae in some cell types and I think rat liver cells are among them. But in five minutes at 4°C you're going to lose microtubules. If you warm the cells up, the microtubules will jump back. So perhaps this experiment should not be totally interpreted in terms of the cytomatrix.

Clegg: Is it reversible in the same time frame?

Osborn: Yes, it is reversible. I don't know what the time of reversal is from, but certainly the time for destruction can be very short.

Masszi: There is a problem in the microwave experiment, that is, the bulk water has one relaxation time, with no spread parameter. The living substance has a longer relaxation time, but it is a question whether it has a spread parameter or not.

Clegg: Yes, it has a spread parameter it is 0.46, at least for water in Artemia cysts. But this is for the dispersion between 800 MHz and 70 GHz. Over that dispersion we see a distribution of relaxation times without doubt, and the spread parameter is 0.46. We find that the majority of cyst water relaxes below 800 MHz.

Masszi: I think that the time is an experimental question. That is, whether there is, or there is not a spread parameter. According to our microwave measurements there is no spread parameter.

Clegg: But did you measure up to 70 GHz?

Masszi: No. We measured at 2-5 GHz.

Clegg: We are not talking about hydration water, that's relaxing at hundreds of MHz or a few GHz. We are talking about water that is relaxing at 10 or 12 GHz instead of 20 GHz. I believe that extrapolation is very risky from low GHz frequencies.

Pollack: You made the calculation that for a cell of about 8 μ m in radius it would take a small molecule about 100 ms to diffuse from the center to the periphery. You could make a similar calculation, if all the enzymes involved in the intermediary metabolic chain were randomly distributed. How long would it take for the entire reaction to occur. And is this completely unreasonable?

Clegg: So the question is, suppose you have all the enzymes dissolved in the same volume, you could calculate how fast things would go, assuming mass action which metabolic models usually do, and then measure the flux through this pathway and see if you can account for the flux, by the calculation. These calculations have been done starting in the 60's. Except for things like yeast cells, which are after all fermentative

machines, you can't account for the rate of most of the enzymes. For example phosphofructokinase is present in cells in very low concentration, and if there weren't some kind of coupling you couldn't account for the rate of glycolysis for instance. People have pointed this out for over twenty years. But once again that's arm waving and the general biochemical community has said: "Prove it, we are not going to make our lives complex until you absolutely compel us to believe this".

Kellermayer, Jr.: You mentioned that the microtrabecular network may be composed of actin filaments and you brought up that enzymes, for example aldolase might be attached to it. But I remind you, that enzymes may be attached to microtubules, too. Several microtubule-associated proteins have enzymatic activities, and among Porter's pictures there was a magnified one. And you pointed out that no fine structure could have been seen. But actin filaments are composed of subunits, so you should have been able to see fine structure, if the microtrabecular network was composed of actin filaments.

Clegg: It would be wonderful, if that were the case. But I don't think that's necessarily the case. You just don't see fine structure, even if you are looking at purified proteins, what you can see are complexes, and things like this if you use the right techniques, but with these high voltage EM techniques you don't see any fine structure in the general case. With regard to why actin can be a major component of the MTL, I believe Schliwa's studies justify that option. Also, I think Mary Osborn hit it right on the head this morning, when she said that the matrix is a very significant mass and volume. There aren't many kinds of cell proteins to find from those that are present in such large amounts. Actin is one of them. We should also remember that actin is a highly conserved protein. It's found essentially in all animal cells, and I assume it's in a lot of plants. It's present in very substantial amounts. And it doesn't matter whether the cells are locomoting or not, whether they have stress bundles or fibers, or not. That tells us, I think, that actin must have some general and ubiquitous role, such as the one proposed here.

Kellermayer, Jr.: Just one sentence of Porter hit me this morning which was that the majority of the proteins inside the cell may form this structure. So it is not necessary that a single kind of generally accepted filamentous protein must be the basic subunit of the microtrabecular network.

Clegg: That sounds fine to me.

DISTRIBUTION OF POTASSIUM AND OTHER ELEMENTS IN LIVING CELLS AS STUDIED BY ELECTRON-PROBE X-RAY MICROANALYSIS

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INTRODUCTION

Cells in the bodies of vertebrates maintain extra- and intracellular concentration gradients of K^+ , Na^+ , Cl^- and other ions. What mechanisms are involved in the maintenance of such ionic gradients? Two main mechanisms have been proposed: (I) that cellular membranes, because of their semipermeability and associated ion selective active transport "pumps", are primarily responsible for the ionic gradients and (II) that intracellular macromolecules with selective ion adsorption properties are responsible for the ionic gradients. There seems no a priori reason why both of these proposed mechanisms and others might not be operational in the same cells or why different cell types might not have greater or lesser reliance on one or the other of these two mechanisms.

How do we decide which of these two possible mechanisms is the main determinant of the observed ionic gradients? To answer this question we have made measurements on the subcellular distribution of ions in a variety of cell types which were subjected to different experimental conditions. Ion gradients were measured by use of electron-probe X-ray microanalysis on cryofixed, cryosectioned and freeze-dried thin sections of the cells. Briefly, this method involves the rapid freezing of cells and tissues in liquid propane at $-169^{\circ}C$. The cells are then thin sectioned at $-100^{\circ}C$ and the water removed from the sections by freeze-drying prior to electron probe X-ray microanalysis for the concentration of elements at the subcellular level. The element distributions and content measurements are essentially those in vivo.

In short, our experimental results, along with reports from the

literature, indicate that the majority of intracellular K^+ is adsorbed onto macromolecules. The findings are not intended to deny a role for ionic pumps in cellular membranes as a mechanism for contributing to the generation of the ionic gradients but they do support the hypothesis that K^+ and perhaps Cl^- adsorption makes a major contribution to maintenance of their measured concentration gradients.

Failure of K^+ to diffuse from but not Na^+ or Cl^- to diffuse into mouse erythrocytes and liver tissue hepatocytes in thin cryosections which are warmed above a temperature which allows diffusion of ions suggests the adsorption of intracellular K^+ in these cells.

A prediction from the hypothesis that the plasma membrane is the responsible mechanism for maintenance of the ion gradient is that its physical disruption will allow the ion concentration gradients to dissipate. Conversely a prediction of the ion adsorption mechanism is that the concentration gradients of ions will not diffuse towards equilibrium upon disruption of the plasma membrane. An electron probe x-ray microanalysis study was designed to test these predictions. An established approach to the localization of diffusible ions in place in tissues is by rapid cryofixation in liquid propane cooled in liquid nitrogen (4). In the present study cryofixed liver and whole heparinized blood were cryosectioned and freeze-dried at two different temperatures. One temperature, $-100^{\circ}C$, prevents the diffusion of ions in tissues, while the other temperature, $-40^{\circ}C$, allows the diffusion of ions (3,4). We therefore subjected (1-2 mm^3) pieces of mouse liver and whole blood samples to cryosectioning and freeze-dried (cryosorption) at either $-100^{\circ}C$ or at $-40^{\circ}C$ prior to x-ray microanalysis to determine if there would be a significant redistribution of Na^+ , Cl^- or K^+ between the extra- and intracellular compartments. Our method of handling blood and pieces of mouse liver were as described previously (2,3,4).

To test that the intracellular K^+ was not free to diffuse from the intra- to the extracellular environment we did x-ray microanalysis of the distribution of K^+ in the cryofixed and cryosectioned erythrocytes in whole heparinized mouse blood. In the case of whole mouse blood the plasma constitutes more than half the total volume, which therefore provides enough extracellular volume to allow for a several fold reduction of the 20 fold higher intracellular K^+ gradient if the intracellular K^+ were free to diffuse.

The effects of cryosectioning and cryosorption temperature on the intra-erythrocyte concentrations of Na^+ , Cl^- and K^+ are listed in table 1 (from 4). As can be seen, the cryosectioning at -40°C as compared to -100°C revealed influx of Na^+ and Cl^- but no significant loss of cellular K^+ . The intra-erythrocyte K^+ did not show significant diffusion down its intra- to extracellular K^+ gradient.

Table 1. Effect of cryosectioning and cryosorption temperature on the Na^+ , Cl^- and K^+ content of mouse erythrocytes in heparinized blood as measured by electron probe x-ray microanalysis (mM/kg dry weight, mean $\pm$ SEM^{a,b})

ion	temperature	
	-100°C	-40°C
Na^+	120 ± 11	393 ± 36
Cl^-	208 ± 18	557 ± 37
K^+	288 ± 12	242 ± 10

The results of one-way analysis of variance showed Na^+ and Cl^- to be significantly higher at -40°C ($p < 0.001$) while the mean K^+ values did not differ significantly.

^a average of 10 erythrocytes

^b Table adapted from references 3 and 4

The results of the studies on liver hepatocytes (not shown) were similar and revealed a significant influx of Na^+ and Cl^- into the hepatocytes down the extra- to intracellular concentration gradient, which was interpreted to support the hypothesis that at least a portion of the normal extra- to intracellular gradient of Na^+ and Cl^- was maintained by the intact plasma membrane. As in the case of the erythrocytes, a significant quantity of K^+ failed to leave the hepatocytes at the higher temperature (-40°C). A second observation on the subcellular distribution of K^+ in hepatocytes suggested that little if any intracellular diffusion of K^+ occurred at the warmer, -40°C , temperature. In this regard, a significant nuclear/cytoplasmic concentration gradient of K^+ , which was measured in sections of hepatocytes processed at -100°C , was not dissipated when processed at the warmer temperature as might be expected if the nuclear K^+ were all free to diffuse between the subcellular compartments of the hepatocyte (3).

From these experiments we conclude that the maintenance of the Na^+ and Cl^- gradients between the extra- and intracellular compartments of

erythrocytes and of hepatocytes was due, at least in part, to the intact plasma membrane but that the maintenance of the extra-intracellular gradient of K^+ in the hepatocyte and erythrocyte was best accounted for by its adsorption to intracellular macromolecules. These findings can now be added to the growing list of evidence that intracellular K^+ ions are not freely dissolved in cell water (1,4-6,8-15).

Development of a nuclear/cytoplasmic K^+ concentration gradient in growing amphibian oocytes.

In addition to extra-/intracellular ion concentration gradients there is good evidence of ion gradients between compartments within the cell. For example there are a number of reports in the literature which indicate that fully grown oocytes of amphibians have a higher nuclear concentration of K^+ than exists in the cytoplasm when expressed on a wet or on a dry weight basis (6,9,22,26,29-32). How is this ionic gradient generated and how is it maintained? Results of our x-ray microanalysis of the concentration of K^+ , Na^+ and Cl^- as a function of the stage of oocyte growth, table 2, shows that pre-yolk stage oocytes do not have a nuclear/cytoplasmic (N/C) gradient of K^+ , Na^+ or Cl^- . During oocyte growth to full size, the nuclear concentration of K^+ increased while the cytoplasmic concentration of K^+ decreased. Thus a significant N/C gradient of K^+ was generated during oocyte growth. One possible mechanism to explain the N/C gradient might be the development of a K^+ pump in the nuclear envelope; however, Paine et al. (30) suggest this mechanism to be unlikely as the fully grown amphibian oocyte's nuclear envelope was found to be too permeable to maintain a N/C gradient of K^+ based on an active transport system. Another possible mechanism is the preferential adsorption of the K^+ to intranuclear sites. Experiments were therefore designed to test this idea, as reported next.

Is the majority of nuclear K^+ free in solution such that it will exchange with its ionic surrogate Rb^+ ?

To answer this question, fully grown and manual defolliculated oocytes of the amphibian Xenopus laevis were exposed to a Ringer's solution where K^+ was replaced by Rb^+ on a molar basis (2). Serial samples of the oocytes were taken over the course of 40 hours and were cryofixed and cryosectioned for elemental analysis by electron probe x-ray microanalysis. Oocyte volume remained constant. Net Rb^+ influx showed a slow exponential increase into the nucleus and the yolk-free cytoplasm.

Table 2. Ion concentration in the nucleoplasm and cytoplasm of growing amphibian oocytes expressed in mM/kg dry weight $\pm$ SEM^{a,b}

Stage of oocyte growth	K ⁺	Na ⁺	Cl ⁻
Pre-yolk			
nucleus	743 $\pm$ 13	83.4 $\pm$ 11.9	194 $\pm$ 0.8
cytoplasm	659 $\pm$ 13	71.5 $\pm$ 5.9	176 $\pm$ 4.8
Intermediate			
nucleus	712 $\pm$ 11	59.6 $\pm$ 12.0	160 $\pm$ 4.8
cytoplasm	554 $\pm$ 8	53.6 $\pm$ 5.9	112 $\pm$ 3.2
Full grown			
nucleus	892 $\pm$ 4	71.5 $\pm$ 11.9	198 $\pm$ 11.2
cytoplasm	300 $\pm$ 8	29.8 $\pm$ 5.9	80 $\pm$ 3.2

^a n=9 to 21

^b Modified from reference 22

Linear regression analyses of the data on K⁺ efflux from the nucleus showed evidence of two exponential components, a small component (about 15-20% of the total K⁺) which effluxed with a half-life of 0.5 hour and a large component with an indeterminately long half-life (table 3). These data therefore suggest that only a small proportion of nuclear K⁺ is lost in exchange for Rb⁺ but the linear regression analysis of the cytoplasm data shows that essentially none of the cytoplasmic K⁺ is lost in exchange for Rb⁺. These results provide strong evidence for the intranuclear adsorption of the majority of nuclear K⁺ and for the adsorption of essentially all of the cytoplasmic K⁺.

Table 3. Linear regression analysis for net potassium efflux in the nuclear and cytoplasmic compartments after the oocytes were placed in rubidium Ringer's solution^a

Compartment	Component(s)	n ^b	Slope $\ln K^+t/K^+(0)$ hr	Y-intercept K/K(0)	Correlation coefficient r	Significance of Slope p value	Size of Compartment (nM)	Half- life (hr)
Nucleus	Fast	8	-138×10^{-2}	.24	.74	<.05	171	.51
	Slow	4	$-.303 \times 10^{-2}$	.81	.38	NS	578	indeterminant
Yolk-free cytoplasm	Single	13	$-.091 \times 10^{-2}$	.877	.10	NS	372	--

^a Modified from reference 2

^b Number of time points analyzed (average of 3-7 oocytes per time).

Evidence for the association of Cl^- with material and structures cleared from the open cytoplasm of viable cells by ultracentrifugation at 100,000 times gravity for one hour.

Studies have shown that the subcellular contents of Euglena gracilis (a flagellate) can be stratified into six layers by means of ultracentrifugation, with the cells still remaining viable (18-20). One of these layers was clear and was reported to be free of a number of components, including membranes, nucleic acids and enzymes, and to be almost free of proteins (18-20). This finding was used to support the idea that all enzyme activity in cells takes place on fixed cytomatrix surfaces (7). We reasoned that a subcellular redistribution of ions associated with specific subcellular components might also be obtained by such an ultracentrifugal stratification procedure. If, for example, an ion, such as Na^+ , K^+ , or Cl^- , were bound to a given macromolecule, the ion might be expected to decrease in concentration in the layer of cytoplasm cleared of cytomatrix, ribosomes, proteins, and enzymes. However, if certain ions were free in solution they would not decrease in concentration in this cleared layer of cytoplasm. By comparing the distribution of ions at the subcellular level in stratified and non-stratified Euglena one can obtain information on the mechanism(s) by which subcellular element concentration gradients are established and maintained (1).

Our ultrastructural observations on cytoplasm in non-stratified and stratified cells showed that a field of open cytoplasm free of almost all recognizable structures was indeed produced by the ultracentrifugation. If any of the Na^+ , K^+ or Cl^- ions were closely associated with the recognizable structures it might be expected that they would be removed or decreased in concentration by ultracentrifugation. Analysis of the data in table 4 (table 4 is modified from reference 1) revealed that the concentration of Cl^- was significantly decreased by this procedure ($p < 0.001$). The fact that the cleared layer of cytoplasm has been shown to contain little if any macromolecular material or enzyme activity (18-20), plus the fact that the Cl^- concentration is significantly decreased to less than half its initial concentration, indicates that more than 60% of the Cl^- in the open cytoplasm of non-stratified Euglena is adsorbed to the material that was removed by ultracentrifugation. The fact that over 60% of the

cellular Cl^- may be closely associated with intracellular material is also supported by recently published Cl^- -efflux studies on human lymphocytes (28).

Table 4.

Two-way analysis of variance statistical evaluation of the subcellular redistribution of various elements due to ultracentrifugation (element concentration in m Mol/kg dry weight $\pm$ S.E.M.)^a

Potassium	Nucleus	Cytoplasm
Non-stratified	92 $\pm$ 24	155 $\pm$ 24
Stratified	348 $\pm$ 48	150 $\pm$ 27
Column mean	208	152
Treatment ^b	9.337 ^c	< 0.01 ^d
Location	1.519 ^c	NS ^d
-TxL	14.320 ^c	< 0.001 ^e
Sodium	Nucleus	Cytoplasm
Non-stratified	39 $\pm$ 10	32 $\pm$ 15
Stratified	35 $\pm$ 1	73 $\pm$ 28
Column mean	37	51
Treatment	1.866 ^c	NS
Location	--	NS
TxL	0.762 ^c	NS
Chlorine	Nucleus	Cytoplasm
Non-stratified	87 $\pm$ 15	107 $\pm$ 15
Stratified	149 $\pm$ 15	42 $\pm$ 15
Column mean	115	77
Treatment	0.711 ^c	NS
Location	3.609 ^c	< 0.05 ^f
TxL	8.363 ^c	< 0.0019

^a 8-12 cells measured for each treatment

^b Results of a two-way ANOVA to determine significant differences due to: treatment (T), subcellular location (L) and interaction between T and L (T $\times$ L).

^c The F value.

^d The probability (p) of significance of the F value; a p value of < 0.05 is considered significant.

NS, not significant.

^{e, f, g} Results of an Student-Newman-Keuls multiple range test for significant T and L. Interaction differences between individual means show: f) Cl^- to be in significantly lower concentration in the cytoplasm of stratified cells as compared to the cytoplasm of non-stratified cells and g) Cl^- to also be significantly increased in the nucleus of the stratified cells compared to the non-stratified cells; e) K^+ to be in significantly higher concentration in the nucleus of the stratified cells compared to the nucleus of the non-stratified cells.

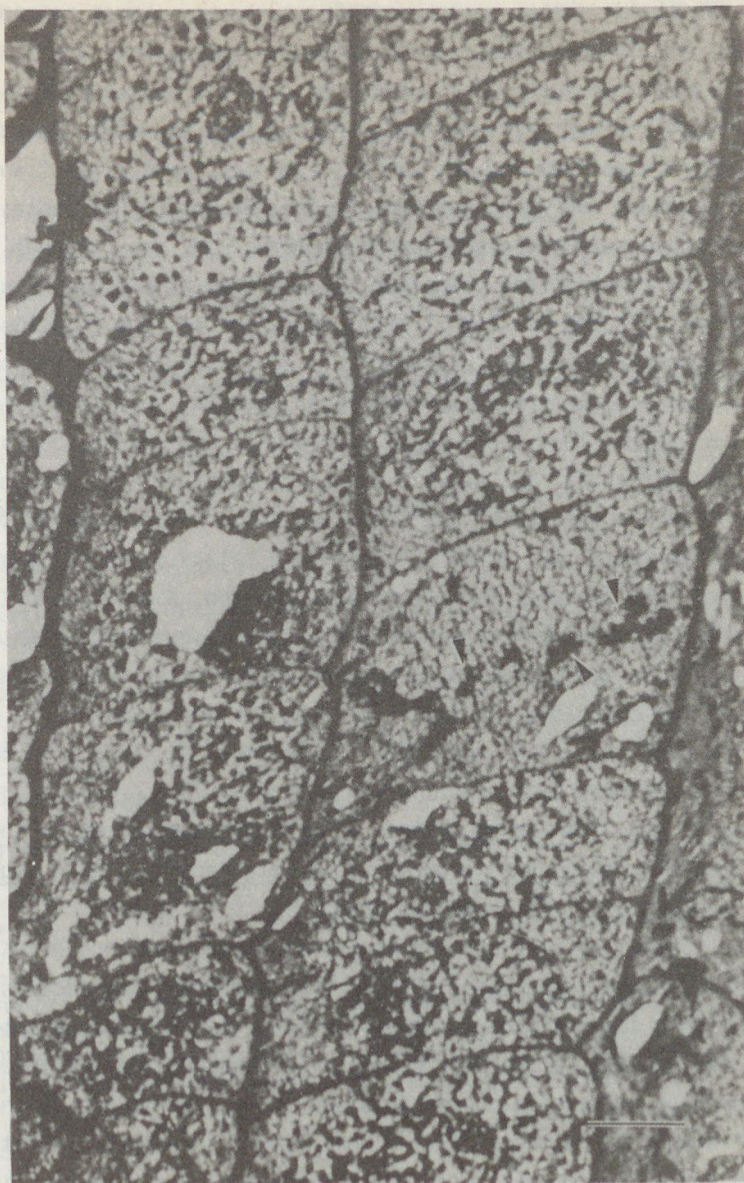


Fig. 1 A scanning transmission electron microscopic image of a freeze-dried unstained cryosection of innermost cortex cells in the meristem region of onion root tip. The nucleus in most cells shows a nucleolus. Areas of condensed chromatin are seen within the interphase nuclei. One cell is in mitosis and shows condensed metaphase chromatin (arrowheads). Bar, 5.0 μ m; X3250.

The possibility that ultracentrifugation may have caused the differences in elemental concentration between the nucleus and the open cytoplasm is a question that cannot be entirely eliminated. In fact table 4 reveals the existence of interactions between the ultracentrifugation treatment and the subcellular distribution of K^+ and Cl^- between the nucleus and the cytoplasm. In the case of Cl^- concentration, centrifugation caused a significant increase in the nucleus and a significant decrease in the cytoplasm. In the case of K^+ concentration, centrifugation caused a significant increase in the nucleus. An increased concentration of nuclear Cl^- and K^+ resulting from active transport by the nuclear envelope membranes does not seem likely, since others have shown that the nuclear envelope of Euglena has pores (25) and is too permeable to serve a transport function (14,30). Another explanation for accumulation of Cl^- and K^+ in the nucleus is that a significant increase in fixed anionic and cationic charge-sites has occurred in the nucleus. Such fixed charge-sites, specific for K^+ and Cl^- , may have occurred either by unmasking such charge sites within the nucleus and/or by the preferential nuclear influx of such fixed charge-sites from the cytoplasm. In fact, evidence for the unmasking of fixed charge-sites and for the selective adsorption of ions onto macromolecules has recently been reported (24).

Evidence for the accumulation of K^+ and phosphorus during the condensation of interphase chromatin into metaphase chromatin suggests the ionic binding of K^+ to phosphate groups.

As suggested by the studies mentioned above, intranuclear K^+ was hypothesized to be associated with or adsorbed to macromolecules. One source of this association might be to fixed negative charge sites on proteins or nucleic acids. For example, it is known that histones H1m and H3 undergo extensive phosphorylation just prior to and during the metaphase stage of mitosis (13). One might therefore predict that phosphorus would preferentially accumulate in metaphase chromatin in the form of added phosphate groups. This would be expected to increase the fixed anionic charge sites. Such fixed anionic charge sites might provide additional ionic binding sites for K^+ . This prediction was evaluated by electron-probe x-ray microanalytical studies on interphase heterochromatin and on metaphase chromatin in meristematic cells of onion root tip. Onion cells were selected for study because of the

relatively large areas of heterochromatin in the interphase cells and because of the large areas of chromatin present at the well-defined metaphase stage of mitosis. Cryofixed root tips were cryosectioned at -100°C at a thickness of $0.1\text{ }\mu\text{m}$. Figure 1 illustrates the morphology seen in such thin cryosections and shows areas of heterochromatin in interphase cells and condensed metaphase chromatin (arrowheads). The x-ray microanalysis of such subcellular areas in thin cryosorbed sections provided the data listed in table 5.

Table 5.
Element concentration (mMol/kg dry $\pm$ SEM) in subcellular compartments of interphase and metaphase cells from the meristem of onion root tip

Stage of Cell Cycle and Compartment	Element					
Interphase	n	p	S	Cl^-	K^+	Na^+
Chromatin	9 ^a	743 ± 62	140 ± 8	63 ± 32	780 ± 29	48 ± 23
Open-cytoplasm	10 ^a	688 ± 38	146 ± 10	39 ± 6	728 ± 39	91 ± 18
Metaphase						
Chromatin	5 ^b	$1101 \pm 46^{\text{C}}$	136 ± 10	39 ± 5	$973 \pm 15^{\text{C}}$	101 ± 22
Open-cytoplasm	2 ^b	541 ± 45	137 ± 22	17 ± 4	639 ± 16	63 ± 44
Spindle-cytoplasm	4 ^b	496 ± 56	119 ± 9	42 ± 16	657 ± 40	22 ± 13

Analysis of variance statistics

F-value	7.392	2.443	0.501	8.523	1.389
p-value	< 0.001	NS	NS	< 0.001	NS

^a n=number of individual cells measured

^b n=number of areas measured on the same metaphase cells

^c a multiple-range test (Student-Newman-Keul's) showed that these mean values were significantly higher than all other mean values in this column. No other means were significantly different.

Table 5 shows that metaphase chromatin had significantly higher concentrations of phosphorus (P) and potassium (K^+) than interphase chromatin. The increased concentration of K^+ found in metaphase chromatin parallels the increase in P and therefore suggests that the K^+ is involved in balancing the negative charge of histone phosphate groups. Thus, the correlations between chromatin condensation, histone phosphorylation and the increase of monovalent cation justified consideration of a role for monovalent cations in chromatin condensation at metaphase. The data also provide support for the hypothesis that K^+ can ionically bond to the phosphate groups in chromatin.

CONCLUSIONS

The electron-probe x-ray microanalysis studies which we have done provide a body of data to support the hypothesis that much of the intracellular K^+ is adsorbed to fixed charge sites within living cells. Our studies add to the growing list of experimental proof that the majority of intracellular K^+ is in the adsorbed state (23, 24). The unequal distributions of K^+ , Cl^- and perhaps Na^+ between the subcellular compartments in perhaps best accounted for by the preferential distribution of macromolecules which bind such ions. This conclusion regarding K^+ is in keeping with Eugene(Jenő)Ernst's conclusion (11) that the bulk of cellular K^+ in cells exists in a nondiffusible form. We conclude from our data and from data in the literature that gradients of K^+ , Cl^- and other ions are maintained, in part at least, by selective adsorption to fixed charge sites on intracellular macromolecules.

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DISCUSSION

Hazlewood: I find the conventional view of the cell can be shown to evolve directly from the assumption that the cellular interior mimics that of dilute solutions. If the ions are adsorbed, that is, out of solution, the assumption of dilute solution theory are violated. Therefore, the two views are mutually exclusive.

Cameron: I agree that much of the cellular potassium, chlorine and probably sodium are not free in solution. I am also aware that much of the cellular water is motionally perturbed from that of bulk water. In a series of studies I have observed that the osmotic property of a substantial portion of the cellular water is reduced from that of bulk water. That is to say a substantial portion of cellular water fails to obey Van't Hoff's equation for osmosis. It is not uncommon to find that a hundred or more grams of water per hundred grams of dry mass in the cell deviates from bulk water in its osmotic behavior. This helps explain the observations of others that various types of cells do not act as a perfect osmometer. We hypothesize that the water of hydration in a cell differs from bulk water in its motional properties (as indicated by pulsed proton NMR studies) and also differs in its osmotic activity. Cellular water, other than hydration water, should have the properties of bulk water and might be expected to obey the assumptions of dilute solution theory. The question for me is how much of the cellular water is hydration water and how much is bulk water? In this regard, I make a plea that we express the amount of water in cells not in the percent of water, but in terms of grams of water per gram of dry mass. If one assumes that for every 100 grams of cellular dry mass there is one hundred and forty grams of water of hydration water one might predict that this amount of water would not participate in the osmotic equation of Van't Hoff under physiological conditions. This much water amounts to all of the cell's water in some cells i.e. in cells with less than 62% water.

Hazlewood: That 62% of the water is osmotically inactive?

Cameron: No a cell up to 62% water, not 62% of the water.

Hazlewood: I don't understand.

Cameron: Let me express it this way: 140 grams of water per 100 grams of dry mass in a cell may appear to be "osmotically inactive" under physiological conditions. That would mean that a cell that had 62% of its

weight as water would have 38% of its weight as dry mass. Such a cell would have no water with the motional and osmotic properties of bulk water.

Hazlewood: That's precisely my point. That is, as far as I can tell violates the fundamental assumption that water is like that of an ideal solution.

Cameron: All right in this example it does but in cells with higher water mass, for example in the unfertilized sea urchin egg we do find evidence for bulk water as measured by our NMR titration method (Merta et al., J. of Cellular Physiology 127:439-447, 1986). Let me point out that the amount of hydration water in the sea urchin egg decreases dramatically shortly after fertilization. Thus, different cells may have different amounts of hydration water, and this amount can change in the same cell in different states.

Clegg: I think we have probably some term problems here, because, I think everyone has a good feeling for what primary water of hydration is. You know, that can be described; you can see a lot of it with X-ray crystallography, in the case of proteins. Its behavior has been pretty well mapped out. Now, as soon as one says free, my feeling is virtually all the water in the cell is free, in the sense that if you put an osmotic gradient, you can suck it out. So we need to know when you say osmotically inactive, and when you say free.

Cameron: All cellular water is strictly speaking osmotically active, because one can get it all out by high vacuum and high temperature if you desired. However, there are degrees of difficulty in getting the water out by osmotic gradients. If one puts viable cells into sucrose solutions of various concentration, one finds that there is a percentage of the cellular water that doesn't participate in the osmotic equation. For example, if one plots the osmotic behavior of frog muscle as the reciprocal of the osmotic pressure of the extracellular solution on one axis and the water content in gm water per gm dry mass on the other axis, you will find that the linear fit of the data in the physiological range extrapolate to one hundred and twenty grams water per hundred grams of dry mass (Ling and Negendank, *Physiol. Chem. Phys.* 14: 137, 1970). This helps explain the failure of a frog muscle to act as a perfect osmometer, as reported by Overton in 1902. I am currently testing the hypothesis that this fraction of apparently "osmotically inactive" water matches what we

measure by our NMR titration method as the hydration water fraction. Let me add that we subdivide hydration water into different categories depending upon its correlation times. Thus hydration water is subdivided into water which is either ionically or hydrogen bound to the surface (bound water) and water that is motionally perturbed but not hydrogen bonded to the surface (structured water). In the case of a globular protein (lysozyme) hydration water amounts to a shell of 2.5 to 3 water molecules thick. Such a shell of 2.5 to 3 water molecules amounts to 140 grams of water per 100 grams of dry weight; this is the same amount of hydration water that we found in the unfertilized sea urchin egg.

Ling: This meeting has been interesting and very exciting to me, and I think if Professor Ernst were here, he would share my feelings. You don't realize, how minority was our view of protoplasm. At Professor Ernst's time there were about a couple others like Martin Fischer, who were talking about the living cell. Fischer suggested that protoplasm represents "a union of protein, salt and water in a giant molecule". Think how the opponents of this view of protoplasm must have disliked that statement, and yet in how many different ways are we gradually converging to this view, even though the word "molecule" has to be scratched out. I feel very encouraged because I think at one time Dr. Cameron and Dr. Clegg had a far less inclination to this minority view. I try to twist their arms but I don't think I succeeded at all; through their own findings they gradually came closer and closer. I might as well add Dr. Hazlewood, I'm very glad that he reached the conclusion also not because I or Professor Ernst twisted his arm, but because he conducted experiments on his own. And for this I'm truly happy. I have disagreement on some small points, but I think this is the right trend. And this symposium is very meaningful, because it allows us to feel that perhaps even sometimes when you feel very much in the minority, maybe you are not all that much in the minority. And that's a good feeling.

Pollack: Well, after Ling's eloquent remarks I hesitate. I'd like to know what classical experiments exist on which the opponents base their views and why are they wrong?

Cameron: It appears that key experiments were done by A.V. Hill, on muscle. He investigated the distribution of urea and found that it went into the cell, and partitioned equally between the inside of the cells and the outside of the cells, ergo it was concluded that all of the water in

the cell was free to dissolve solute (urea in this case). We have not in the symposium discussed the possible ion excluding possibilities of bound water. This is a controversial subject. I am convinced that the degree of solute exclusion of water is dependent upon the probe used. Eric Ponder, famous for his erythrocyte studies, used ethylene glycol as a probe and he too, found it equally distributed between the water inside and water outside of the cell. Well, from that time most physiologists immediately accepted that all of the water inside the cell was like bulk water. Professor Huber apparently had been holding with Dr. Ernst's ideas on the nature of protoplasm but the data from A.V. Hill, changed his mind radically. And the conclusions of Hill and Ponder dominated the field until now with the exception of a few doubtful scientists who held divergent views. It was the particular properties of the probes that Hill and Ponder used for their experiments that led them to these conclusions. Had they done their experiments with other probes the story might be different. Other researchers have subsequently done studies with probes of different sized molecular weight and different chain lengths and have found evidence of solute exclusion in cells (Bobo, J. Gen. Physiol. 50: 2547, 1967). It's a complex problem requiring further examination.

Pollack: Well, weren't there experiments on the change of potassium, I think by Hodgkin and Keynes?

Ling: Hill was a very powerful person. So when he said something, everyone listened. And Hill's experiments actually dramatically changed the entire outlook, as Dr. Cameron correctly described. I owe the detailed knowledge of these events to Professor Ernst (see his Biophysics of Striated Muscle for details). Hodgkin and Keynes did report near normal mobility of K^+ in muscle as did later Kushmerick and Podolsky. However, in 1973, Margaret Ochsenfeld and I used a different technique, and we were able to show the K^+ mobility in frog muscle cells is reduced much more than expected were K^+ to be free in solution.

Cameron: Regarding potassium, the data Nancy Smith and I presented as well as other data from the literature strongly support the idea that a large proportion of cellular K^+ is not free to diffuse in cells. I have not thrown the Na^+/K^+ ATPase pump completely out of my thinking. So I'm not a complete convert, at least not yet.

Ling: In a few more years!

Keszthelyi: Now I understood that potassium is limited in its diffusion,

because the data show that. What limits the diffusion?

Cameron: I am proposing what Dr. Ling has proposed, it is associated with charged sites on proteins.

Keszthelyi: And could you show it outside of a cell?

Cameron: Yes, there are physical techniques for looking at whether K^+ is adsorbed or is not adsorbed. There is such data to show if K^+ is or is not free in cells or solution and I offer the x-ray adsorption edge fine structure of K^+ in frog erythrocytes as an example (Huang et al. Sci. 204: 191, 1979).

Keszthelyi: And does the adsorption or binding of potassium change some characteristics of proteins?

Cameron: I have just done some studies with solutions of actin monomers (5 mg/ml) which were electrophoretically purified. As one increases KCl concentration to 50 mM, there was a lengthening in the proton NMR T_1 relaxation time suggesting a release of hydration water to become free water which I also could detect by an increase in the size of ice crystal growth under controlled freezing conditions. When I further increased the K^+ concentration to 100-200 mM, which is a concentration often cited inside a living cell the purified actin at 12 mg/ml forms protein crystals! Indeed, K^+ does change the protein characteristics.

Keszthelyi: I asked this question because the protein I study, bacteriorhodopsin, does not function if there are no ions bound to it.

ATP DEPENDENT CO-COMPARTMENTATION OF PROTEINS AND K^+ WITHIN THE LIVING CELL

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INTRODUCTION

Twenty five years ago, when I was a first year medical student here in Pécs, Professor Ernst taught us his facts like these: 1.) "Potassium is not freely dissolved in the cell water". 2.) "In living cells, not certain hypothetical pumps supposedly localized in the cell membrane, but rather the intracellular proteins are responsible for the selective accumulation of K^+ and exclusion of Na^+ " (6,7). Then he added: "If we knew more about the mechanism of the protein -dependent selective K^+ accumulation, we would know much more about the major unknown, i.e. much more about the organization of the living mass".

Later I came to realize that Professor Ernst's statements which were facts for him and also for his students, were unknown facts for others in different scientific communities. It was a great luck for me that my very first experimental findings about the organization of DNA inside the nucleus of a living cell supported his statements and I could, and I can still consider Professor Ernst's statements as facts (14,17). Nevertheless, the mechanism is the one that has been keeping me alert in my continuous reserach including those experiments that I am going to present here.

In the experiments selected for this paper, thymus lymphocytes in suspension and cultured cells in monolayer were treated with Triton X 100 and Brij 58 non-ionic detergents and the release kinetics of lipid membranes, K^+ , ATP and proteins were studied. According to our working hypothesis, if K^+ ions were freely dissolved in the cell water and only the lipid membranes were separating the free intracellular K^+ -solution from the free extracellular electrolyte solution, a rapid immediate outward diffusion of K^+ would take place after the detergent induced removal of lipid membranes.

On the basis of the diffusion coefficient of K^+ in free solutions, the outward diffusion kinetics of the intracellular K^+ could be well predicted, but the actual findings were very different from the predictions. Our experimental findings are not only supporting the fact that K^+ is not freely dissolved in the cell water but also, they are offering newer insights into the mechanism of the protein-dependent intracellular K^+ -accumulation.

Observations on thymus lymphocytes

Lymphocytes were obtained from thymus glands of young calves, suspended in their own sera. The experimental steps of the detergent treatment, electron microscopy, protein and K^+ measurements were described in detail in our previous publication (Kellermayer et al., 1984).

The electron micrographs showed clearly that the lipid membranes were removed completely from lymphocytes which were exposed for 5 minutes to Triton X 100 or Brij 58 detergents (Table 1).

These observations are consistent with the observations of Schliwa et al (1981), who showed that the lipolytic effect of Triton X 100 and Brij 58 detergents are similar in that both remove all lipid membranes of separated cells within 5 minutes.

The K^+ concentration in the pellets of lymphocytes treated with Triton X 100 for 15 minutes was 15 mM per liter of water. With longer incubation times, there was no major change in the K^+ concentrations in the pellets (Table 1). The cells treated with Brij 58 for 15 minutes also lost their lipid membranes, however, the electrolyte concentrations followed a different pattern. The K^+ concentration was 81 mM per liter of water at 15 minute exposure time. After 20 minutes of Brij 58 treatment, the K^+ concentration was 52 mM per liter of water and only after 30 minutes decreased the K^+ concentration to the equilibrium level (Table 1, and Fig. 1).

The ratio of the external, or supernatant K^+ concentrations (mmol/l H_2O) to the K^+ concentrations in the pellets (mmol/l H_2O) has been defined as the ρ - (ρ) - value; Ling, 1984. In the case of the Triton X 100 treatment, the K^+ ρ -values were between 1 and 2 at every incubation time point (Fig. 1). On the other hand, after 15 minutes of Brij 58 treatment, the ρ -value for K^+ was 19.3. This value was 6.2 after 20 minutes, and it became 2.1 only after 30 minutes (Fig. 1). The protein concentration in

TABLE 1 Summary of findings on detergent treated thymus lymphocytes

Exp. ^x min.	Det. ^{xx}	n	EM (lipid membranes)	H ₂ O % +	K mmol/kg H ₂ O +
0	None	8	Intact	78.2±1.3	148±8.3
15	T.	16	Removed	73.5±0.9	15±0.8
	B.	16	Removed	76.7±1.6	81±4.8
20	T.	12	Removed	72.1±0.7	15±0.2
	B.	12	Removed	74.2±1.3	52±3.3
30	T.	12	Removed	71.3±0.8	10±0.5
	B.	11	Removed	72.2±1.1	21±1.8

^x Exposure time includes 10 minutes centrifugation time at 40.000 g

^{xx}Both detergents (T=Triton X 100; B=Nrij 58) were used in 0.2% concentration

+ All data are given as mean + standard error of the mean.

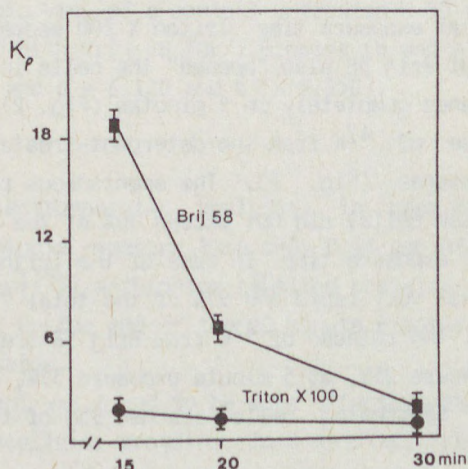


Fig. 1. The ordinate is the ratio of the concentration of K⁺ in the supernatant to the concentration of K⁺ in the pellet, referred to as rho-value. The abscissa is the time of exposure to Brij 58 and Triton X 100 detergents.

the supernatant reached a plateau in 15 minutes in case of Triton X 100 treatment, while a gradual increase was observed in the case of Brij 58 (not shown here).

The release of K from detergent treated lymphocytes.

Observations on monolayer tissue culture cells .

In these experiments an SV40 virus transformed H-50 cell line, HEp2 human carcinoma cell line and human fibroblast (HEF) cultures were used. The cells were treated with Triton X 100 and Brij 58 non-ionic detergents and four different analyses were performed on the detergent-"opened" cells: a.) electron microscopy, b.) ^{42}K release, c.) release of ^{35}S methionine labelled proteins, and d.) chemiluminescence measurement of ATP released from the cells (4). The technical details for the electron microscopy, ^{42}K , ^{35}S methionine-labelled protein release have already been published in detail (19).

The method for the chemiluminescence determination of ATP released from detergent treated cells will be published separately (20, 21).

The electron microscopic observations of the detergent-treated cells indicated that both detergents expressed considerable membranolytic effect in a very short exposure time. Triton X 100 seemed to be more effective than Brij 58 but Brij 58 also "opened" the cells in 2 minutes and removed the lipid membranes completely in 5 minutes (Fig. 2).

The release of ^{42}K from the detergent-treated cells showed several interesting features (Fig. 3). The spontaneous release of ^{42}K from the control (untreated cells) did not exceed 30% of the total ^{42}K , even at the longest (20 min) exposure time. In case of the Triton X 100-treatment, the release of ^{42}K was very rapid and 97% of the total ^{42}K was released in the first 2 minutes. The release of ^{42}K from Brij 58-treated cells was slower. At 2 minute exposure 25%, at 5 minute exposure 37%, and the release of ^{42}K from the Brij 58-treated cells reached 95% of the total only after 20 minutes (Fig. 3).

Analyzing the Brij 58-induced ^{42}K -release at more than three time intervals a sharp increase was observed between the 5 and 11 minute exposure times (Fig. 3B).

The exposure of the cells to ^{35}S methionine during an entire generation cycle gave us an excellent tool to measure the detergent-induced release of labelled proteins and also to use these proteins for

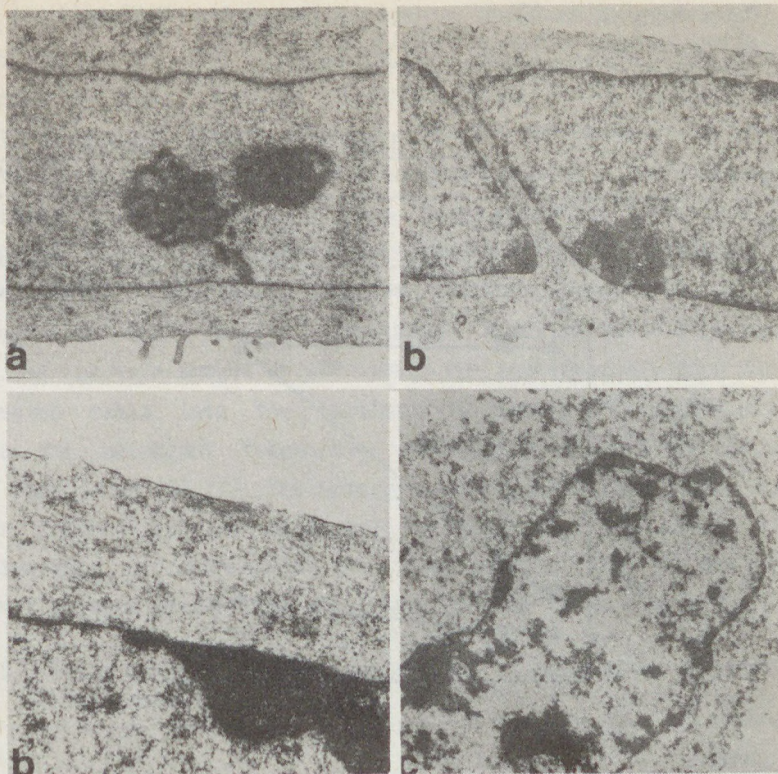


Fig 2. Electron microscopic view of a control untreated H-50 cell (a) and H-50 cells treated with Brij 58 for 2 minutes (b and b') and 5 minutes (c). (a,b and c x 6.120 and b' x 9.550).

two dimensional gel electrophoretic analyses. In case of the Brij 58 treatment, at the 2 minute exposure time only 0.5% and after 5 minutes still only 1.2% of the [35 S] methionine labelled proteins were released from the cells (Table 2). By the end of the 20 minute exposure the Brij 58 mobilized 45% of the proteins.

The Triton X 100 detergent was found to be more effective than Brij 58 in mobilization of the intracellular proteins. In 2 minutes, Triton X 100 removed as much protein, as Brij 58 did in 20 minutes. The amount of Triton X 100-mobilized proteins was about 54% of the total labelled proteins by the end of the 20 minute exposure (Table 2).

TABLE 2 Percentage of labelled proteins released from H-50 cells after various exposure to Brij 58 and Triton 100 detergents for different time intervals.

Detergents	Time of detergent treatment		
	2 min	5 min	20 min
Brij 58	0.5	1.2	44.4
Triton X 100	44.9	51.3	53.6

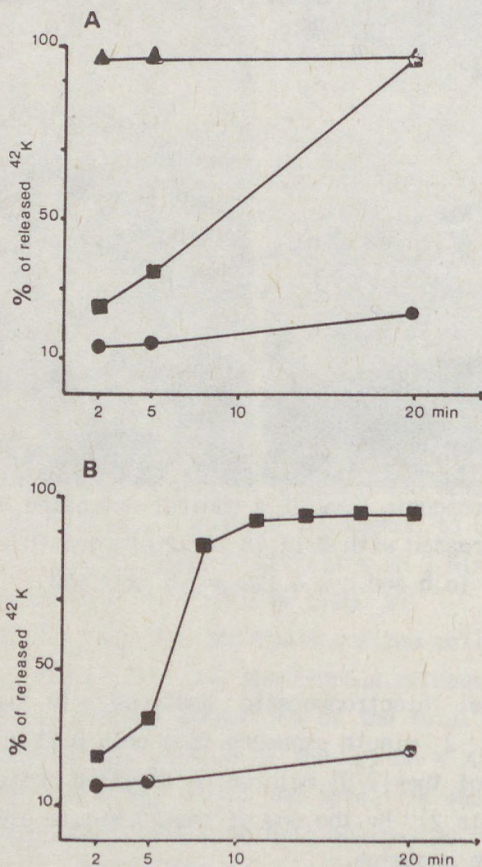


Fig. 3. Release of ^{42}K from H-50 cells previously equilibrated with ^{42}K , then treated either with Brij 58 (squares) or with Triton X 100 (triangles) or with no detergents, control (dots).

In A, exposure times were 2, 5 and 20 minutes. In B, the ^{42}K release from Brij 58 treated cells was analysed at four more exposure times (8,11,14,17 minutes).

For the chemiluminescence measurements of ATP released from the cultured cells, first we had to probe the Brij 58 and Triton X 100 detergents in respect to their possible inhibitory effect on the luciferin-luciferase reaction. As we found, neither Triton X 100 nor Brij 58 inhibited the reaction (20,21). For the ATP release studies, the cells, i.e. human carcinoma cell line (HEp-2) and human embryonal fibroblast (HEF) were grown on 0.5x2.4 cm sized cover glasses. The cover glass cultures were placed into a specially modified stirred sample cell of an Applied Photophysics SP8X Spectro Fluorimeter supplied with a photon counting unit. A certain amount of ATP leaked out spontaneously from the control, untreated cells into the luciferin-luciferase ATP reagent mixture, but after the detergent treatment, much higher released ATP concentrations were registered (20,21). The detergent-induced ATP release was much faster after Triton X 100 than after Brij 58 treatment. Furthermore, there was a considerable difference in the time required for the Brij 58-induced maximal release of ATP from HEp-2 and from HEF cells (Table 3).

TABLE 3 Time required for the maximal ATP release after detergent treatment.^x

	Minutes	
	Triton X 100	Brij 58
HEp-2	2.3 ± 7	7.4 ± 1.3
	n = 9	n = 13
HEF	3.2 ± 1.1	11.3 ± 0.9
	n = 6	n = 8

^x All data are given as mean $\pm$ standard error of the mean.

Evaluation of findings on the detergent-induced release kinetics of intracellular K⁺, ATP and proteins.

In the last 20 years, detergents, mainly the non-ionic detergents have been succesfully used in the discovery of the filamentous cytomatrix, frequently called detergent-resistant cytoskeleton (11,12,15,16,26,27,32). While the detergent-resistant cellular structures hold about 25-35% of the total cellular proteins, the larger sum, the detergent mobile proteins (65-75% of total proteins) and several other cellular components have been less frequently studied, and their release kinetics is practically unknown. Now, our findings indicate that the analysis of the detergent induced release kinetics of several major cellular components like

proteins, ATP, and K^+ can lead us to newer insights into their dynamic associations, in other words into the organization of the living mass. The findings what we are referring to here, can be summarized briefly as the followings: The cells are "opened" by the detergent molecules because their lipid membranes are removed, but the intracellular K^+ , ATP and proteins, previously declared as soluble components, are not released immediately. The time gap between the detergent-induced removal of the lipid membranes and the release of the listed major intracellular components is not only falsifying the free intracellular solution theories, but support the association concepts by direct experimental findings. If K^+ , ATP (Mg-ATP) and proteins were freely dissolved in the cell water they would have been released more rapidly after removal of the lipid membrane than we observed. On the basis of the diffusion coefficients of K^+ , ATP or even that of a 40.-80.000 dalton size protein molecule, the equilibrium should take place in seconds from a lymphocyte of 4 μm in diameter or from a 2-4 μm thick monolayer culture cell after "opening" them by the detergent-induced removal of their lipid membranes. The release of K^+ , ATP and proteins is definitely not a passive process following the removal of the lipid membranes. As we observed, the K^+ -, ATP- and protein-mobilizing effect of the two detergents showed considerable differences (18,19). The Triton X 100-induced release of K^+ , ATP and proteins was so rapid that there was not time for study of their release kinetics, but Brij 58 allowed us time to study the release kinetics of these three major cellular components very carefully. The release kinetics of K^+ showed a sigmoid curve with a sharp increase between 5 and 8 minutes. Similarly there was hardly any protein released from Brij 58-treated cells after 2 and 5 minutes, but practically all of the detergent-mobile proteins were released after 20 minutes. The Brij 58-induced ATP release reached the maximum after 7.4 and 11.3 minutes in case of HEp-2 and HEF cells, respectively. From these observations, we hypothesize that proteins, ATP, and K^+ are dynamically co-compartmentalized inside the living cell. Although, the structure and function of such protein - ATP - K^+ co-compartments have not been well clarified yet, there are several findings supporting the existence of such co-compartments inside the living cell.

Dynamic protein - ATP - K^+ co-compartments inside the living cell.

J.S. Fruton wrote in 1979: "Throughout the nineteenth century, the energy-rich character of protoplasmic proteins was emphasized. - A common feature of the hypotheses was the assumption that upon the death of the cell, the energy-rich living proteins are converted to dead proteins which represent the well-known materials isolated from biological sources" (8). Now it is tempting to believe that our hypothetical dynamic protein - ATP - K^+ co-compartments and the "energy-rich living proteins" discussed in Fruton's paper are the same. Nevertheless, there are several questions to be answered.

Is it possible that proteins inside the living cell are in a different state than in the test tube after isolation?

Can it be that proteins released from the cell are converted into a condition different from that previously existing inside the intact cell?

According to our findings, the answer to both questions is yes. Certainly, the selective K^+ -accumulation is lost, once the mobile proteins are released from the cells cytomatrix.

To understand Fruton's "energy-rich living proteins" which have the capability of selective K-accumulation, we would need to know, how the dynamic protein - ATP - K^+ co-compartments are built up within the living cell. Now, we will try to bring together the solid observations which indicate that the mobile proteins, the K^+ and the ATP molecules are co-compartmentalized with the proteins of the cytomatrix.

1. The proteins of a living cell can be divided into two groups:

a.) Twenty five - thirty percent of proteins of a living cell resists the non-ionic detergent treatment and always exists in an extended, fibrillar form (12,15,26,29,32).

b.) The rest of the intracellular proteins (60-70%) is also not freely dissolved in the cell water (16,19,27). This larger sum of proteins can either be loosely bound to the skeleton in a form of the three-dimensional microtrabecular lattice (29,30,31) or can be condensed in "coacervate" forms surrounded by the three dimensionally arranged resistant protein filaments (33,34).

2.) The ATP and some other molecules "energize" the proteins by their association or adsorption and not by spliced, high-energy phosphate groups (1,22,24,25).

3.) The adsorbed ATP and other "energizing" molecules induce high electron density sites in the supramolecularly arranged protein complexes (9,22,23,25,36,37).

4.) There are short range electrostatic interactions between proteins and K^+ at the high electron density sites (3,5,10,18,19,23,25).

5.) The H_2O molecules are structured on the surface of proteins in a way that the more the proteins are in the extended form the higher is the structural stability of H_2O (2,10,22,23,28,38).

6.) ATP can enhance the water-structuring capability of proteins (22,23,25,38).

In summary, the "energy-rich living proteins" of Fruton or the supramolecular organization of the intracellular proteins, as we can see it now, requires both types of proteins, i.e. the fibrillar protein network and the potentially mobile proteins. The co-compartments that we hypothesize in this paper are the three-dimensional arrangements of these two types of proteins, ATP, H_2O and K^+ in close association with them. The anatomical basis of the micro compartments for K^+ is the three-dimensional protein skeleton with changeable packing density and the potentially mobile proteins with the capability of translocation, accumulation, association and transition from a globular to a fibrillar state.

Because the ATP molecules are associating with the proteins, high electron density sites are induced and the water-structuring capability of proteins are enhanced (22,23). This results in strong protein dependent water structures with low dielectric constant. In such a low-dielectric constant environment the "unhydrated" potassium ions can participate in a short-range electrostatic interaction like a "ping-pong" ball with the electrons at the high electron density sites of the proteins (13) and by this the potassium ions or potassium in a "quasi-atomic state" become the central elements of the feed back loops dynamically preserving the high electron density sites, i.e. the high energy state of the protein structures. Our protein - ATP - K^+ co-compartments can be considered as self-organized systems where the elements interact in a highly co-operative manner (35). A very schematic summary of the view of the dynamic protein - ATP - K^+ co-compartments with indications to the energy production, energy dissipation, and prevention of the energy dissipation is placed here at the very end of the paper (Fig. 4).

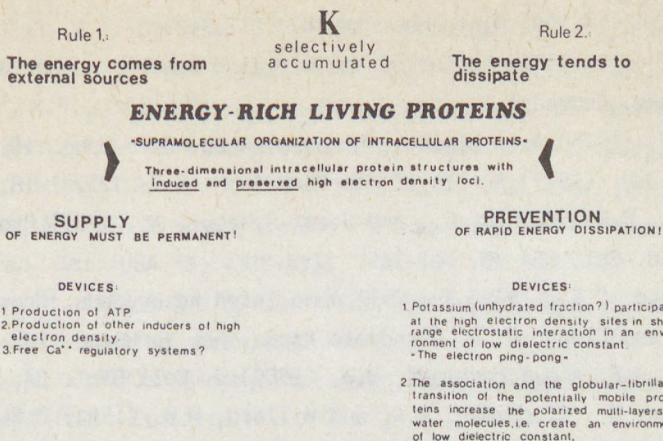


Fig. 4. A summarized "picture" of the "energized" protein structures capable of selective K^+ accumulation.

Conclusion: Neither K^+ , ATP, nor the previously considered soluble proteins are freely dissolved in the cell water. Two types of intracellular proteins, the stabilized fibrillar proteins and the potentially mobile proteins along with H_2O , ATP, and K^+ are co-compartmentalized inside the living cell.

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DISCUSSION

Clegg: One of the problems we've had with this kind of approach, which is a good one, is the kind of ionic environment you provide to the cells. How much data do you have on the kinds of environments you present to these detergent-treated preparations.

Kellermayer: In these experiments the medium we used had 1-1.5 mM potassium, 18-20 mM NaCl, and a pH of 7.2. We did some different experiments, too, but in general the selected concentrations for K^+ and Na^+ were close to one tenth of the concentration values of the extracellular space. This is what we think might be close to free potassium and sodium concentrations inside the cell.

Clegg: Don't you think you'd be better advised to use an environment that's more like, the intracellular one? Because as soon as that detergent hits that cell surface the cell is looking at an effectively extracellular circumstance. Whatever you provide out there, is going to go in.

Kellermayer: We did many experiments with the steady state studies, either with detergent resisant cytoskeleton or with the mobilized proteins. The concentration range of K^+ and Na^+ was from 0 up to 100 mM. We just didn't have enough time to apply buffers with larger concentration variations of K^+ and Na^+ in these release kinetics studies. Rather we went on to study different cellular components. For example, if you change the Ca^{2+} concentration, you can have significant influence on the diffusion of molecules. So, that's again a beautiful experiment, to change the Ca^{2+} concentration.

Osborn: Have you looked at the effect of drugs, I mean cytochalasin, for example.

Kellermayer: No, I have not yet looked at the effect of drugs.

Németh: Are the Brij-treated cells dead cells, or are the effects reversible?

Kellermayer: We had three types of approaches: 1. To study the release kinetics, 2. To extend the decomposition time by dilution of the detergent and change the medium. 3. To try to put the released components all back together. In other words, not to break up the cells to a great extent, and then try to reconstruct the cell. This third approach is called the "giant cell" model. At the beginning, or before the detergent molecules reach the cells they are living, intact cells. I don't know at what moment one can

call the cell (attacked by the detergents) still alive. If the selective potassium accumulation is a sign of the organization of the living material, then I would say they are still maintaining an elementary life phenomenon.

Németh: You called this "agony", right?

Kellermayer: Yes, probably they are "agonizing".

Cameron: On the last slide you had something I considered rather speculative and you provided no support for it, that is, that globular proteins make a transition to fibrillar proteins. That's very important. What kind of evidence you have for that?

Kellermayer: There is not much definitive evidence for the globular-fibrillar transition inside the living cell. Bradbury's finding about the H_1 histone can be considered as such kind of transition. One end of the H_1 histone molecule is always fibrillar but the other end is globular. The globular end opens, when its association occurs at a given ionic strength. Another bit of evidence may be the microtrabecular lattice itself. If you accept that the same proteins are in fibrillar form in the lattice and globular after their isolation from the cell. Finally, the microtubule-associated proteins show certain transitions from globular to fibrillar arrangements in fine EM studies.

Ling: More and more goodies! Really a pleasure to listen to all of this. I think you have produced, and with more work you will further extend the time, during which this prolonged accumulation will persist. And it reaffirmed some findings which really have been in the literature for quite some time. One is a negative experiment that is always hard to handle, because, it could be negative because you are not skilled enough, or it could be simply that it could not be done. This experiment was a beauty and, if it had been successfully carried out, would have proven the membrane pump theory. Namely, you can remove all the cytoplasm from a squid axon. And the squid axon membrane retains all the normal activities indicating sensitivity to ouabain, action potentials and time constants exactly the same as usual. In that case, if you put sea water and energizing materials like ATP and some creatine phosphate, then you tie both ends and you incubate this little sack in sea water. And you would anticipate the pump to work. And in this case gradually the sea water will be replaced by isotonic potassium. This experiment had been extensively tried, by a group of the most skilled scientists at the physiological

laboratory in Cambridge University. And this was disclosed at the Johnson Foundation in 1963 by Professor Richard Keynes. Now this experiment had been tried by many others, and yet never failed to reproduce the failure. Just imagine, if you have half a cell, an apple cut into one half. You expose the cut end alone to this outside world, the solution, and then instead of letting the other half also be exposed to the solution, you suspend in air with a gasket. The air will completely make the pump nonfunctional, because a pump needs either a sink, or a source, and air doesn't provide either of them. And it was done. And it accumulates potassium and excludes sodium exactly the same. But I think there is nothing more wonderful about simply removing the membrane altogether. And that was what you did. And well, I'm very happy to hear it.

Kellermayer: Thank you!

Pollack: You were talking mostly about potassium, a monovalent cation. What about divalent cations, for example in muscle, Ca^{2+} is released and binds to other sites. Obviously it needs to be somewhat diffusible to get from one site to the other. So, would you comment on the relative diffusibility of divalent cations.

Kellermayer: I like this question. Yes, after "opening" the cells by detergent treatment we can expect different release kinetics for Ca^{2+} , Mg^{2+} and many inorganic and organic constituents. We have already done experiments on the detergent-treated lymphocytes but the actual finding on Ca^{2+} and Mg^{2+} are very much dependent on the Ca^{2+} and Mg^{2+} concentration in the medium the detergent was solubilized in. You might find accumulation instead of release.

DETERMINATION OF FREE AND BOUND POTASSIUM IN MUSCLE BY ELECTRON MICROSCOPY

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A basic feature of virtually all living cells is their ability to accumulate K^+ ions selectively and to exclude Na^+ ions partly: in the extracellular environment of a cell the Na^+ concentration is high and the K^+ concentration is low whereas the cell contains much K^+ and small amounts of Na^+ . Until today two totally different explanations of this phenomenon are discussed. The commonly taught membrane pump theory maintains that a very thin cell membrane separates the extracellular space from the intracellular pool and that membrane situated pumps are responsible for the asymmetric ion distribution. The membrane pump theory is based on the assumption that virtually all cellular K^+ is freely dissolved in free cellular water. Opposite to this view are the theories which try to explain cellular ion accumulation and exclusion by properties of the entire cytoplasm (Troschin, 1958; Ling, 1962; Ernst, 1963). Among these the association-induction hypothesis (AIH) of Ling (1962, 1984) is the most advanced from both a theoretical and experimental standpoint. This theory is based on the assumption that cellular proteins, ions and water exist in close cooperative association maintained in a metastable high energy state; according to this theory K^+ is accumulated in living cells because it is weakly bound (adsorbed) to negative charged sites of cytoplasmic proteins with high selectivity over Na^+ and cellular Na^+ is kept at low concentrations because of a low partition coefficient between protein-influenced cell water and extracellular water.

Stimulated by this controversy and mainly impressed by Ling's demonstration that under certain conditions the frog muscle cell commands less than one tenth of the energy needed to operate only one of the postulated pumps (the Na^+ pump, see Ling, 1962, pp. 189-212) I looked for

experiments suitable to test the alternative theories. From the following established facts I concluded that a precise localization of K^+ in the striated muscle could answer the question whether cell K^+ is free or bound: 1) In the striated muscle the water is not evenly distributed; the water content is about 10 % higher in the I band compared to the A band (Huxley and Niedergerke, 1958). 2) The putative binding sites for K^+ are β - and γ -carboxyl groups; myosin contributes more than 60% of the β - and

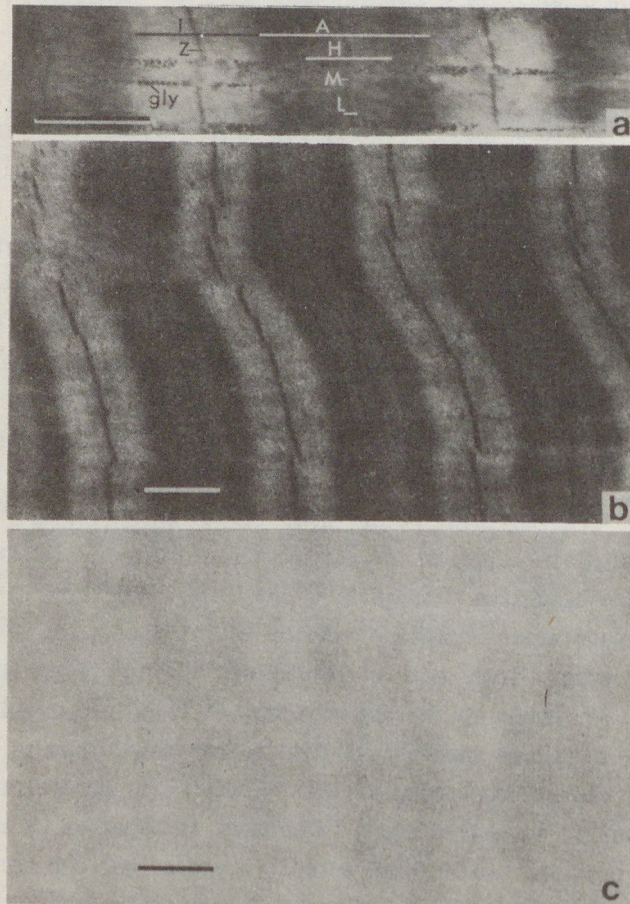


Fig. 1: Electron micrographs of dry-cut, unstained sections of freeze-dried frog sartorius muscles. Living muscles had been loaded with Cs^+ (a) and Tl^+ (b) prior to freeze-fixation, freeze-drying and embedding. (c) Normal K^+ containing muscle. Abbreviations: A=A band; H=H zone; M=M line; L=L zone; I=I band; Z=Z line; glycogen. Scale bars = 1 μm . (a) from Edelmann (1977b), (b, c) from Edelmann (1984a). Reprinted by permissions.

γ -carboxyl chains of all the muscle proteins and is found primarily within the A bands of the striated muscle (Ling, 1977b). Hence the membrane pump theory would predict a slightly higher K^+ concentration in the I band whereas the AIH postulates a preferential K^+ accumulation in the A band.

Now the following question had to be answered: Which method can be used to detect the "true" subcellular K^+ distribution of a living muscle? It was evident that the results of earlier trials of K^+ localization in striated muscle (e.g. Macallum, 1905; Nesterov and Tigyi-Sebes, 1965; Kállay and Tigyi-Sebes, 1969) had not been accepted because dislocation of the ions during preparative steps could not be ruled out. Hence it was necessary to develop new independent techniques for electron microscopy and to check whether ion redistribution artifacts during preparative procedures can be avoided. Associated with this goal was the problem how to detect the cellular K^+ distribution in the most effective way. Having in mind the following considerations I decided to use the method of transmission electron microscopy: It is an established fact that living cells accumulate Rb^+ , Cs^+ , and Tl^+ according to the same mechanism as K^+ i.e. by a pumping mechanism or by adsorption to negative fixed charges (Numerous investigators have demonstrated the similarity of cellular uptake of K^+ and of the K^+ surrogates Rb^+ , Cs^+ , and Tl^+ . See e.g. Epstein and Hagen, 1952; Lubin and Schneider, 1957; Mullins and Moore, 1960; Ling and Ochsenfeld, 1966; Bange and Van Iren, 1970; Van Iren and Van der Spiegel, 1975). Specifically, it has been shown with living frog sartorius muscle that alkali-metal ions and Tl^+ replace each other reversibly and in a mole-for-mole fashion under physiological conditions (Ling and Bohr, 1971; Ling, 1977a). The subcellular localization of Rb^+ , Cs^+ , or Tl^+ would therefore imply detection of sites normal occupied by K^+ . Especially Cs^+ and Tl^+ with the atomic weights of 133 and 204 respectively should be directly visible under the transmission electron microscope if these electron dense ions are unevenly distributed in a muscle preparation.

In 1976 (Edelmann, 1977a, 1977b), the first electron microscopic visualization of Cs^+ and Tl^+ in striated muscle was possible after developing a new freeze-drying and embedding technique (Edelmann, 1978, 1979, 1986); dry-cut sections from Cs^+ or Tl^+ loaded muscles, prepared according to this cryomethod, showed a preferential accumulation of the electron dense ions in the A bands especially at the outer edges and at Z lines (Fig. 1). In the following years this finding was confirmed in

different laboratories by additional new independent methods (see review by Edelmann, 1984a): autoradiography of air-dried single fibers using ^{134}Cs and ^{204}Tl (Ling, 1977a); electron probe x-ray microanalysis (EPM) of K in air-dried myofibrils (Tromit s and Tigyi-Sebes, 1979); autoradiography of frozen hydrated single fibers at liquid nitrogen temperature using ^{86}Rb and ^{134}Cs (Edelmann, 1980); EPM of freeze-dried cryosections of muscle containing the normal concentration of K^+ or loaded with Rb^+ , Cs^+ or Tl^+ (Edelmann, 1983); visualization of Tl in frozen hydrated cryosections (Edelmann, 1984b). In addition, it could be shown with sections of freeze-dried, embedded muscle that the cellular proteins can be stained with Cs^+ (Fig. 2; Edelmann, 1984a, 1986a): Cs^+ is preferentially bound at the same sites which accumulate this ion in the living muscle (compare Fig. 1a with Fig. 2a). The binding capacity of the muscle proteins for Cs^+ is lost in glutaraldehyde fixed preparations (Fig. 2c).

Taken together, these results fulfill the expectations of the AIH and are not in harmony with the predictions of the membrane pump theory; it must be concluded that K^+ accumulation in living muscle is mainly due to K^+ binding at cellular proteins (Edelmann, 1984a).

However, even if it would be possible to demonstrate quantitatively the correspondence of the Cs^+ distribution in "stained" sections and in sections of freeze-dried and embedded Cs^+ loaded muscle (or freeze-dried cryosections), one could argue that it is not yet proven that still a considerable amount of the K^+ surrogate is dissolved in cellular water. In order to prove by analytical electron microscopy that the Cs^+ or Tl^+ concentration in cell water is at least as low as in the extracellular solution (as postulated by the AIH) it would be necessary to analyse cellular areas which contain only water or only water and solid components (proteins) which do not bind the ions. The following experiments with frozen hydrated cryosections suggest that this difficult problem may eventually be resolved in the future:

Studies in which most of cellular K^+ of frog sartorius muscle has been replaced with Tl^+ as described by Ling (1977a), the Tl^+ loaded muscles and normal K^+ containing muscles have been frozen on a LN_2 cooled Cu-block and cryosectioned at -160°C in a REICHERT FC 4 cryoultramicrotome. The frozen hydrated sections have been transferred into a ZEISS cryo transmission electron microscope (EM 10 CR) and photographed while still in the frozen hydrated state (-160°C). The

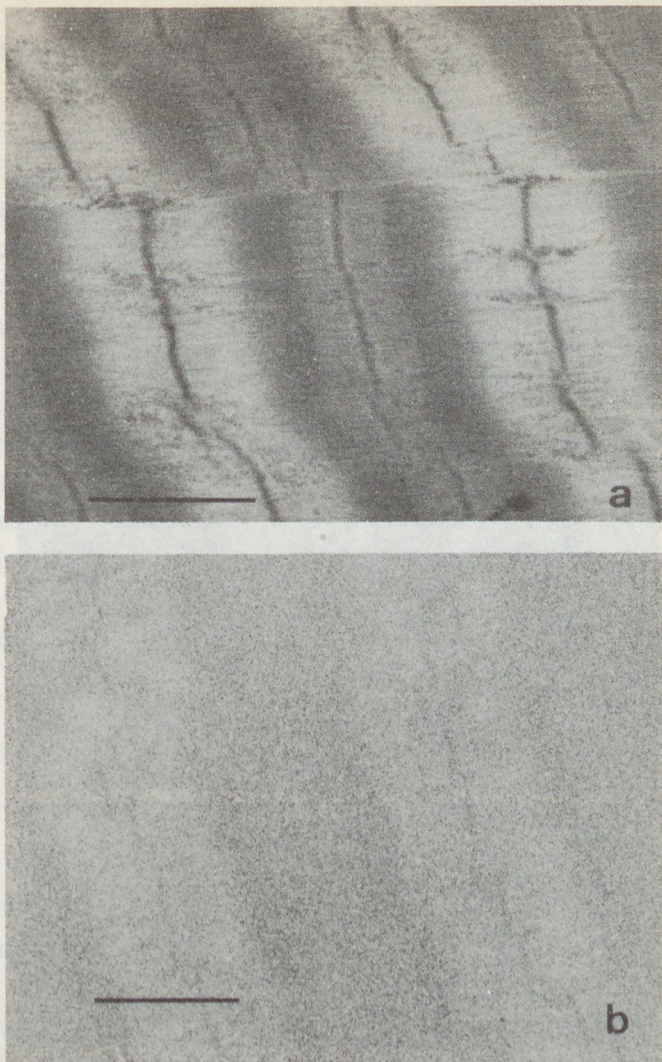


Fig. 2: Muscle sections "stained" with a solution containing 100 mM LiCl and 10 mM CsCl as described by Edelmann (1984a). (a) Freeze-dried and embedded muscle. (b) Glutaraldehyde fixed and embedded muscle. Cs^+ binding sites are only visualized in the freeze-dried preparation. Scale bars = 1 μm . From Edelmann (1986a). Reprinted by permission.

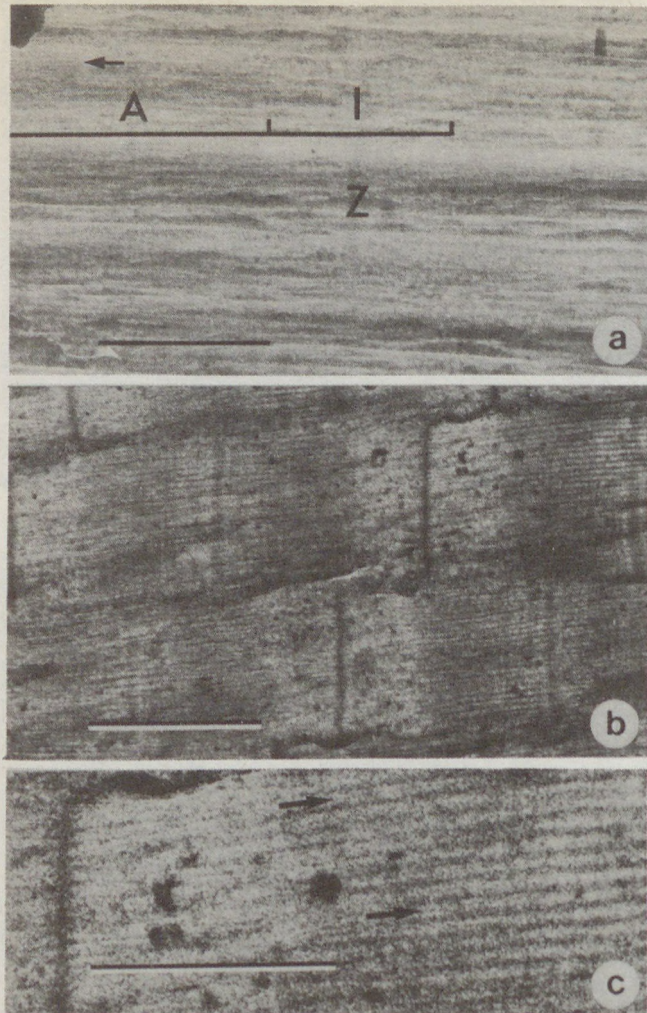


Fig. 3: Frozen hydrated cryosections of frog sartorius muscle.

(a) Normal K^+ containing muscle. Only very faint ultrastructural details can be seen. A = A band; I = I band; Z = Z line. Arrow points to a myosin filament. (b) Tl^+ containing muscle (see text). Dark A bands and dark Z lines indicate sites of preferential Tl^+ accumulation in the living cell. The Tl^+ accumulation pattern is similar to the Cs^+ and Tl^+ patterns shown in Fig. 1 and Fig. 2. Higher magnification (c) shows that darkening of the A band is mainly due to Tl^+ accumulation at thick myosin filaments between which thin actin filaments (arrows) can be seen. Scale bars = $1\ \mu m$ in (a) and (b), $0.5\ \mu m$ in (c).

sections of normal K^+ containing muscles show only very faint ultrastructural details such as Z lines and myofilaments (Fig. 3a). On the other hand, the frozen hydrated cryosections of Tl^+ loaded muscle are well stained (Fig. 3b). The visualized Tl^+ accumulation pattern of the living muscle confirms again the results obtained with the above mentioned independent techniques namely that K^+ and K^+ surrogates are preferentially accumulated in the A bands and at Z lines. In addition, Fig. 3c shows that Tl^+ is accumulated at myosin and actin filaments; between the visualized protein filaments we see water (and protein?) filled spaces which apparently contain rather few of the electron dense Tl . This finding strongly supports the view that most of cellular Tl^+ is bound and that the Tl^+ concentration in cell water is very low. It is expected that microdensitometry will be suitable to quantitate the visualized Tl^+ distribution; hence, a quantitative determination of K^+ binding sites at different proteins of the living muscle seems to be possible.

Acknowledgement

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DISCUSSION

Pollack. You showed some really beautiful pictures. As you pointed out, the concentrations of potassium, cesium, and rubidium, seem to be highest in the flanking regions of the A-band, not in the central region. And it seemed to me that those regions with the highest concentration corresponded to the regions of overlap of thick and thin filaments.

Edelman: Yes, that's precisely what it is. The pictures showing the accumulation of rubidium, cesium or thallium are very similar to the well known pictures obtained from uranium stained sections of chemically fixed and conventionally prepared muscle; in both cases the regions of overlap appear relatively dark due to a preferential accumulation of electron dense ions. It is assumed that in both cases the heavy ions are bound to fixed negative charges of proteins. The binding of alkali-metals however, is much weaker than that of uranium and can only be demonstrated with chemically unfixed preparations.

Kellermayer, Jr: As I understood from your lecture, the ions thallium, rubidium, and cesium behave quite similarly as potassium. I ask whether you have an idea or theory why potassium is the unique element chosen by the living cell.

Edelmann: Because it is more available at the surface of the earth. If e.g. rubidium would be available at a similar concentration as potassium we would expect organisms with high cellular rubidium and potassium concentrations.

Kellermayer, Jr: Selective accumulation is an experimental fact. But when you conclude that it is specifically created by actin or myosin, you make a jump (from reality to hypothesis). You can characterize actin and myosin in a biochemical sense, by electrophoresis, but separated in an electrophoretic gel, neither actin nor myosin will selectively accumulate ions although the amino acid side chains are there. I am trying to point out the importance of location, because actin and myosin in muscle differ from the separated ones in that they exist in a "high-energy state" in the intact muscle, according to Dr. Ling.

Edelmann: The question is: how can I maintain the high-energy state and demonstrate binding of alkalimetal ions at proteins in vitro? It seems, that the high-energy state can be maintained at least partly by fast freezing, freeze-drying and embedding.

Mátrai: Do your ions accumulate at membrane, as well?

Edelmann: I haven't looked very carefully at the membranes. In the muscle preparations I have never seen a particular high accumulation of electron dense ions at the cell surface. So I cannot yet answer the question if it is possible to detect potassium binding sites at membranes.

Cameron: I'm thoroughly impressed with the beauty of the contrast in fully hydrated muscle treated with thallium as we have never seen anything with such excellent morphology, so I would like to ask why is there such a good contrast, and has the treatment with thallium influenced the function of that muscle in any way?

Edelmann: Thallium has an atomic weight of 204 which is close to that of e.g. lead which has 207. If the electron dense ions are bound (concentrated) at certain cellular structures we must obtain a good contrast even with frozen hydrated sections. Physiological studies with thallium have been made by Gilbert Ling; he showed that you can replace 80% of potassium in about 6-8 hours with thallium, and the muscle is still alive and contractable.

Ling: The replacement is reversible, you can replace thallium back again with potassium, by increasing the extracellular potassium and decreasing the extracellular thallium.

Tigyí: Your pictures are very attractive, and convincing, but they are indirect evidence. The electronmicroscopic radioautography, which was developed by me is direct evidence, if you use the proper fixation method. If you use osmium-vapor fixation, not liquid, then even after the procedure, you will dilute no more than 10% of the radioactive potassium from the muscle fiber. Therefore it is a more direct evidence for the specific accumulation of potassium. This is only my opinion.

Edelmann: My work was stimulated by findings of your and other's. However, since I could not exclude the possibility of ion redistribution after any chemical fixation I tried new cryomethods and avoided completely chemical fixation.

Pollack: In response to my question about the higher concentrations in the area of overlap, one way to be sure that that's the reason for the differences of concentration is to change the degree of overlap.

Edelmann: I agree. I have found that muscles with smaller sarcomere lengths show wider ion accumulation bands at the outer edges of the A-bands corresponding to the wider overlapping zones.

Pollack: Have you done the same experiments with a muscle that's either contracting or in rigor and do you find any difference?

Edelmann: I have done some experiments with contracting muscle. I found A-band shortening, and also shortening of the ion accumulation bands.

Pollack: What does the accumulation band look like? Is it uniform?

Edelmann: It looked uniform, and not so sharp at the edges.

THE K⁺ EFFLUX AND SWELLING DURING GLYCEROL TREATMENT OF MUSCLE

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My original assumption inherited from Professor Ernst (1) with whom I began my research work, was that some part of K⁺ in the muscle was somehow "bound". I have carried out experiments on muscles with membranes destroyed by different methods (2-5) and all results reinforced the view about the existence of the bound K fraction. After glycerol treatments the muscle could not be regarded any more as a living matter, but some conclusions about the state of ions might be drawn as the muscle proteins kept their ability for contraction in this unique condition.

After the pretreatment of 11.6 M glycerol at +2 °C, one sartorius muscle of a frog was put in 5.8 M glycerol solution containing 55 mM NaCl, and the other in ion-free 5.8 M glycerol solution at -12°C. At different time intervals (from 1 to 216 hours) the muscles were removed and their K contents were determined by flame photometry.

K⁺ and Na⁺ content of intact sartorius muscles were 76 meq.K⁺ and 24 meq.Na⁺ per kg wet weight. The initial K⁺ and Na⁺ concentrations, following the 11.6 M glycerol treatment, were 69 and 16 meq./kg wet wt., respectively. The K⁺ concentration of the muscle as a function of time of exposure to the 5.8 M glycerol treatment was approximated by the sum of two exponential terms with different rate constants, namely as

$$K^+(t) = A \exp(Bt) + C \exp(Dt).$$

The experimental K⁺ concentrations fit very well this type of theoretical curve with the correlation index of greater than 0.98 (Fig. 1). Two fractions of potassium were found: one fraction, with a half-life of almost 1 hour; and a second (slower) fraction with half-life of about 75 hours. The difference between the two rate constants was obviously significant (Table 1).

Table 1

	Faster potassium		Slower potassium		correlation index
	A	B	C	D	
	Potassium conc. (meq. K ⁺ /kg wet wt.)	Rate constant (hour ⁻¹)	Potassium conc. (meq. K ⁺ /kg wet wt.)	Rate constant (hour ⁻¹)	
Na ⁺ containing glycerol	33.5	-7.69x10 ⁻¹	34.9	-9.55x10 ⁻³	0.989
ion-free glycerol	22.3	-7.89x10 ⁻¹	44.4	-8.99x10 ⁻³	0.984

$$K^+(t) = A.exp(B.t) + C.exp(D.t)$$

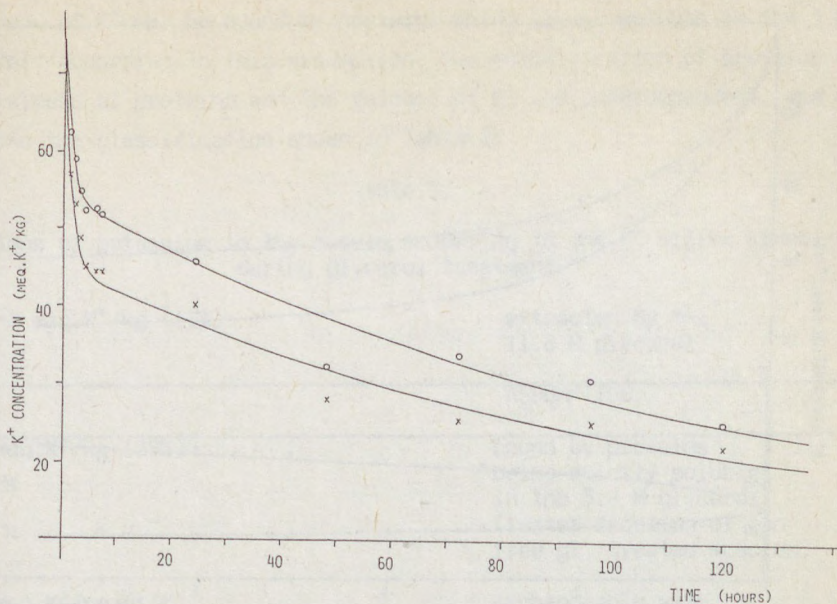


Fig. 1. K^+ concentration (meq. K^+ /kg wet wt.) of frog sartorius muscle as a function of time exposed to glycerol solutions. Each value of the measured concentrations is mean (relative error = 5%) of 20 different muscles. The theoretical curves are sums of two exponential terms: $K^+(t) = A \exp(Bt) + C \exp(Dt)$. The parameters of this equation are computed by means of non linear regression analysis. X=measured K^+ concentrations after Na^+ containing glycerol treatment; O=measured K^+ concentrations after ion-free glycerol treatment.

The faster fraction of K^+ in the muscle treated with Na^+ containing glycerol was larger by 11.0 meq. K^+ /kg wet wt. than that in the muscle in ion-free glycerol. In Fig. 2, the difference in asymptotes of the two faster fractions of K^+ concentration curves are pronounced.

The diffusion coefficient of K^+ in glycerol solution at $-12^\circ C$ is calculated on the basis of viscosity measurement (6), the result of the calculation: $1.92 \times 10^{-6} \text{ cm}^2/\text{s}$. Taking this value for the mobility of free K^+ one would expect the half-life of K^+ in the muscles to be of the order of within one hour. The faster fraction of K^+ corresponds to this expectation. However, one can say definitely about the slower fraction that rate of loss of potassium in cells with damaged membranes is significantly slower than that of free diffusion. This finding supports the view that a significant part of muscle potassium is somehow bound.

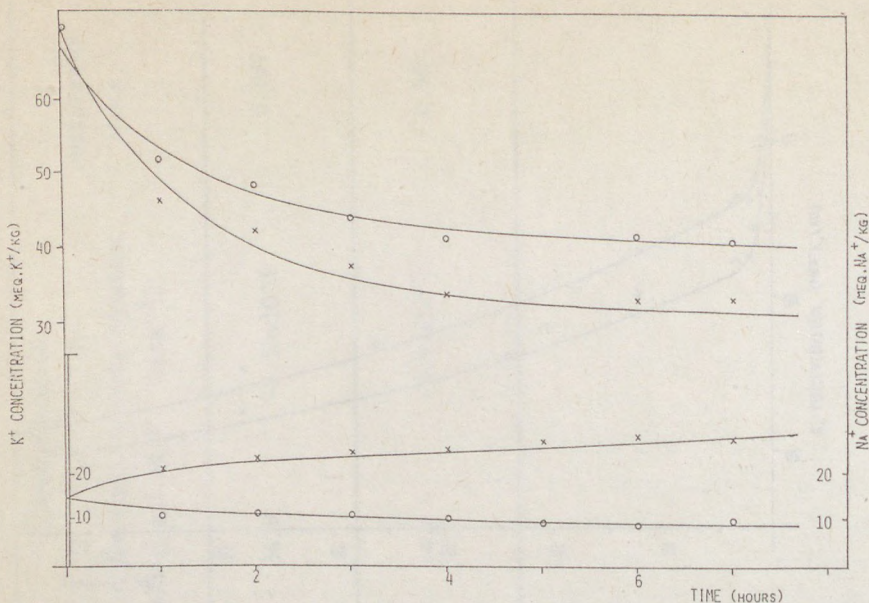


Fig. 2. K^+ and Na^+ concentrations as a function of time of exposure to glycerol solutions. The first seven hours of Fig. 1 are expanded so that the two asymptotes of K^+ concentration curves of the Na^+ containing and ion-free glycerol treatments can be seen. The two lower curves are the theoretical Na^+ concentration curves computed as sums of two exponential terms, too. X=measured K^+ and Na^+ concentrations after Na^+ containing glycerol treatment; O= measured K^+ and Na^+ concentrations after ion-free glycerol

Investigating the effects of different alkali metal ions on the release of the faster K^+ fraction I found that Li^+ and Na^+ ions had similar effect, in the presence of these ions the magnitude of the faster fraction increased. Rb^+ ion, however, caused a retention of K^+ ion (5). The magnitude of the faster fraction of K^+ decreased. Cs^+ ion had no significant effect on the potassium release.

Kellermayer and his coworkers found that the decrease of K^+ ion in Brij 58 treated thymus lymphocytes (7) and H-50 tissue culture cells (8) was correlated with the solubilization of proteins. I assume that in my experiment the two K^+ fractions with different rate constant correspond with the free and bound K^+ fractions of the glycerol treated muscle. The free fraction of K^+ corresponds to the sum of K^+ fraction exchangeable with Na^+ during glycerol treatment, and that K^+ fraction which was originally bound but was quickly soluble in the 5.8 M glycerol. The slower

fraction of K^+ may be bound by proteins being slowly soluble in the 5.8 M glycerol. According to this assumption, the solubilization of proteins and the release of proteins and the release of K^+ are interdependent, and one can make the classification shown in Table 2.

Table 2.

Fractions of potassium in the muscle according to the K^+ efflux kinetics during glycerol treatment

8+2 meq. K^+ /kg (11%)	extracted by the 11.6 M glycerol
ASSUMPTION	
22+3 meq. K^+ /kg (29%)	bound by proteins being quickly soluble in the 5.8 M glycerol (faster fraction of ion free gl. treated muscles)
11+2 meq. K^+ /kg (14%)	exchangeable with Na^+ during glycerol treatment (difference between the asymptotes of ion-free and Na^+ gl. treated muscle)
35+4 meq. K^+ /kg (46%)	bound by proteins being soluble in the 5.8 M glycerol (slower fraction of the gl. treated muscles)

INTACT MUSCLE: 76+8 meq. K^+ /kg (100%)

Ling (9) and Negendank (10) obtained that the maintenance of cell volume in a normal or a swollen state did not require an intact membrane, but the intra- or intermolecular salt linkages of macromolecules are responsible for the regulation of cell volume. These salt linkages are formed primarily between cationic and anionic side chains.

In the present experiment, the muscles swelled in both the ion-free and Na^+ containing glycerol of 5.8 M during the whole time of exposure.

Besides the salt linkages of fixed cationic and anionic sites of proteins I suggest that the bound K^+ fraction partly exists in another type of salt linkage, which has the ability to link the similarly charged sites of proteins. The central cation shares its charge between the resonance anionic (carboxylate, phosphate, sulphate) groups. Similarly,

negative ions could be sandwiched between two positively charged (α -amino, ϵ -amino, guanidyl) groups. According to Lewin (11) interposed water molecules support the bridge called by him as "ion-water-bridging". This type of linkage is very weak and can easily be broken by thermal agitation. While these ion bridges dissociate, swelling occurs in the system. Assuming the existence of this type of K^+ bridge, the K^+ loss and muscle swelling observed in this study may result from the breakage of K^+ bridges.

From the schematic representation of the interior of the glycerol treated muscle (Fig. 3), one can see that during the time of drying the evaporated water originates from two different parts: (i) from the glycerol solution and (ii) from the structured water of hydration shell around the extended random coil of protein. The dry weight of the control intact muscle was 20% of its original wet weight. When the 5.8 M glycerol solution was dried alone, its dry weight was 56% of its original wet weight after overnight drying. Thus, the glycerol solution lost 44% of its original weight when it is dried. The dried glycerol treated muscle should contain the pure protein substance and some dried glycerol being 56% of the total glycerol-water solution penetrated into the muscle during the treatment. "Relative weight" in Fig. 4 means the weight after glycerol treatment divided by the wet weight of the intact muscle, and the dry weight of the glycerol treated muscle after overnight drying divided by the wet weight of the intact muscle in the case of the upper and the lower curve, respectively.

Why should I indicate the part of structured water in Fig. 3? If only the glycerol solution penetrated into the swelling muscle one could get the lower curve from the upper curve (Fig. 4) by a simple calculation. The vertical distance between the upper curve and the horizontal line of 0.20 would equal the glycerol solution in kg gained by the muscle being one kg originally in its intact state. In the graph of Fig. 4 between the horizontal line of 0.20 and the curve of dry weight must be the 56% of the total penetrated glycerol solution, but the dry curve plus 0.44 times vertical distance between the 0.20 and the upper curve does not reach the upper curve. Thus, there must be some extra water, titled by me as the structured water, which could be evaporated.

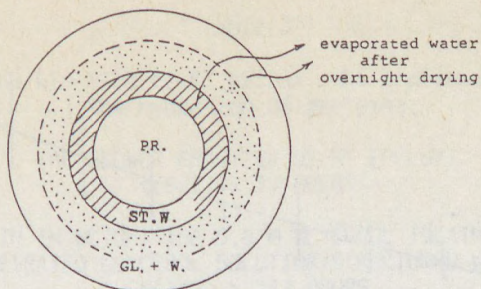


Fig. 3. Schematic representation of the interior of the glycerol treated muscle. During the time of drying the evaporated water originates from two different parts: (i) from the glycerol solution and (ii) from the hydration shell of macromolecules (or structured water). Keys: PR. = pure protein substance; ST.W. = structured water; GL+W. = glycerol solution penetrated the muscle (the dotted part does not evaporate).

The increasing lower curve in Fig. 4 means the increasing amount of glycerol penetrated into the muscle, which is in close relationship with the swelling of glycerol treated muscle.

Using the exponential regression analysis on the curves of wet and dry weights of the swelling muscles, the rate of glycerol and water influx can be calculated.

Comparing the half-lives of K^+ efflux and the glycerol+water influx (Table 3), it is remarkable that the K^+ efflux is about four times faster than the penetration of glycerol or swelling process. It means that there is some part in the muscle which there has been K^+ release, yet not penetrated by glycerol. There must be a rate limiting step after the K^+ release, which retards the processes of glycerol penetration and swelling.

The half-life of the development of the structured water (or hydration shells) is about 267 hours. However, the half-life of the swelling itself (gain in the penetrated glycerol solution) is about 324 hours. Studying the dependence of an effect upon a cause in this swelling system one can say that the cause is the hydration of macromolecules which precedes the effect i.e. the swelling.

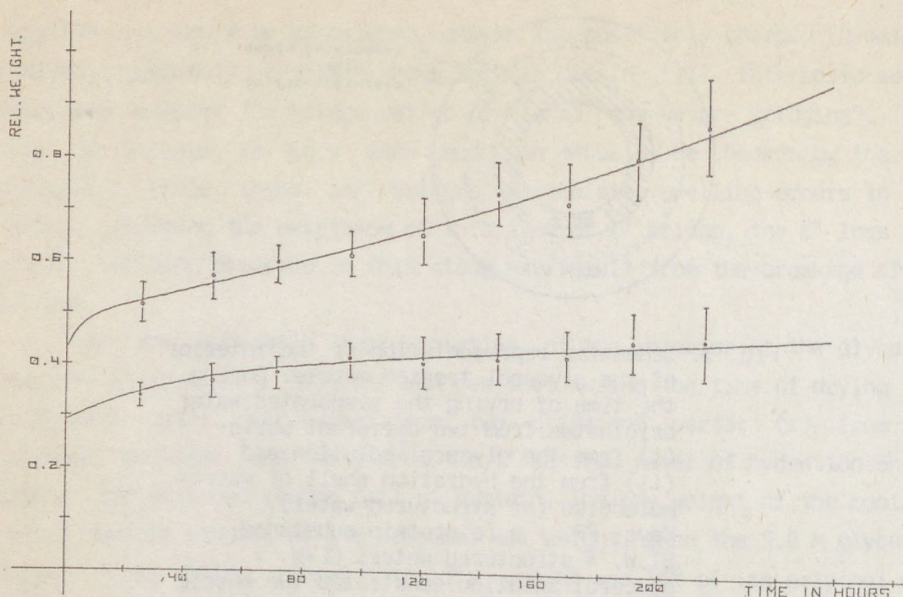


Fig. 4. Wet and dry relative weights of swelling muscles as a function of time of the 5.8 M glycerol treatment. "Relative weight" means the weight after glycerol treatment divided by the wet weight of the intact muscle, and the dry weight of the glycerol treated muscle after overnight drying divided by the wet weight of the intact muscle in the case of the upper and the lower curve, respectively. The horizontal line of 0.20 indicates the relative weight of the dried intact muscle.

In Fig. 5, the simplest model of macromolecular swelling is depicted. In the folded macromolecules, there are many intra- or intermolecular linkages which keep the assembly of macromolecules being compact. However, if the intra- or intermolecular linkages are broken down, hydration process can start along the released thread of macromolecule. The extended rod-like macromolecules expand the intra- and intermolecular space into which more solvent can penetrate. The apparent specific volume of macromolecules is increased. This may be the most primitive biological system which transforms the chemical energy into mechanical work. Expansion work was done by the system at the expense of chemical change.

Table 3

Half-lives of the kinetics of K^+ efflux, the processes of swelling and the hydration of proteins.

THE SLOWER FRACTION OF K^+ EFFLUX:
 $t(1/2) = 77$ HOURS

GAIN IN WEIGHT THE 5.8 M GLYCEROL TREATMENT
 (PENETRATED GLYCEROL SOLUTION+STRUCTURED WATER):
 $t(1/2) = 267$ HOURS

GAIN IN DRY WEIGHT OF THE GLYCEROL TREATED MUSCLE
 AFTER OVERNIGHT DRYING
 (PENETRATED GLYCEROL SOLUTION)
 $t(1/2) = 324$ HOURS

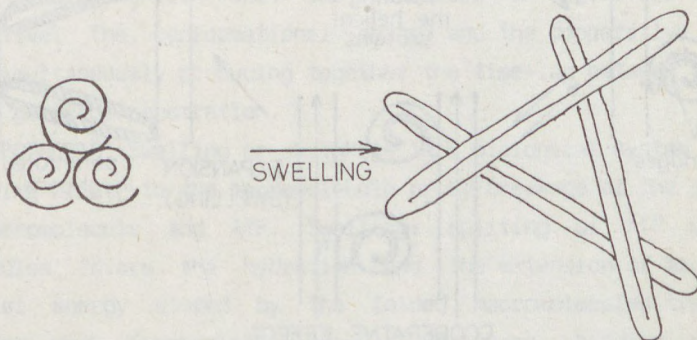


Fig. 5. The simplest model of macromolecular swelling due to hydration process along macromolecules. The hydration extends the folded macromolecule and transforms the chemical energy into mechanical one.

Summarizing, the K^+ efflux precedes the hydration process of macromolecules and this slightly precedes the swelling. Thus, one rate limiting step is the development of the structured water along the macromolecules which takes a longer time after the breakage of intra- and intermolecular linkages of K^+ bridges. The second rate limiting step is the expansion work done by the extended rod-like macromolecules. The expansion of the system (or swelling) is delayed by elastic forces of the

system itself and by the slower penetration of the solvent into the system and, of course, by the external pressure.

In Fig. 6, a schematic model is shown, in which the rate limiting step may be the extension of folded proteins. The result is: more hydrated water and expansion of intermolecular space traversed by the extended rod-like macromolecules. Into this space, more glycerol solution can penetrate.

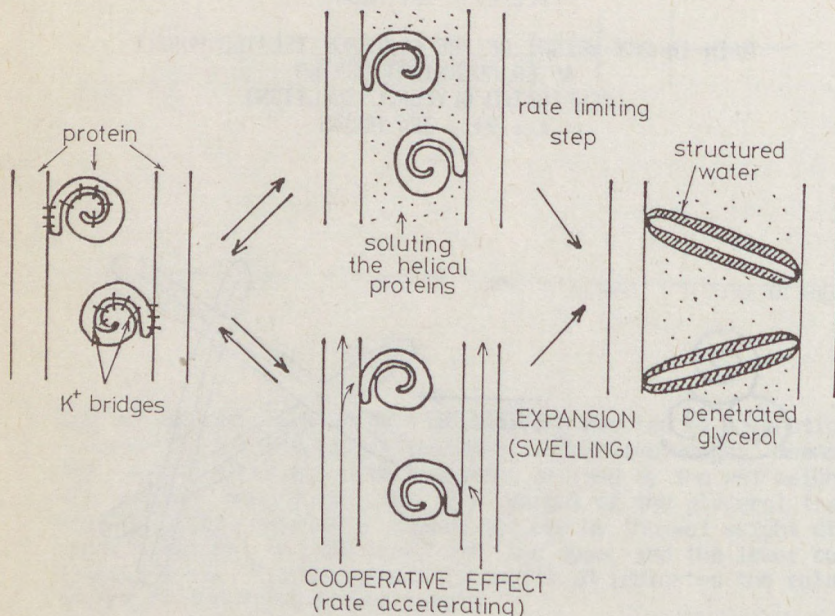


Fig. 6. The time-lag between the K^+ efflux and swelling is interpreted by this schematic model. In the upper route the rate limiting step may be the extension of folded proteins. The lower route shows that the penetration (or swelling) has normal kinetics but the K release is accelerated by cooperative phenomenon.

However, there is another possibility for interpreting the time-lag between the K^+ release and the glycerol penetration (the lower route in Fig. 6). If one assume that the penetration or swelling have normal kinetics but the K^+ release is accelerated somehow. The schematic drawing in Fig. 6 depicts a K^+ release to be an all-or-none phenomenon with the glycerol serving to induce cooperative changes along macromolecular structure. Thus, K^+ release occurs before the glycerol has penetrated the whole fiber. The protein threads in Fig. 6 could be any type of fibrous

filament of the striated muscle or MTL (microtrabecular lattice) of any cell.

Cooperative phenomenon can arise in any connected structure in which the states of all members of the community are interdependent. Any change in the system follows the all-or-none law. It is reminiscent of phase-transition in solid-state physics, and Ling's association-induction hypothesis in biophysics (9). The existence of the cooperative effect would be tested by the existence of the S-shape like K^+ efflux curve. For this purpose the change of K^+ concentration is to be measured in the micro-region of the glycerol treated muscle cell. Due to glycerol treatment there was not any connection among the cells. In the present experiment the resultant K^+ efflux from the structures of independent cells was measured.

I should mention that the processes in Fig. 6 are not certainly alternative. The conformational change and the cooperative effect could occur simultaneously producing together the time-lag between the K^+ efflux and the glycerol penetration.

HYPOTHESIS. Swelling or shrinkage in a biological system is regulated by binding of ATP to the macromolecule or by breakage of the bonds between the macromolecule and ATP. Swelling: splitting of ATP upon the macromolecules favors the hydration and the extension of macromolecules. Potential energy stored by the folded macromolecules transforms into expansion work (mechanical energy). Shrinkage: binding of ATP to the hydrated random coil of a macromolecule causes the release of hydrated water molecules due to temperature effect (reaching the critical temperature of phase-transition in the structured water phase). Having released the hydration shell the macromolecule is forced by its surroundings (external pressure and elastic forces of the system itself) to collapse. The intramolecular linkages are favorable again and the system becomes to be more compact. Compression work is done by the external and elastic forces and by intra- and intermolecular linkages. This energy is stored as the potential energy of the system and looks as it were chemical energy. The real chemical energy is liberated upon the binding of ATP which forces the bound water molecules in hydration shells to release.

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DISCUSSION

Pollack: What happens if you immerse the fiber in glycerol. Does the fiber begin to contract or not?

Hummel: Yes! It does.

Pollack: Then the potassium efflux or the changes that you are describing on your slides are somehow associated with the contractile process. The question is if you disrupt the membrane in a different way that doesn't involve contraction, do you get similar potassium efflux kinetics?

Hummel: There were two different consecutive glycerol treatments. Contraction occurred only with the first treatment. Potassium efflux kinetics was investigated only during the second treatment.

Pollack: Just one related question. If we suppose that during contraction the proteins undergo a transition from a helix to a random coil, this randomness induces perhaps more randomness of water and the release of potassium. Is that a possible consistent kind of idea?

Hummel: It's very much possible.

BLOOD CELL RHEOLOGY AS RELATED TO STRUCTURE

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INTRODUCTION

Blood cells are the natural choice of studying cell mechanics, because they normally float freely in a suspension, and their deformability is vital for the circulation. The latter is obvious, if internal diameters of nutritive capillaries and sizes of blood cells are compared (Table I.)

TABLE I.

Comparison of blood cell and capillary sizes

cell type	diameter	excess	cytoplasmic
	μm	area	viscosity
granulocytes	7.0-8.0	80-160%	13 Pas
monocytes	7.5-8.5	60-110%	-
lymphocytes	6.0-6.2	60-80%	-
erythrocytes	7.0-7.6	60%	0.0060 Pas
blood plasma (37°C)			0.0012 Pas
water (37°C)			0.0007 Pas

Pas (pascal-second = Ns/m^2) is the SI unit of viscosity

Diameters of muscle capillaries are about $5\mu\text{m}$, but may be as small as $3.8\mu\text{m}$ (9).

RHEOLOGICAL PRINCIPLES

The response of a material body to external forces is determined by its structure. Therefore, mechanical studies may disclose important details about the structure of any physical object.

External forces may compress, shear or bend a body, depending on

their direction, and are quantified as compressive shear or bending stresses (31). In any case, the response of a material body may be viscous or elastic deformation. Viscous deformation continues as long as the stress acts, and its rate is proportional to the stress. On cessation of the stress, the body remains deformed. Such response implies no internal structure of the material, only friction between its building particles (examples: water, honey).

The deformation of an elastic material is proportional to the stress applied, and remains constant as long as the force is there. When the stress is removed, an elastic body fully recovers its original shape. Such a material stores energy when deformed, and returns it on recovery. Elastic deformation is always the consequence of an internal network-structure. In a network, the relative positions of the particles within the body are fixed by inter-particle bonds. When subjected to deforming stress, these bonds allow a limited change of distances between particles. As long as the bonds are not broken, the material can recover its original shape. The strength of these bonds may vary enormously. However, the principle of the structure is identical to that in the Eiffel tower, in the spiders web and in a diamant cristal. Material bodies built of more complicated substances (such as living cells), usually have combined elastic and viscous properties, they are more or less viscoelastic. The two basic types of viscoelasticity are shown in Fig. 1.

Adhesivity is one of the most important aspects of cell mechanics, however, it has been only recently analyzed in detail (11).

Although mechanics may look dry, and for some biologists it may appear as irrelevant to life sciences, rheological studies can interpret microscopic and biochemical data related to structure. Some examples in the field of blood cell rheology may illustrate this.

ERYTHROCYTES

The fluidity of erythrocytes is of vital significance keeping blood in flow. This can be visualized by considering a suspension of solid particles of red cell size and shape. At a particle concentration of 0.66, this suspension would be a solid, similar to drying mud.

The well known picture of resting erythrocyte with two symmetrical dimples giving a typical biconcave shape is absent in flowing blood.

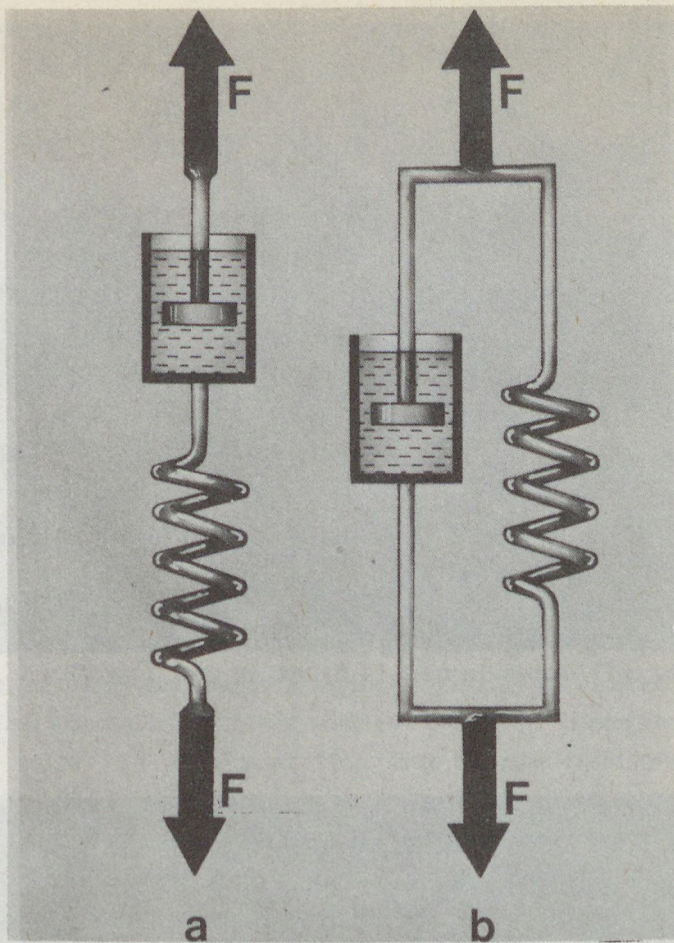


Fig. 1. Viscoelastic bodies.

- a: serially coupled viscous and elastic element. Here the elastic component does not reach across the whole body. Deforming such a structure will result in an initial elastic response, followed by a continuous viscous deformation. Removal of the stress will be followed by only partial recovery of the original shape.
- b: parallel viscous and elastic components. This body responds to stress by an initially viscous deformation, but as deformation proceeds, elastic properties will be more pronounced. Cessation of stress results in slow, but full recovery of the original shape.

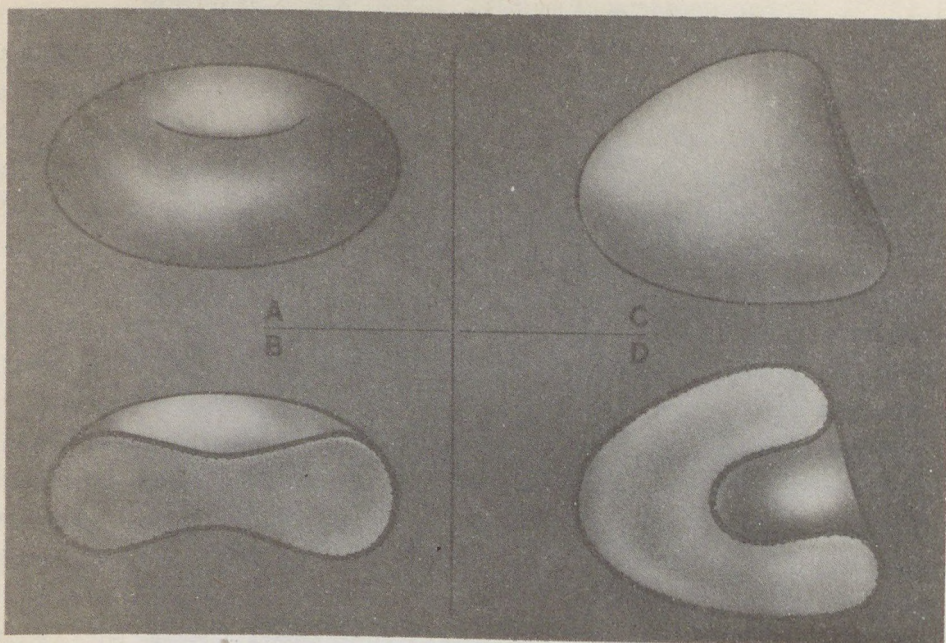
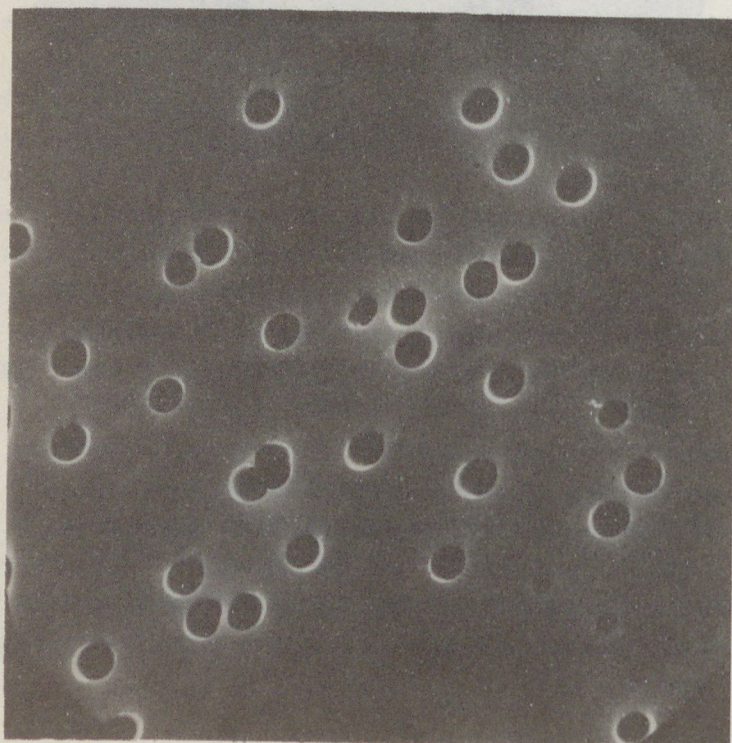


Fig. 2. Red cell shapes in rest (A,B) and in capillary flow (C,D).



There, they are elongated, oriented parallel to streamlines, and the membrane rotates around the cell interior (13). In narrow tubes, such as capillaries, red cell deformations are more complicated (Fig. 2). The internal diameter of the narrowest tube, which red cells can enter, is 2.8 μm . Short holes (e.g. endothelial slits) as narrow as 1 μm may be negotiated by normal erythrocytes.

The mechanics of erythrocytes is determined by 3 basic features:

a: excess surface area

b: viscosity of the cell interior

c: membrane viscoelasticity.

ad a: Red blood cells possess a membrane, larger than needed to cover their body. This is referred to as "excess surface area". The volume of an erythrocyte is about 90 μm^3 , and it has a surface area of 150 μm^2 . However, a sphere of 150 μm^2 surface area can be filled with a volume of 180 μm^3 . The difference (approx. 90 μm^3) allows the flaccidity of the membrane and is essential for cell deformability.

ad b: Red cells contain a hemoglobin solution of about 300 g/l concentration, and this cell interior is usually considered as a simple liquid. The viscosity of this solution (measured as membrane-depleted, hemolysed cells) is 6 mPas at 37°C. From the pure rheological point of view, encapsulating hemoglobin in the red cells does not make much sense, since the overall viscosity of hemolysed blood is about the same as that of intact blood. However, this may be a good method to keep hemoglobin intravascular, and has some biochemical advantages as well.

ad c: The lipid bilayer of erythrocyte membrane is underlined by a skeletal protein network composed of spectrin, actin, protein 4.1 and other macromolecules (29,41). These build up a complex, integral system, essential to the stability of the cell, and responsible for most of its mechanical and other properties (e.g. 23).

Erythrocyte mechanics can be studied as the flow rate of red cells through narrow channels (filtration methods, Fig. 3.). Such methods (e.g. 30) give information about the overall deformability of the cells, but no detailed knowledge about its components. More specific information can be



Fig. 3. Blood cell deformability can be measured by using filters of regular channel size (Nuclepore, 5 μm pore diameter, 250 000 pores/ cm^2).

derived from micropipette studies, however, these methods are less suitable for routine use. Fig. 4 shows the principle of testing erythrocyte membrane deformation, without area dilatation. On application of a suction pressure, the cell flows into the pipette, the flow rate depending on surface shear viscosity of the membrane. When pressure equilibrium is restored (deforming stress released), the membrane elasticity removes the tongue from the pipette. Fig. 5 shows testing membrane of an osmotically swollen cell. Here suction pressure results in membrane area dilatation and membrane bending. Another method is to deform the cell, release it thereafter, and measure the rate of shape recovery (as demonstrated with a leukocyte in Fig. 8).

Micropipette studies have shown, that red cell membrane has a characteristic anisotropy (Table II): it is viscoelastic in surface shear

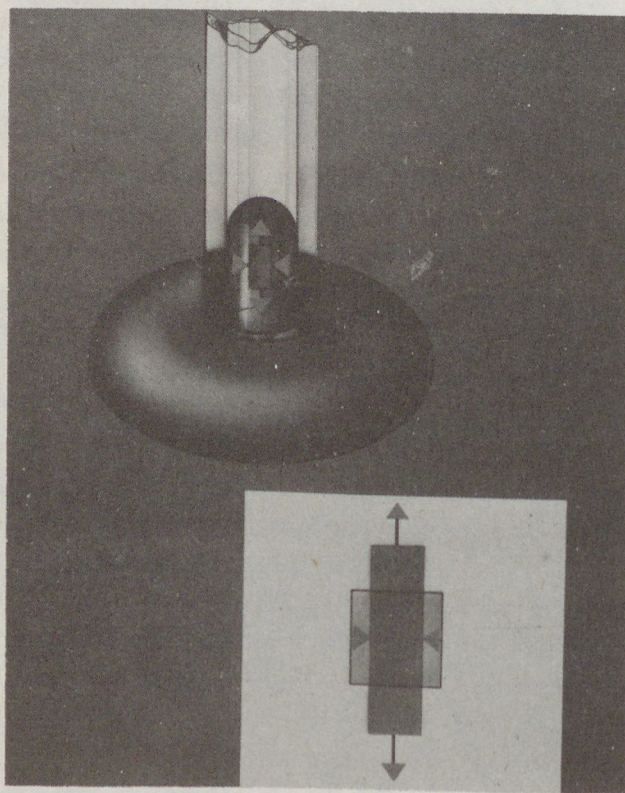


Fig. 4. Micropipette testing of flaccid cell membrane. Surface area remains constant, but shape of the surface element changes. Surface shear viscosity and bending elasticity can be inferred from such experiments.

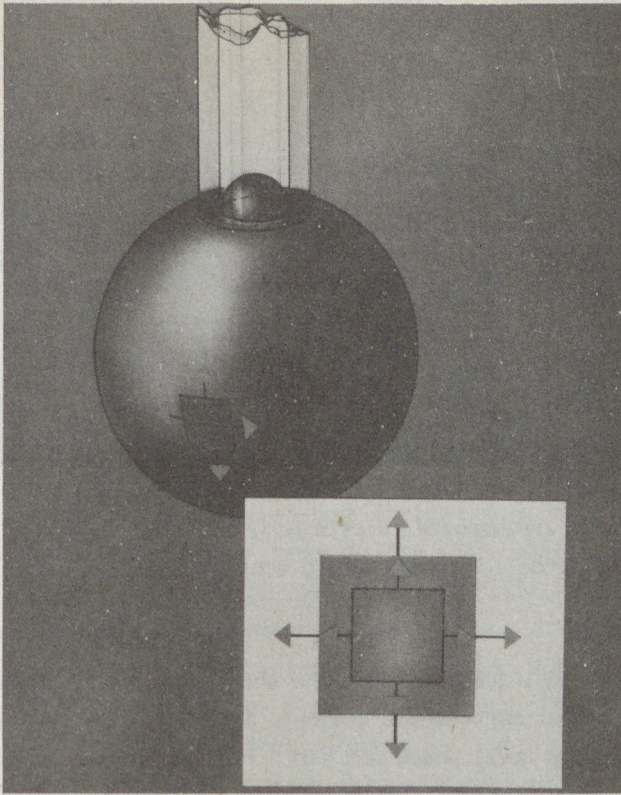


Fig. 5. Micropipette testing of cell membrane by extending surface area of a spherical cell. Dilatation elasticity and viscosity can be inferred from such studies.

deformation (as shown by the model in Fig. 1a), in other words, flows easily around the cell; but it has a defined bending elasticity and strongly resists area expansion.

Table II.
Rheological anisotropy of the red cell membrane

deformation	elastic modulus
surface shear	4×10^{-6} N/m
bending	3×10^{-18} Nm
dilatation	0.1 N/m
deformation	viscosity
surface shear	5×10^{-7} Ns/m
dilatation	3×10^{-2} Ns/m

Ref. (10) and (19)



Fig. 6. Erythrocyte and leukocyte in a blood smear. The shape of erythrocyte is retained, but the leukocyte is flattened, probable due to adhesion.

It has to be pointed out, that the shear flow of the membrane does not have a conventional macroscopic analogy. If a piece of textile or paper is deformed, as the cell membrane in Fig. 4, it must wrinkle to make a tongue, because it cannot undergo surface shear deformation, due to permanently crosslinked network structure. A thin layer of rubber can extend a tongue, apparently identical to the one in Fig. 4, but in this case tongue extension is associated with more area dilatation, and less surface shear. In contrast to this, if deformation is slow enough, the cell membrane perfectly adopts to the pipette shape with a smooth surface, without increasing surface area. In a very fast deformation, however, the membrane may wrinkle, because reorganization of the cytoskeleton into the new shape takes some time. The structure responsible for these properties may be outlined as follows: the membrane is a fluid, with a non-crosslinked network structure. The units building the membrane always have the same count of neighbours, with the same distance between them, and there are strong bonds between the neighbours, as implicated by the dilatation and bending rigidity. Although the strong bonds are continuously present, the partners in the bonds can be replaced by others, resulting in surface shear flow.

Erythrocyte shape

Erythrocytes deform into various shapes if external force is applied, and normal erythrocytes always recover the biconcave shape at rest. However, the position of the dimple has no preferred location, as demonstrated in experiments with red cells containing Heinz bodies or labelled by small attached latex particles. This suggests, that the biconcave shape is the consequence of membrane bending elasticity and excess surface area, rather than a cytoplasmatic structure. The biconcave shape represents the configuration of membrane bending with minimum energy storage (e.g. 14).

Membrane components

There are numerous studies about the relationship between membrane components and blood rheology. Crosslinking 5 to 50% of the red cell membrane spectrin by diamide reduces the deformability of the cells proportionally (28). In spectrin deficient red cells, outward bending of the membrane prevails, suggesting contraction of the internal membrane layer. This results in bizarre cell forms with pseudopods and evaginations. If subjected to high shear, these protrusions get lost, producing cell fragments and spherocytes, with decreased surface area/colume ratio. If cell surface charge is decreased to near zero (by enzymatic removal of sialic acid residues of the glycophorin), the membrane of these spectrin deficient cells lose elasticity, and do not recover the biconcave shape after deformation. They also fragment already at very low shear stress (3 Pa), whereas normal cells resist stresses up to 3000 Pa (40).

In addition to intrinsic membrane components, other non-membrane-proteins may also affect cell membrane properties. There is probably some interaction between red cell membrane and the hemoglobin molecules, since adhesivity and surface charge distribution of sickle cells is different from HbAA cells (18,34). Protein adsorption at the external surface of red cells may also alter the mechanical properties, as described in case of plasma proteins (8,21) and other macromolecules (20).

Pathological changes of human erythrocytes

Older red cells (separated by their higher density) have decreased deformability. This is due to increased membrane viscosity and elastic modulus, and also loss of excess surface area (44). Disorders of cell

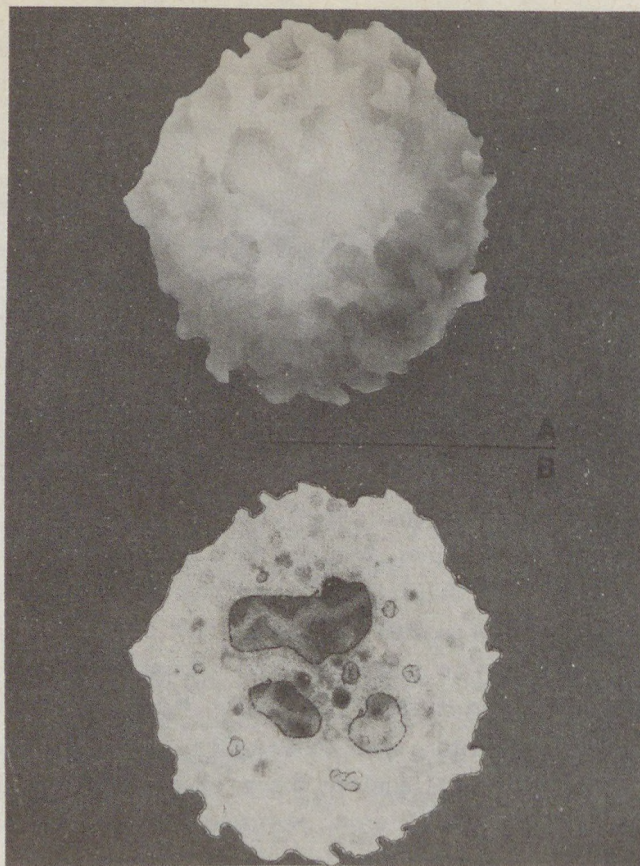


Fig. 7. Three-dimensional view (A) and cross-section (B) of a granulocyte. The membrane has a large number of ruffles, extending its surface area.

membrane, hemoglobin molecule or metabolism are often reflected in decreased erythrocyte deformability (e.g. 23). Sick cell disease (genetic error in the amino-acid sequence of the hemoglobin molecule) is probably the most extensively studied. When subjected to low oxygen tension (< 30 mmHg), these cells undergo typical morphological changes, and become 5-50 times more rigid, as compared to normal HbAA cells or oxygenated HbSS cells (35). Defects of membrane-associated proteins, such as spectrin (26) or protein 4.1, are also associated with changed membrane rheology (45).

LEUKOCYTES

Krogh has already observed, that white cells appear to be less deformable in the circulation than red cells (22). Circulating leukocytes have a shape rather different from the one known from histology books. In vivo, they are usually spherical bodies, but they are flat in blood films (Fig. 6). Leukocytes also have an excess membrane surface area, however, in contrast to the red cells, this builds up ruffles (Fig. 7), capable to unfold during cell deformation (2). The narrowest tube, which granulocytes can enter with their full length, is of $2\text{ }\mu\text{m}$ diameter. This limit is due to the stiff nucleus, because excess membrane surface area of granulocytes is larger than that of erythrocytes. Short holes of even smaller diameter ($1\text{ }\mu\text{m}$) may be negotiated.

In vivo studies in humans were carried out by Bagge and Branemark (1), using the skin tube chamber technique, a chronic implantate suitable for in vivo microscopic observation of the microcirculation. According to their observations, the passage of a leukocyte through a muscle capillary may be described as follows: The entrance time of a granulocyte into a narrow capillary is about 1 s (red cell: 0.002 s). This involves initial plugging and tongue formation, and slow deformation. When elongated and narrowed to the diameter of capillary, the granulocyte begins an intermittent advance, followed by a train of red cells. In wider capillaries, some of them may overtake the granulocytes. This train slows down where endothelial nuclei protrude into the capillary. Reaching the postcapillary venule, red cells push the leukocyte aside, where it rolls along the vessel wall, recovering its spherical shape. In case of local inflammation, leukocytes adhere to the endothelium and stay there, decreasing the diameter and increasing flow resistance. In capillaries, leukocyte adherence may result in complete blockage.

Entry times into narrow tubes were also measured in vitro. To fully enter a glass tube ($3.6\text{ }\mu\text{m}$ I.D.) at a pressure head of 300 Pa ($=3\text{ cm}$ water column), lymphocytes required $26\pm 15\text{ s}$, neutrophils about 25% less, lymphoblasts and myeloblasts were somewhat slower than lymphocytes (23). Lymphocytes did not fully enter narrower tubes, because the nucleus did not deform sufficiently.

In an other study (36), neutrophils entered glass tubes of $5\text{ }\mu\text{m}$ internal diameter, driven by pressure heads of 200, 300 and 500 Pa in 9, 4 and 2 seconds, respectively. Lymphocytes entered this tube about 10 % faster.

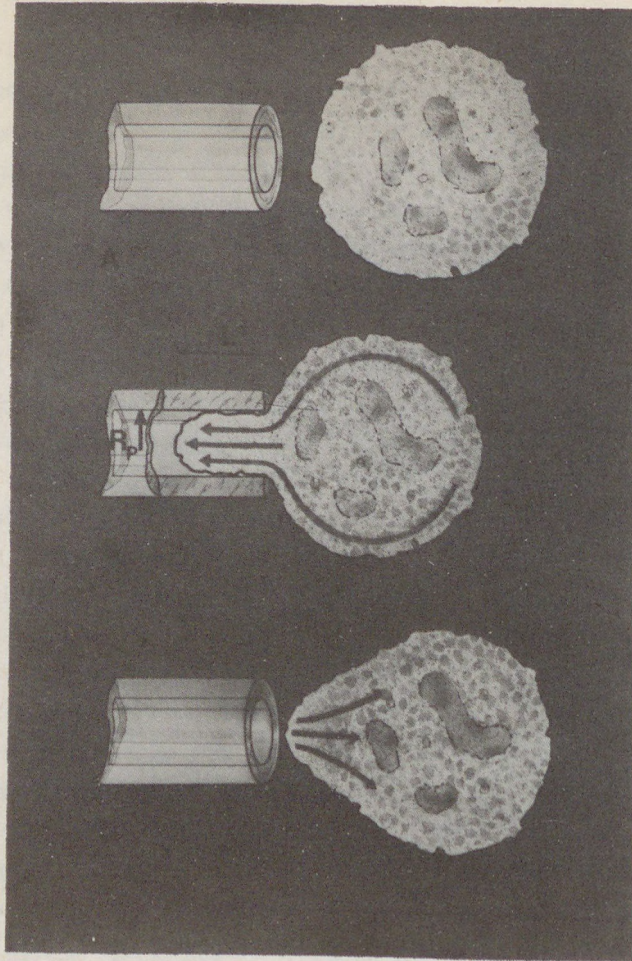


Fig. 8. On application of suction, leukocyte extend a tongue into the micropipette (B), which continues growing as long as the pressure is applied. When removed from the pipette by a positive pressure step, the cell recovers slowly its original shape (C). Typical recovery times: 20-30 s at 23°C, 10-20 s at 37°C. Mechanical properties may be calculated from pipette radius (R_p), tongue length (L) and rate of change of length (D).

The threshold pressures required to push the full cell into tubes of 4, 5 and 6 μm I.D. were found to be 300, 150 and 30 Pa for neutrophils, and 800, 150 and 30 Pa for lymphocytes, respectively.

Compared to erythrocytes, the internal structure of leukocytes is complicated, nucleus, granules and mitochondria filling 37% of the cell volume. The rest is homogeneous cell matrix (37). This cell matrix has a pronounced viscoelasticity. As revealed by micropipette studies (Fig. 8). application of a stress step results in a fast, small deformation, suggesting an elastic component in the structure (k_2 in Fig. 9). This is followed by continuous deformation (flow), showing the presence of a serial viscous component (η in Fig. 9). Releasing the cell (no external stress acting any more) results in a slow, but complete recovery of the original spherical shape, suggesting a parallel elastic component (k_1 in Fig. 9). This behavior can be summarized in the mechanical model (3,38) of standard solid (Fig. 9). In this model membrane properties are ignored, as well as granules and nucleus, therefore it applies only to deformations, where these are not involved. However, it is very useful to interpret experimental data.

Treatment of granulocytes with cytoskeleton disrupting agents (colchicin, cytochalasin) results in a decreased elasticity, suggesting, that cell elasticity is associated with the cytoskeletal network (5). This is also supported by morphological and biochemical evidences (42).

Temperature dependence of granulocyte mechanics was studied in the range of 9-40 $^{\circ}\text{C}$ (43). The cytoplasmatic viscosity followed the temperature dependence of water and water-based solutions (approx. 3-fold increase with temperature decreasing from 40 to 9 $^{\circ}\text{C}$). The elasticity parameters remained constant within this temperature range.

Leukocyte adhesion

Granulocytes frequently adhere to vessel walls. Electron microscopic studies showed a gap of 60 nm between the endothel and granulocyte (17). In animal experiments, 0.4-2 Pa wall shear stress was required to mobilize leukocytes adhered at the venular wall (25). In an other study (12), adhered leukocytes resisted shear stresses of 50 Pa in mild inflammation, and 500 to 5000 Pa if strong activation was present. Adhesive forces were close to zero if inactive cells were investigated. In rats, sulfinpyrazone (a platelet aggregation inhibitor) reduced leukocyte adhesion to vascular

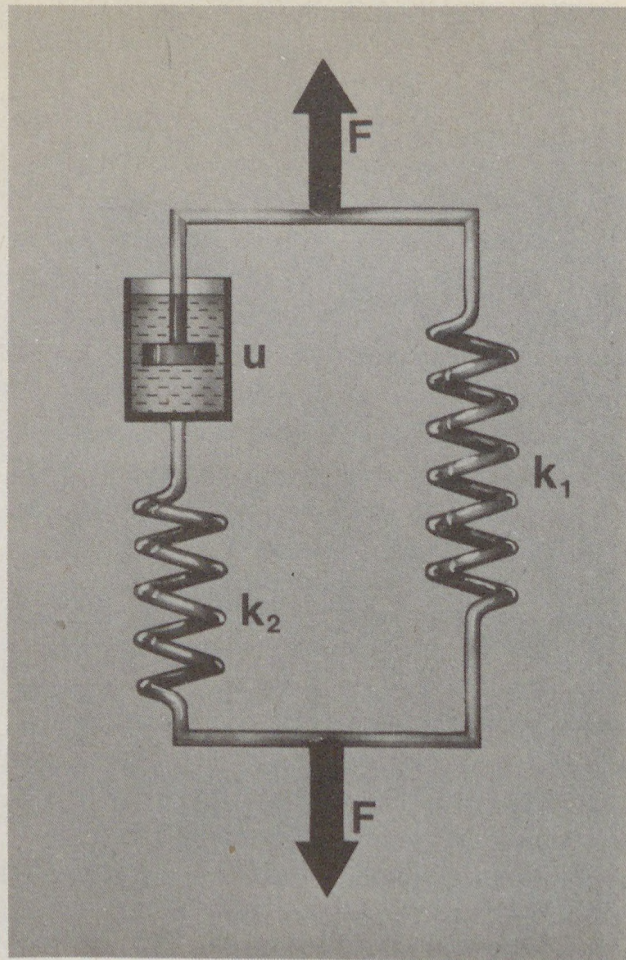


Fig. 9. Mechanical model of a leukocyte.

The serial elastic (k_2) and viscous (u) components are responsible for the initial fast deformation and for the continuous flow when entering narrow tubes, respectively. The parallel elastic component (k_1), which is weaker than k_2 , recovers the cell in the spherical resting shape.

injury sites, if given intraperitoneally for two days before the study, but not if acutely given only (46). Adhesivity of granulocyte precursors (promyelocytes) to plastic and cultured endothelial surfaces was less than that of granulocytes, and increased parallel with cell maturation (7).



Fig. 10. Leukocyte extending a pseudopod (P); N: nucleus.

Pathological white blood cells

Mechanics of leukemic cells was also studied. The rheological properties of granulocytes and chronic myeloid leukemia cells are similar, as well as those of lymphocytes and chronic lymphoid leukemia cells. In contrast to this, lymphoblasts and leukoblasts are 10-100 times stiffer than lymphocytes and leukocytes (24).

ACTIVE DEFORMATION

Leukocytes emigrate from venules only. Although there is a closer contact between vessel wall and leukocyte in capillaries, but no excess surface area left, due to forced deformation. In venules, wall shear stresses are lower, and the spherical shape with ruffled membrane is recovered, allowing deformation required to negotiate the narrow gaps in the wall. Emigration of leukocytes from venules is slow, taking 2-9 minutes (6). Leukocyte egress into marrow sinuses was also related to increase of deformability with maturation (15).

Circulating granulocytes are in passive state. If activated (e.g. in heparin anticoagulated blood at temperatures of 34°C and above), they show phagocytosis and chemotaxis. These activities are associated with pseudopod growth. Pseudopods initiate at the membrane, grow for 20-40 s,

followed by retraction at a similar rate. The pseudopod is free of cell organelles, and has a fine fibrillar ultrastructure (Fig. 10). In a retracting pseudopod, granules return into the region by Brownian motion. The membrane is unfolded on the pseudopod, and this cell region can be characterised as an elastic solid (assumed to be a polymerized actin gel). In micropipette experiments, (pressure head 80 Pa, pipette diameter 2.5 μm) the suction of a 2.5 μm long portion of the cell body into the pipette took 6 s, whereas the pseudopod region protruded 0.5 μm in 60 ms, and no more deformation was observed during the following 6 s (39). Activated leukocytes have an increased tendency to aggregate (16), and show decreased deformability in filtration tests (4). It has been suggested, that activated leukocytes may contribute to ischemic tissue lesions (e.g. 27).

Acknowledgement

Figs. 1, 2 and 4-10 were drawn by András Pázmány.

SUMMARY

Rheological studies may provide additional information about the structure of the living cells, since the latter determines their mechanical properties. Such studies, carried out on blood cells, showed that erythrocytes were fluid droplets encapsulated in a viscoelastic cell membrane. This membrane had anisotropic rheological properties, characterized by little resistance to surface shear deformation, considerable bending elasticity, and very large resistance to surface area dilatation. The membrane surface area is much larger than necessary to cover a sphere of red cell volume, and the excess surface area is responsible for the high deformability of erythrocytes. Changes of composition or structure of the membrane-associated cytoskeleton are usually reflected by altered erythrocyte mechanics.

Leukocytes also have an excess surface area in form of membrane ruffles. This makes large shape changes possible. White blood cells are viscoelastic bodies, showing slow deformation and shape recovery under physiological stresses. Consequently, they may interfere with blood flow in the microcirculation. Viscoelasticity of leukocytes is probable due to the cytoskeletal network. Activated leukocytes are even more rigid, due to pseudopods, which are probably composed of crystallized actin gel.

Studies on other but blood cells are not known at present. However, interpretation of ultrastructural and biochemical data of other soliter cell types (e.g. non-adherent cultured cells) may be facilitated by appropriate rheological studies.

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DISCUSSION

Hazlewood: I'm curious to make sure I understand the time period. Whenever you pulled one cell in the pipette, and then were able to release it and then measured the time that it took to reach its original shape. Did you say 30 seconds?

Mátrai: For leukocytes it's around that. For erythrocytes it's around 10 ms. Leukocytes have a large internal viscosity. Do you find it too much?

Hazlewood: No, it's very rapid!

Mátrai: We shouldn't forget that these cells must go through tubes which are half as wide as they are, throughout our life.

Ling: Red blood cell has been historically a model of the theory of lipid bilayers. Gother and Grindall analyzed the total lipid contents and calculated that there is just enough bilayer to cover the cell. If so, then the force holding the membrane is essentially the lipid interaction, primarily van der Waals forces. And these van der Waals forces are very-very weak. And the question is, how can you demonstrate such a great tensional strength in dilataion.

Mátrai: The problem is that it's not just a lipid bilayer. There are lots of proteins associated with it, and there is an actin and spectrin meshwork under this lipid layer. If you deplete ATP from the cells, then there is a change in the actin-spectrin network, somehow it contacts a bit, and that's the cause of these crenated erythrocytes. But if you give chlorpromazine that extends this layer under the membrane, it will build up force inside, so you get a stomatocyte, and if you apply both drugs, then you stay where you started.

Ling: I think that's fascinating, and that's exactly what I am trying to drive at. That is, I'm not the first one by any means to say that the membrane contains much larger proportion of protein. In fact if you analyze all the membranes that have been analyzed, you see that the consistent proportion is actually protein, about 60% of dry weight. This indicates that the chances, that there are large regions of blood cell membrane that are entirely held together by the van der Waals forces of lipid, which is a necessary condition of Singer-Nicholson model, are very low. It will just not behave like that. On the other hand, there is suggestion that there is a proteinaceous network, filled with phospholipid, but when you begin to stretch it, the strongest point you are baring against is not the lipid, but the protein.

Mátrai: I'd like to add that hemoglobin is also involved in this. And another thing is that outside there is always an adsorbed layer of proteins, part of which may be removed by washing, but large part of it cannot be removed, and it has some interesting pathological connection, because the amount of these proteins is changed in some diseases.

Osborn: I just wanted to comment on the recent work of Dan Brandon's lab. They used different detergents to solubilize the erythrocyte membrane selectively. They made very beautiful pictures of spectrin-actin-ankyrin and band 3. And this work is notable for a number of reasons. First of all this is a situation where the model existed long before the picture. And the picture really confirms the model. You see the arrangement of the proteins and you can identify which protein is which. You see short filaments of actin coming off the membrane. Second, it's interesting in terms of what Dr. Kellermayer spoke and showed about the use of different detergents, because it is a very soft extraction of the membrane, and one just wonders what part of this submembraneous cytoskeleton plays, because it's very different from the cytoskeleton. It must be important for the experiments that you are describing. Finally, what one wants to know is of course how the corresponding thing in a cell type other than the red blood cell would look. I suppose nobody has produced such pictures yet.

Kellermayer: Probably it's not absolutely true that the red blood cell doesn't have an internal skeleton.

ENERGY AND MOLECULAR MOTIONS

ABSTRACT

The energy and molecular motions of a system of particles are studied. The energy is defined as the average of the square of the velocity of the particles. The molecular motions are studied by means of the correlation function. The energy and molecular motions are studied for a system of particles in a fluid. The energy is defined as the average of the square of the velocity of the particles. The molecular motions are studied by means of the correlation function. The energy and molecular motions are studied for a system of particles in a fluid.

1. INTRODUCTION

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BIODYNAMICS AND NMR

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INTRODUCTION

Biodynamics is a subdomain of biophysics, which attempts to characterize a living system by the investigation of random motion. Theoretically it is based on the time-dependent statistical mechanics, using the experimental results of Nuclear Magnetic Resonance (NMR). The aim of this contribution is to show how the NMR data can yield information concerning the state of a living system.

DYNAMICS OF THE LIVING SYSTEMS

Biological molecular systems are characterized by a great number of possible configurations, high entropy and significant Brownian (random) movements. Four kinds of molecular motion can be considered in biological systems: 1) Fast rotational and translational fluctuations of small molecules. 2) Restricted rotations and vibrations of small entities of macromolecules. 3) Chemical exchange and "tunnelling" effect. 4) Slow fluctuations, which are associated with the cooperativity of the macromolecular chains.

Recently, we have introduced a new concept of the "dynamic entropy" (entropy of fluctuations) (1-3). This concept can be supported by the following idea: As the internal energy in fluids, polymers and cellular systems is given in terms of the fast molecular kinetics, entropy must also be somewhat related with molecular motion. In this context we have derived (1) a simple expression for the "entropy of fluctuations", and we have related it to the correlation time, τ_c . In the language of NMR, this approach allows us to relate entropy with the NMR parameters (relaxation time, T_1 , or the spectral line width). Consequently we obtain the

following entropy ratio of a system, passing from a state "A" to the state "B"

$$S_A/S_B = (T_{1A}/T_{1B})^2 \quad (1)$$

Alternatively, the entropy ratio can be given in terms of the spectral-line width Δ

$$S_A/S_B = (\Delta_B/\Delta_A)^2 \quad (2)$$

APPLICATIONS

A) Tumor and cancer biology. During the past 10 years, NMR and spin relaxation have been used extensively for the investigation of relaxation times in healthy and tumor tissues, as early detection of internal neoplasms is hampered by the relatively high permeability of many tumors to X-rays. One of the difficulties in treating patients with cancer is the fact that the neoplasm is often discovered late. The major causes of cancer related deaths are due to tumors of the lung, colon and breast.

In his pioneer work, DAMADIAN (4) reported that in some tumor tissues the spin relaxation times are longer than in the corresponding normal tissues. This phenomenon, confirmed also by other workers, extends not only to the tumor, but also to other organs. It acquired the name: the "systematic effect".

Some of DAMADIAN's results are reviewed in Table 1. It is clear that in all cases the ratio $T_{1\text{tum}}/T_{1\text{norm}} > 1$. This result is related with the SZENT-GYÖRGYI's assumption (5) that cancerous tissue has a lower degree of organization and less water structure than normal tissue. Following this interpretation, the disorder and entropy must increase during the formation of a tumor. This is also consistent with the LING's theory of the "polarized multilayers" of cell water (6). Therefore, the slower spin relaxation in cancer tissues can arise from a loosening up of the ordered water in normal cells, as predicted by the differences in the patterns of metabolism between cancer cells and normal tissue. Furthermore, cancer tissues are also characterized by an increased water content. This contributes to the increase of the molecular dynamics and higher entropy in the sample.

Table 1. T_1 -relaxation and entropy ratios in normal and tumor tissues

SYSTEM	T_1 (sec)		$S_A/S_B \sim T_{1A}/T_{1B}$
	A-TUMOR	B-NORMAL	
BREASTS	1.08	0.367	2.94
NERVE	1.20	0.567	2.16
BONE	1.02	0.554	1.85
SKIN	1.04	0.616	1.69
STOMACH	1.23	0.765	1.61
SPLEEN	1.11	0.701	1.60
LIVES	0.83	0.570	1.50
LUNG	1.11	0.788	1.40

DAMADIAN (4)

On the other hand, the longer relaxation times of cancer water protons cannot be interpreted as being due to the decrease in the content of paramagnetic ions. A survey of the literature shows that results of studies of the paramagnetic ion effect are largely negative (7).

Several spin relaxation parameters can be combined into a single NMR malignancy index.

The "systemic" effect has also been confirmed in tissues of tumor bearing animals (see the review paper (8)). In all types of cancer in a number of animal models, at least one involved organ and sometimes a number of organs exhibit elevated T_1 values. It was also found that the "systemic" effect can be extended to the blood serum as well, since the cancer cells might secrete some substances into the blood that would

travel to distant organs and imply the elevated spin-lattice relaxation times.

B) Biophysical phase transitions. Frequently, the physiological and morphological transformations give rise to changes in the state of the investigated biological system. A striking example of this change is the above mentioned transition from "healthy tissue" to "tumorous tissue". In all cases it was found that $T_{1\text{tum}}/T_{1\text{norm}} > 1$, as shown in Table 1. The application of eq. (1) to the DAMADIAN's results demonstrates that, for example the tumor of skin leads to the increase of entropy by the factor of 2,886. The increase of entropy in the tumorous tissue, also confirmed by the calorimetry is given by the increased molecular disorder, due to the lack of biological regulation.

Another example of the increase of entropy is the action of cholera toxin (9). In this case it was found that the relaxation times, T_1 and T_2 of water in cholera infected tissues were longer than in control tissue samples. This result suggests that cholera treated tissues exhibit greater motional freedom of water than do control samples and supports the general view that cholera enterotoxin acts by influencing intracellular protein-water interactions. Otherwise said, the increased water permeability of cells yields the enhanced secretory activity of small intestines.

A number of physiological "phase transitions" are given in plant biology. The complex physiological transition during Bean seeds (*Phaseolus vulgaris* L.) maturation, situated toward the twentieth day of development, is characterized by a significant maximum of the T_1 relaxation (10). The maturation of the Bean seeds is accompanied by an important metabolic and structural transformation. This is perfectly reflected by the dynamics of water molecules due to the interactive properties of water and proteins in the spirit of LING's "association-induction" hypothesis (6). The sudden increase in molecular dynamics has an autocatalytic character and is probably derived from molecular destabilization of the system.

Another example of a sudden "phase transition" in plant biology is flowering. In this domain one has the early "vegetative" (V) state and the derived "floral" state (F). The physiological transition between these states can be detected by NMR (1,2). It has been established that the biophysical and biochemical changes in the leaves during floral induction seem to be closely related to the fast energy and entropy changes, which may result in a rapid modification of the membrane permeability

properties. It is supposed that a new balance in the fluxes of matter and energy may result from these changes and thus give rise to a thermodynamically more stable "floral" state (2). The latter is characterized by a partial blocking of molecular dynamics, by a decrease in diffusivity of small molecules and by an increase in structure and of molecular order. Therefore, the flowering plants have lower levels of internal energy and entropy. The physiological states can also be identified by the ^{31}P -NMR, as presented in Fig. 1. This figure shows two ^{31}P -NMR spectra of the fresh spinach leaves. The "vegetative" state exhibits a narrow line, given by the inorganic phosphate (P_i), while the "floral" state yields a broader NMR line. The ratio of these line widths is approximately 3.7. Furthermore, the interpretation of the chemical shifts shows that this state transition is also characterized by the increase of the intramolecular pH by the value of $\Delta\text{pH}=0.5$.

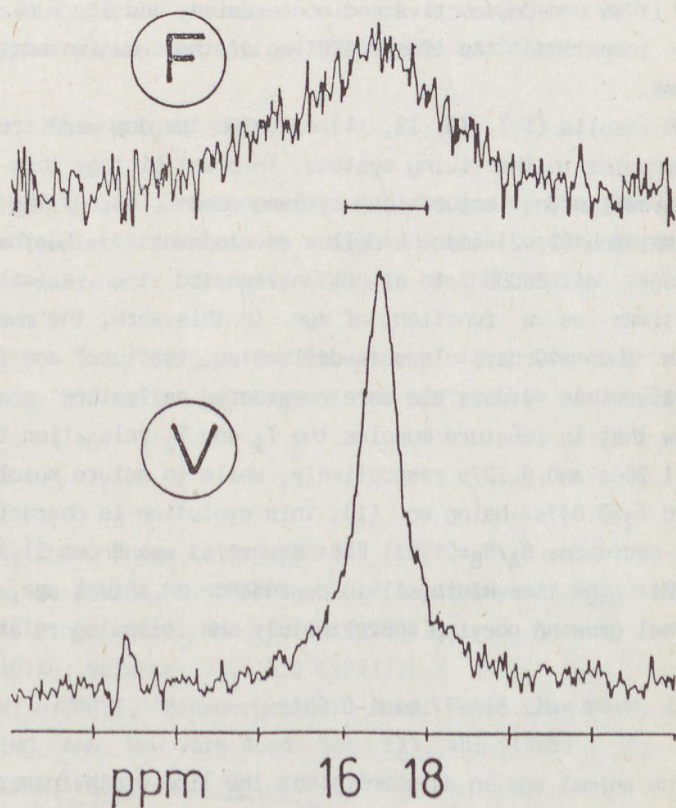


Fig 1.

ENTROPY EVOLUTION AND THE BIOPHYSICAL DEVELOPMENT

From the biophysical point of view, irreversibility is one of the fundamental properties of nature. The observation of the gradual evolution of all things leads to the definition of the "time's arrow". In biology, death is an inevitable and irreversible event. It reveals the inexorable movement of time from the past to the future.

It has been well established that the irreversible evolution in biology is fundamentally different from that in physics (11). In a system "in-vitro" the gradual deterioration has the tendency to lapse order into disorder, while the "self-organizing" property of living organisms implies an increase in order during biological development. As life is a consequence of microscopic rather than macroscopic organization, its irreversible evolution must be explained at the atomic and molecular level, using the new spectroscopic methods such as NMR. This method is important as it is non-destructive and non-evasive, and it is particularly adequate to investigate the time-evolution of the Brownian motion in the living systems.

Some NMR results (1-3, 10, 12, 14) revealed the downward trend of the molecular dynamics in the living systems. In plant biology this trend was found by investigating maturation of Bean seeds (10), of the flowering process in spinach (1, 2) and the callus development (3). Furthermore, in animal biology, HAZLEWOOD et al (13) reported the variation of the relaxation times as a function of age. In this work, the muscles from animals less than 40 days old were defined as "immature" and those from animals greater than 40 days old were considered as "mature" muscles. The results show that in immature muscles the T_1 and T_2 relaxation times have the values 1.206s and 0.127s respectively, while in mature muscle one has $T_1=0.723$ s and $T_2=0.047$ s. Using eq. (1), this evolution is characterized by the entropy decrease: $S_A/S_B=2.782$. Furthermore it was shown (14) that the evolution that the line-width, Δ , in dependence on animal age, increased with the normal growing obeying approximately the following relation

$$\Delta = 12.81 - 7.\exp(-0.55t) \quad (3)$$

where t is the animal age in days and Δ is the line-width in Hertz.

The interpretation of these results shows that there is an increase

in the molecular order and an decrease of the molecular dynamics in muscle water and in the whole system.

Furthermore, our recent results have shown that the ^{31}P -NMR spectral line, corresponding to the inorganic phosphate (Pi) in the tissue culture (calus) goes to a doublet during the biological development.

The "microscopic" (molecular) reasons for the entropy decrease ("negentropy") during the biological development are the following: 1) Ordering of the system due to the biological morphogenesis. 2) Configurational and conformational changes. 3) Creation of the "dissipative" structures (11).

The biological morphogenesis has the following stages: a) Formation of proteins and ADN from monomers. b) Formation of cells from biopolymers. c) Creation of multicellular organisms.

The decrease of entropy due to the elementary morphogenesis can be simply illustrated by the probabilistic treatment of entropy: The creation of one biological structure out of N elements with N! possible configurations yields the entropy difference as follows

$$\Delta S \approx k \cdot \lg N! \approx N \cdot k \cdot \lg N \quad (4)$$

Configurational and conformational changes of biomolecules are given by the molecular transition to a higher spatial order, given for example the random-coil/helix transition and/or by the creation of the molecular "cross-links".

Finally, the "dissipative structures" (the dynamic order) are given by the energy and matter exchange of the system with its environment.

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DISCUSSION

Tigyi: You demonstrated us a very attractive biophysics program. But as you emphasized in the beginning, it is a special one. Different from all others. It's theoretical biophysics, and I'm very happy, because it's a new one in biophysics education. I have a proposal, if you agree. I'm in charge of organizing the international biophysics in the frame of International Union of Applied Biophysics. And this Union has a Commission of Education and Development of Biophysics. This will have a Symposium next year in Jerusalem. Fuller is the head of this commission. We are just preparing the program. It would be nice if you participated there, because we are discussing the different curriculum and programs in biophysics, and it would be nice to discuss it there in front of a wider audience.

Lenk: Thank you very much!

Ling: It's a great pleasure to hear professor Lenk, to talk about NMR and biology. As you have explained it theoretically, the tool is so new and so powerful. And, indeed, it seems to be so new, and yet it's already some years back, when NMR was first applied and produced results that you have shown, by professor Hazlewood. And what I want to emphasize is that this is always the case. When you first make your observation many times it's extremely exciting, it seems to be going in the direction you want it.

Then the second step is that you find complications. And after time it is at this time, the sceptics shoot you down. Because you have oversimplified in the first step, not that it was wrong, but it was just more complicated. And I would illustrate this with the study of T_1 of normal and tumor tissue. And as people might have heard that this discovery of Damadian led to the invention of NMR scanning. And now it's a big industry. And I'm also happy to hear that Professor Damadian is now the head of a very prosperous company. A very few times do you see a biophysicist make out. If you follow the original simple hypothesis that we are detecting mostly water protons, and if the water is structured, then you expect the T_1 of certain size, and then if you have a more random structure, like in liquid water, then the T_1 will become longer. And, so the original conclusion which we all received with great delight was, cancer has less water structure, very simple, as you can see. But on second examination it didn't turn to be so simple. For example if you cook the tissue, the water should be now more normal and the T_1 should be longer. Or if you treat it with poison, that should also be the expectation. The result is sometimes no change, and in other tissues the T_1 actually becomes shorter. How can you expect dead should have negative entropy? And so when you look deeper into it, then you will find it finally; this work was only reported in a short abstract of ours, only a year ago. The fact is that much of the difference between the cancerous and normal tissue you observe in T_1 is due to a contaminant. The amount of iron and copper. Therefore, for some reason iron and copper content of cancer tissue are consistently lower. However, the water is also less structured. But this happened to be revealed more easily by a different probe. And this is what we called equilibrium distribution studies. The main point to emphasize is, not to be discouraged by the first round of apparent inconsistency. That's where most of our NMR friends have withdrawn. When our critics have withdrawn, it is then when it becomes really interesting. And, so in general comment to Professor Lenk's very knowledgable theory and the reports of earlier work, I say we must proceed and reveal really the beautiful thing that we call life.

Lenk: Briefly to reply to Professor Ling's comment. I've been very honoured to be invited here, of course, and particularly to read the names of Professor Gilbert Ling and Professor Hazlewood, and other Gentlemen here, because in our laboratory the name of Professor Ling is very well

known. We agree with your theories. Your theories are very close to the theories that we have elaborated. There is only some complementation.

Ling: I mentioned paramagnetic ion impurities. It's a bad word, because they have very important function, only I've not completely understood it. And so it is impurity only in the sense that we are looking for a pure system that doesn't exist. Now, however, you can produce a model. This would take a long discussion to introduce to you why it is relevant. In other words with this model, you can have, what we believe is the type of water we find in the living cell. Under this condition you can eliminate all the contaminants. Including the proteins, and paramagnetic ions. In this case the relaxation time is indeed of such that the correlation time is indeed different from that of liquid water. But much smaller factor. By a factor of not much more than 3 or 2. I hope I have time to discuss with you in further detail.

Clegg: I don't understand the point that you are making, or what it tells about biology that we didn't know before. I wonder if you could simply tell us. The notion that entropy changes accompany advancing the universe we know about. What I'd like to ask is what is your particular crystallized view of biology from your studies.

Lenk: Of course this crystallized view is to project our problem of biology on the molecular level. This is very simple.

Clegg: But a lot of us are trying to do this.

Hazlewood: Jim, I'd like to address this subject just a little bit, because I couldn't hardly believe my ears, when I heard Professor Lenk to start with statements about entropy. Particularly in the developing organism. It seemed reasonable to me twenty years ago, that we were observing changes in the entropy of the system in the tissue of developing organisms. That is, in the early postnatal development of the organism the system was going from "less order" to a state of "more order". It seemed, that during development, the order within the system, particularly the order of the water molecules within cells had to increase and that this is essential for differentiation. At also occurred to me at that time that, if you suddenly had something to intervene which would disrupt this order, then you would increase entropy of the system and that might be what is happening when transformation occurs. Now they are putting this apparently to mathematics, or pursuing this. I think life is an attempt to reduce the entropy, to sustain the reduced entropy throughout life, and then we give

it all back to universe when we die. I also have a question I wanted to ask. I had not thought about relating this to the correlation time of atomic nuclei. Can you comment on how the entropy is related to the correlation time? Is it correct that you are relating it to entropy?

Lenk: That is crucial problem. Entropy is the lack of integrity, the degree of disorder, of course. And we have in any time two entropies, configuration entropy, i.e. ordering entropy, and also the other that we have introduced here that is the dynamic entropy. Why? If you are puring into a train and the train goes very quickly, you cannot recognize the face of your friend in the train. If the train goes very slowly you can recognize his face. The internal energy is given by the kinetic energy of the molecules. If the internal energy is given by the kinetics, the dynamics of molecules, the entropy also must be related to it. You have the kinetic energy divided by T and it is the entropy. It is from the simple thermodynamics, if we have very fast vibration, the face, the image is dissipated, isn't it? There was a very hard discussion in an NMR meeting in Chicago, where I had a post, and there was a very hard discussion about this concept, how the motion is related to entropy.

Hazlewood: The point that I'm concerned about is: Are you relating entropy to the correlation time? If yes, then you are relating the entropy to a bulk property of water.

Lenk: Yes. So it is mathematically very easy. The starting point, of course, in any case the entropy formula for logarithm of probability, as I've very quickly presented, so there is no problem mathematically, but physically sometimes there have been some problems. What does it mean physically then? I have made this statement that if you have water, if you have ice, and if you have biological bound water, and vapor. The lowest entropy is ice, it is organized, of course, but also the motion in ice is frozen. And if you take bound water in tissue, it has high entropy, and its motion is more important than in ice. This water is entropically more important.

MOLECULAR DYNAMICS OF SPIN-LABELLED PROTEINS IN ACTIN-MYOSIN SYSTEMS

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In the current theories of muscle contraction, it is believed that the shortening is achieved by the sliding motion of thick and thin filaments past one another (Huxley, 1969; Huxley, Simmons, 1973). The molecules of actin and myosin which constitute the thin and thick filaments are organized in striated muscle in a crystal-like, very highly ordered structure. At resting sarcomere length, in the range of 2.3-2.6 μm , the two kinds of filaments overlap. The enzymatically active portions of the myosin molecules, called cross-bridges, protrude as projections from the thick filaments, interact with the actin molecules in the thin filaments and pull them towards the center of the sarcomeres. This contraction process is driven by the energy from the ATP splitting, and controlled by the presence or absence of calcium ions. The cyclic formation and dissociation of cross-bridges requires the large-scale motion of protein domains, and the interaction effects on the conformational state of the protein system.

Direct measurements of rotational motion of proteins by motion-sensitive probe molecules provide some insight into the molecular dynamics of contractile proteins during the ATP driven force generating mechanism. Nitroxide spin labels tightly bound to proteins have been widely used to study molecular motions in actin, myosin and its fragments and in supramolecular complexes (Barnett, Thomas, 1984; Belágyi et al. 1979; Belágyi, Gróf, 1984; Ishiwata et al. 1986; Thomas et al. 1980).

Potentially, on the basis of the structural model of muscle two possibilities can be suggested: the probe molecules may be located on the myosin filaments, or they may be attached to the thin filaments in order to sense the protein motion and the transduction of energy arising from ATP.

I. Thin filament model

It is known from earlier experiments that actin can be specifically labelled on Cys 374, and the EPR spectrum from actin reflects whether the actin is in monomer or in polymerized form (Cook, Morales 1971, see Fig. 1). Moreover, the EPR spectrum of the actin filaments depends on orientation of filament axis relative to the applied magnetic field. It was reported by Burley and coworkers (1971) on oriented F-actin gel from skeletal muscle, that the z axis of the molecular reference system is oriented rather parallel than perpendicular to the long axis of the actin filaments. (The z axis of the magnetic tensors is defined to coincide with the $2p\pi$ molecular orbital of the unpaired electron.)

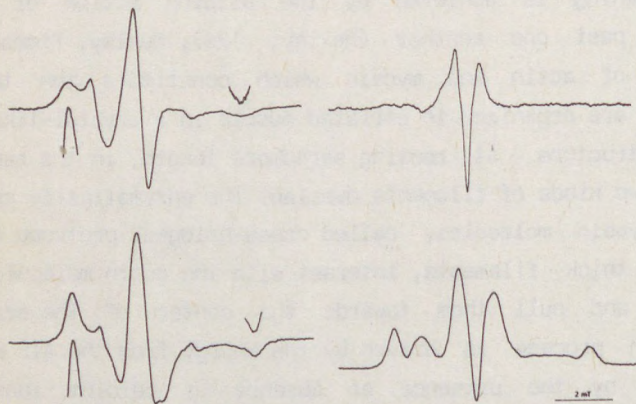


Fig. 1. EPR spectra of spin-labelled actin. Actin was labelled with maleimide spin label in F-form for two hours at 0°C . Degree of labelling: 0.4 mol label/mol actin. Left: conventional EPR spectra of actin, G-actin (upper) and F-actin (bottom). Right: saturation transfer EPR spectra of actin, G-actin (upper) and F-actin (bottom). Concentration of actin was 4.2 mg/ml.

Comparing the apparent rotational correlation time for G-actin with the rotational diffusion tensor components we obtained that the $2p\pi$ orbital of the unpaired electron is approximately perpendicular to the longer axis of the prolate geometry of G-monomer. Therefore, it can be concluded that the longer axis of the monomer is in the plane perpendicular to the long axis of the actin filaments. This finding supports the model reviewed by Engelman rather than the filament model of Fowler and Aeby in which the

longer axis of the monomers coincide nearly with the plane parallel to the long axis of the filament (Engelman, 1985; Fowler, Aebi, 1985).

Introducing a simple parameter it is possible to measure the degree of order of spin labels in different supramolecular complexes (Fig. 2).

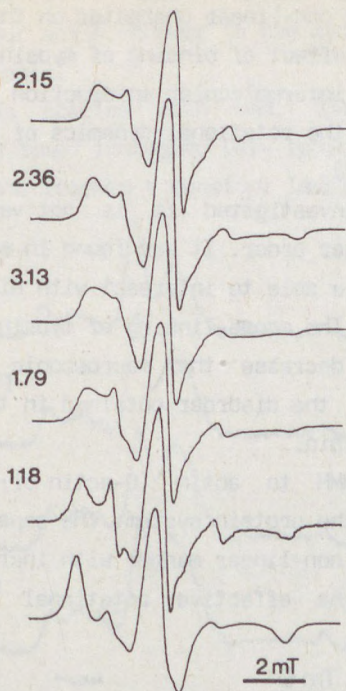


Fig. 2. EPR spectra of oriented actin samples: F-actin; F-actin + tropomyosin (molar ratio of actin to tropomyosin 7:1); F-actin + caldesmon (5:1); (F-actin + tropomyosin) + heavy meromyosin (8:1); F-actin + HMM (8:1). The long axis of the filaments was oriented parallel to the magnetic field. Bottom: EPR spectrum of randomly oriented F-actin is shown. The numbers indicate the calculated order parameter.

The largest degree of order of spin labels was obtained in the case of F-actin-caldesmon complex. Caldesmon, a calmodulin- and actin-binding protein, is an elongated, flexible molecule localized in the actomyosin domains of smooth muscle (Sobue et al. 1981). Its binding to actin induces the polymerization and causes the bundling of the actin filaments (Galazkiewicz et al. 1985). It can be assumed that caldesmon forces the thin filaments for a stronger packing and this is accompanied with a much

perfect macroscopic alignment of the thin filaments. In contrast, the measurements on the acto-HMM complex exhibited a spectrum which resembled to the spectrum of randomly oriented actin filaments. The changes in the degree of order of spin labels induced by binding of heavy meromyosin (HMM) to F-actin show a non-linear character on the molar ratio of myosin fragments to actin. The effect of binding of myosin heads propagates along the actin filaments by intermolecular interaction inducing changes in the intrinsic order and in the rotational dynamics of F-actin (Belágyi et al. 1979; Thomas et al. 1979).

In the samples investigated it is not very easy to separate the macroscopic and molecular order. It was found in striated muscle that the two heads of myosin were able to interact with different actin filaments (Trinick, Offer, 1979). The cross-linking of myosin heads between adjacent F-actin filament can decrease the macroscopic order by interfilament interaction, therefore the disorder obtained in the experiments may have partly a macroscopic origin.

The binding of HMM to actin (G-actin or F-actin) influences the motional dynamics of the protein system. The separation of the outermost extrema increased in a non-linear manner with increasing concentration of the added HMM, and the effective rotational correlation time of the

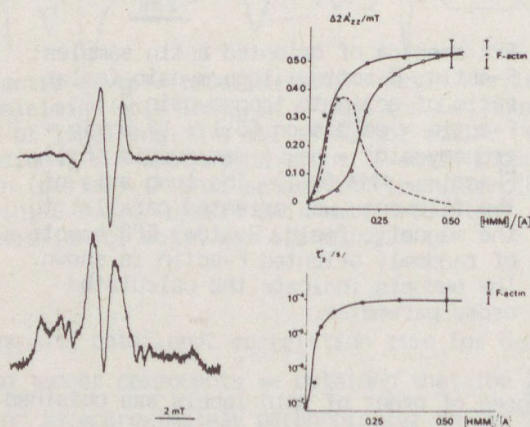


Fig. 3. Interaction of HMM with G-actin. Left: ST-EPR spectra of G-actin (upper) and acto-HMM complex (molar ratio of actin to HMM was 3:1). Right: dependence of hyperfine splitting and rotational correlation time on molar ratio of HMM to actin. Symbols: $\bullet$ - $\bullet$ - experimental data; $\circ$ - $\circ$ - simulated data. The difference curve is the measure of the cooperativity at a given ratio of HMM to actin.

supramolecular complex approached the τ_2 value of F-actin being already at low molar ratio of HMM to G-actin (Fig. 3). Postulating two assumptions, the EPR spectra of the actin-HMM complexes were simulated by computer. The difference curve in Fig. 3 indicates that the maximum effect on hyperfine splitting was observed at a molar ratio of 8:1, that is, the maximum response occurred at four actin monomer to one myosin head, and the nature of the response is biphasic.

When F-actin was reacted with HMM, the L''/L parameter (the rotational correlation time) increased very rapidly with increasing molar ratio of HMM to actin and reached a constant level of 1.03 ($\tau_2 \sim 300 \mu s$).

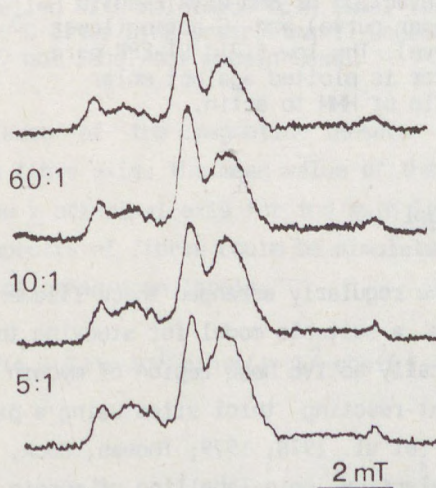


Fig. 4. ST-EPR spectra of F-actin-HMM complexes. Upper curve: F-actin alone. The numbers show the molar ratios of F-actin to HMM.

The results are shown in Fig. 4. The spectral parameter attained to its maximum at about 8:1 molar ratio of actin to HMM which is the same ratio when monomer actin was reacted with HMM (Fig. 5). The two curves show nearly parallel runnings which may indicate that the effect of HMM is independent from the molecular form of actin i.e. monomer or polymerized, and the interaction extends to the same number of monomers around the reaction center. The cooperative effect is intensified by the specific property of the myosin heads that they have two binding regions, one of them between the SH_1 and SH_2 thiol groups, and the other in the junction region of the 50K and 20K domains (Labbe, 1982).

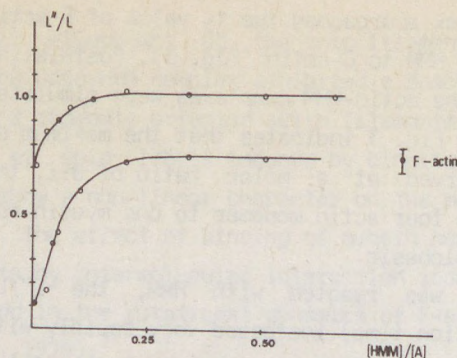


Fig. 5. Interaction of HMM with F-actin (upper curve) and G-actin (lower curve). The low-field ST-EPR parameter is plotted against molar ratio of HMM to actin.

II. Thick filament model

The system of the regularly arranged thick filaments in glycerinated muscle fibres offers a suitable model for studying the force generation because the enzymatically active head region of myosin can be selectively labelled at the fast-reacting thiol sites using a piperidine maleimide spin label (Belágyi et al. 1978; 1979; Thomas, Cook, 1980). In order to achieve a highly selective spin-labelling of myosin with the maleimide spin label, the glycerinated muscle fibres obtained from rabbit m. psoas were pretreated with dithionitrobenzene (DTNB) before spin-labelling. As the results in Fig. 6 indicate, there is no significant difference in the mechanical properties of the fibres which were investigated after spin-labelling following DTNB-EDTA treatment and of the fibres, which were bathed in rigor solution. The ATPase activity of the spin-labelled fibres was $0.028 \pm 0.007 \mu\text{M P}_i/\text{mg protein} \cdot \text{min}$ in the absence of Ca^{2+} which is a little greater than the same activity in control fibres ($0.023 \pm 0.005 \mu\text{M P}_i$ liberated/mg protein.min). In the presence of Ca^{2+} (0.1 mM CaCl_2) the ratio of the ATPase activity of the spin-labelled and control fibres was 0.86 ± 0.15 . The number of spin-labelled sulfhydryl sites was $0.17 \pm 0.08 \text{ mol label/mol myosin head}$ assuming that the spin labels were reacted only with myosin.

The EPR spectra of spin-labelled muscle fibres exhibit a very strong dependence on orientation (Fig. 7). In rigor state of the muscle fibres

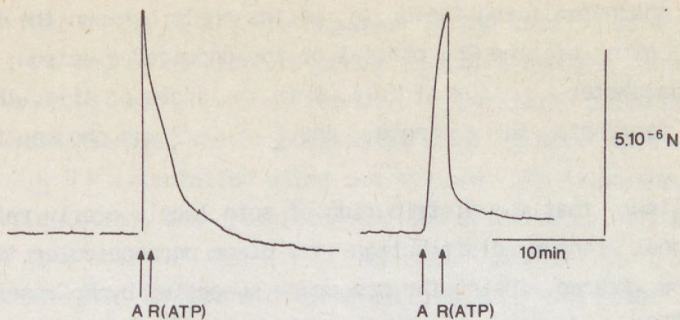


Fig. 6. Mechanical activity of spin-labelled (right) and control muscle fibres (left). The fibres were spin-labelled with maleimide spin label for two hours at 0°C after DTNB pretreatment. Degree of labelling: 0.17 mol label/mol myosin head.

the principal axes of the magnetic tensors were uniformly oriented relative to the fibre axis; the mean value of the angle between the fibre axis $\underline{k}$ and the $\underline{z}$ principal axis for the spin label was calculated to be 84°. The EPR spectra of fibres could be simulated using the distribution function of the attached spin labels,

$$f(\phi, \psi, \theta) = N^{-1} \sin \theta (1 - \lambda^2 \cos^2(\theta - \bar{\theta}))^{-1}$$

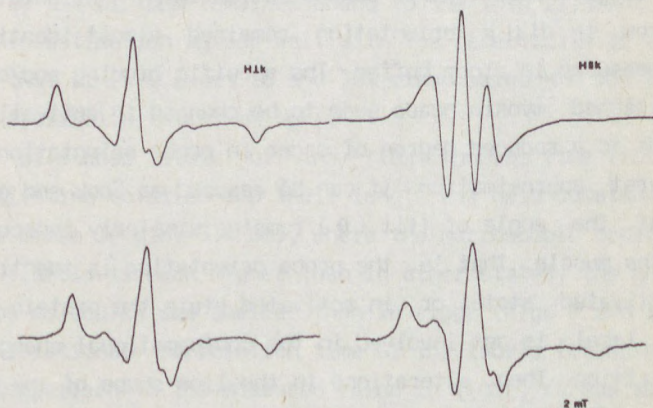


Fig. 7. Conventional EPR spectra of glycerinated muscle fibres in two different orientations as indicated in the figure. The fibres were spin-labelled with maleimide spin label after DTNB treatment. Upper spectra: muscle fibres in rigor; bottom: muscle fibres in activating solution.

where N is the normalizing factor, θ is the angle between the fibre axis and the z axis of the $2p\pi$ orbital of the unpaired electron; λ^2 is the anisotropy-parameter ($0 \leq \lambda^2 \leq 1$), θ is the angle of tilt. The distribution is isotropic in ϕ angle, and $\psi = \pi/2$ was chosen for muscle fibres.

It follows that the distribution of spin labels nearly related to a two-dimensional random distribution in a plane perpendicular to the long axis of the fibres. Using the procedure suggested by McConnell and co-workers (1970), it was possible to estimate the degree of order of spin labels in the different state of the muscle fibres.

The hyperfine splitting constant was $2A'_{zz} = 7.066 \pm 0.03$ mT in rigor which is very near to the rigid limit value of $2A'_{zz}$ evidencing that in rigor state the rotational dynamics of the probe molecules (cross-bridges) is strongly restricted on the time scale of the conventional EPR. These results correspond to the generally accepted fact that almost all myosin cross-bridges are rigidly attached to the thin filaments (Lowell, Harrington, 1980).

In activated state (the fibres were kept in solution containing the component of the rigor buffer plus 5 mM ATP and 0.1 mM CaCl_2 for 10 minutes at 0°C before EPR measurements, and the measurement was also taken in the same buffer), the degree of order of spin labels (cross-bridges) showed a large change towards randomization in $H||k$ orientation, whereas the spectrum in $H||k$ orientation remained almost identical with the spectrum measured in rigor buffer. The specific binding and/or the number of the attached myosin heads seem to be changed in activating solution, and it leads to a reduced degree of order in probe orientation.

At first approximation it can be assumed as Cook and coworkers did (1982) that the angle of tilt (θ) remains similarly independent of the state of the muscle. That is, the probe orientation is identical in rigor and in activated state, or, in activated state the protein segment that holds the label is not involved in the conformational change induced by the ATP splitting. Then, alterations in the line shape of the EPR spectrum can be described solely with the reduced number of the attached cross-bridges (changes only in the anisotropy-parameter). To complete and confirm this assumption it is necessary to monitor the rate of motion of the probe molecules in the different state of the fibres.

The results of spectral simulation showed that significant changes

might be expected in the ST EPR spectra even in the absence of any motion, inasmuch as the degree of order of probe molecules changes during muscle activity. However, it was shown earlier that in partially oriented system, like the muscle sample, the orientational order of spin labels might be estimated in $H \parallel k$ orientation using conventional EPR technique, whereas the $H \perp k$ orientation was promising for the calculation of the rate of motion on the time scale of the ST EPR technique (Belágyi, Gróf, 1984). It means it is possible to separate the order and motion of probe molecules using different fibre orientations and different measuring techniques.

In rigor buffer at resting length of the sarcomers, the L''/L spectral parameter was greater than 1.2 (which indicated a very little sub-millisecond rotational motion ($\tau_2 \sim 1$ ms)). In contrast, in pyrophosphate-relaxing solution (10 mM potassium-pyrophosphate plus the components of the rigor buffer) at about the resting length of the fibres, the orientational order of spin labels is reduced (Fig. 8), and simultaneously the ST EPR spectrum showed the increased rotational mobility of the probe molecules ($\tau_2 \sim 200 \mu s$). Accepting the two-state model of cross-bridges in relaxing medium, it was possible to calculate the fraction of cross-bridges bound to the thin filaments (Ishiwata et al. 1986). From the EPR spectra we obtained $f=0.60-0.65$, that is at resting length in pyrophosphate-relaxing solution 35-40% of the cross-bridges rotated quite freely, whereas 60-65% of them remained bound to the thin filament at room temperature. This estimation agrees well with the recent data of Chen and Reisler (1984) that at 24°C about 20 and 30% dissociated HMM was found in rabbit skeletal myofibrils in 1-5 mM MgP_i .

In highly stretched state of the fibre bundles (the fibres were stretched in activating solution and their length was held constant in the tissue cell, sarcomere length: $3.2 \mu m$), where the macroscopic order of the filaments and fibrils is much higher than in other states, the molecular ordering of probe molecules was smaller than in rigor (Figs 9 and 10). The effective rotational correlation time of the labels (cross-bridges) decreased in comparison to the measured value in rigor, τ_2 was about 200 μs and 50 μs (estimated from L''/L and C'/C spectral parameters), respectively, and τ_2 remained constant even after depletion of ATP (Fig. 11). The difference in the two estimations of the rotational correlation time can be explained by the anisotropic motion of the labels in a restricted area. The increased rotational mobility indicates that the

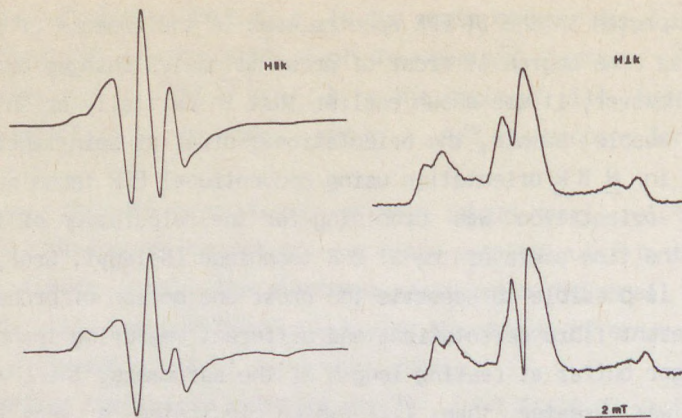


Fig. 8. EPR spectra of spin-labelled muscle fibres in rigor (upper spectra) and in relaxin solution (bottom). Right: conventional EPR spectra; right: ST-EPR spectra.

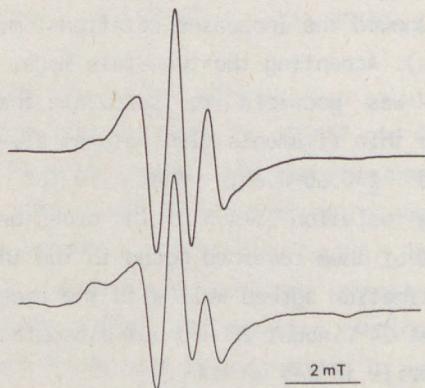


Fig. 9. Conventional EPR spectra of spin-labelled muscle fibres in rigor (upper spectrum) and in stretched state (bottom). The fibre axis was oriented parallel to the magnetic field.

detached heads in the overlapping and in the non-overlapping region contribute significantly to the lineshape of the ST EPR spectra. Recent results of Barnett and Thomas (1984) show that increased rotational mobility is expected even in rigor when the filament overlap is not complete, the EPR spectrum is the superposition of spectra arising from myosin heads in the non-overlapping region which rotate quite freely, and from heads in rigor complex in the overlapping region.

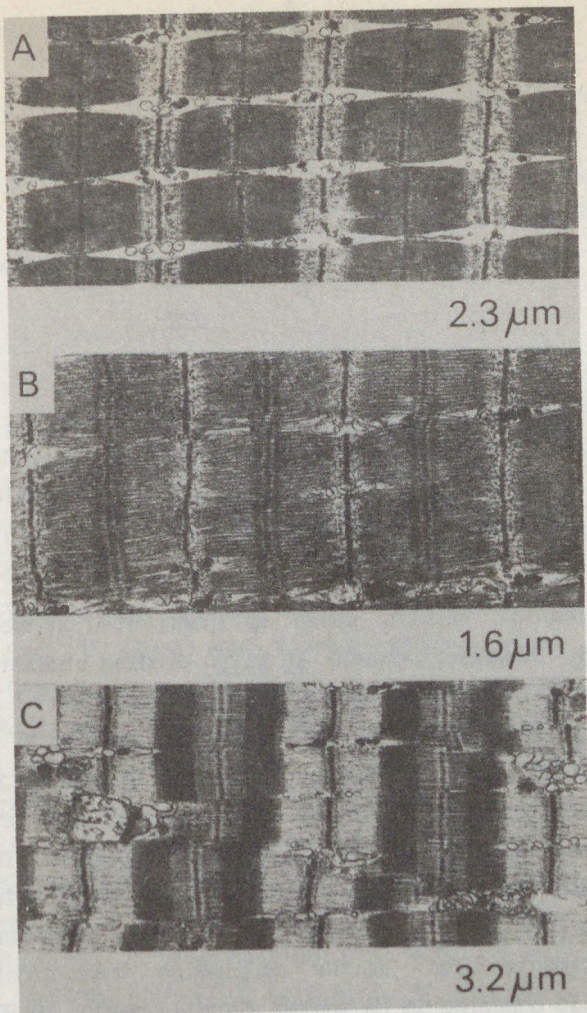


Fig. 10 Electron micrographs of the spin-labelled muscle fibres; A: rigor; B: shortened muscle fibres; C: muscle fibres stretched in activating solution. In the figures the sarcomere lengths are given.

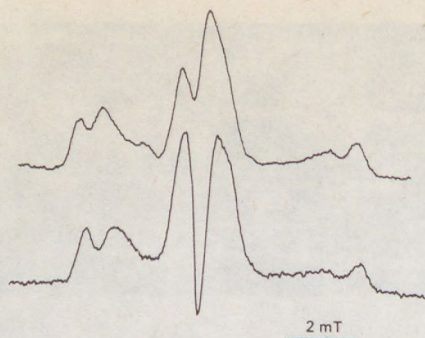


Fig. 11. ST-EPR spectra of spin-labelled muscle fibres in rigor (upper spectrum) and in stretched state (bottom). The fibre axis was oriented perpendicular to the applied magnetic field.

The experimental results could be interpreted on the basis of the two-state model. However, recent in vitro binding studies at low ionic strength suggest the attachment of a significant fraction of cross-bridges to the thin filament even during relaxation (Brenner et al. 1982); it cannot be excluded that a smaller fraction of myosin heads remains attached to the thin filaments at the generally used ionic strength. Moreover, it was also shown by Suzuki and Pollack (1986) that a significant number of bridge-like interconnections exist between adjacent thick filaments in the non-overlap region. On the other hand, rapid freezing experiments led to the conclusion that cross-bridges in contracting muscle appeared to be angled slightly nearer to 90° than rigor cross bridges (Tsukita, Yano, 1985), and it should also be considered that the communication between myosin and actin is likely not one-way system, the energy from ATP splitting can perturb the motional state of actin which in turn may influence the conformational state and binding properties of myosin head during contraction.

These facts provide a basis for further speculation as regards the details of the contraction process and the explanation of the EPR results.

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DISCUSSION

Pollack: I was wondering with regard to your EPR experiments with the S_1 labels. Perhaps you are aware of the controversy between Reedy's group and Heuser's group, as to whether the angles of bridges are close to 90° or 45° . Reedy found the typical rigor angle, but Heuser found that it was closer to 90° . We have some evidence with Charles Trombitás also, that it is closer to 90° . I wonder if your experiments bare on this question that they can distinguish the two. I realize you can tell order and disorder, but can you interpret the experiments in terms of particular crossbridge angles, or not?

Belágyi: No, I can speak only about the orientation of the molecular axis of the probe molecule, but I can't speak about the orientation of the crossbridges.

Pollack: Can you infer something from it or not.

Belágyi: No, not yet!

Ling: We have been brought up on thin filaments and heavy filaments. And then we began to read, as you pointed out, that in many cells such as smooth muscle, some of the actin doesn't exist in the filamentous form. And actin is found in almost every kind of cell, and yet every kind of cell doesn't have thin filaments. The question that came about years ago when I read one of Professor Szent-Györgyi's books, and many others too, have confirmed that the G-actin-F-actin transformation occurs in the presence of electrolytes, potassium, at 0.1 M, for example. Now, there is increasing evidence, that the potassium is not free in the cell. And so, the question is, whether the G-F transformation occur, or doesn't occur, how does it relate? I'd like to hear your comments!

Belágyi: I think I cannot answer it! At very low ionic strength, actin exists solely in its monomeric form, but divalent cations, such as Ca and Mg can induce G-actin - F-actin transformation already in millimolar range of concentration. It is believed that polymerization of actin is due to binding of a divalent cation to the low affinity binding site of actin if the monomer concentration is higher than the critical concentration.

THE ROLE OF WATER IN PUMPING ACTIVITY OF BACTERIO-RHODOPSIN

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INTRODUCTION

In order to understand the role of water in the structure and function of proteins, many studies have been performed on different water soluble proteins using various methods (for review see ref. (1,2)). In our laboratory we have studied the role of water in the membrane bound protein bacteriorhodopsin (bR). Bacteriorhodopsin uses the energy from sunlight to transfer protons from the internal to the external side of the plasma membrane of Halobacterium halobium cells. This way an electrochemical potential is generated which is used for ATP synthesis. We study the mechanism of proton translocation by orienting the bR-containing purple membranes and measuring the displacement current induced by charges moving inside the protein after light excitation (3,4).

The purple membranes can be deposited in oriented way by electrophoresis onto a transparent electrode and then dried (5). These samples remain stable and suitable for study for years. Their humidity may be varied from zero to one hundred percent and this way the role of water in the functioning of bR may be studied. In this paper the results of kinetic measurements of charge motion (6, 7), and visible and infrared absorption studies (8) to gain information on the changes of the protein related to water content are reviewed.

Water uptake by purple membranes

A simple measuring setup (Fig. 1) was constructed which was good to measure changes of light absorption and electric signals after a laser flash. The dried oriented sample was held in a closed chamber and the

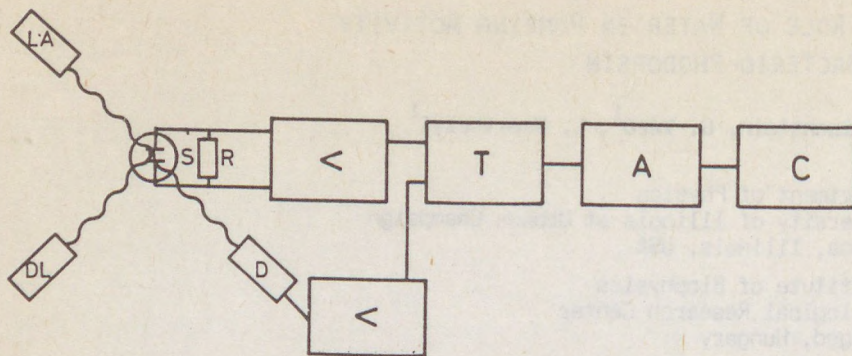


Fig 1. Scheme of the measuring system.

humidity set by appropriate saturated salt solutions. The quantity of water taken up at a given humidity was determined by a micro balance that continuously registered the weight of the sample in the incubation chamber. Equilibrium was reached within 1-2 h. From the results (Fig. 2) it may be seen that the curves between relative humidity $P/P_0 = 0.6-0.7$ tend to a saturation of $W_s \approx 0.05 \text{ g H}_2\text{O/g bR}$ and $S_s \approx 0.1 \text{ nm}^2$, which is approximately the area of one water molecule. Therefore we conclude that at $P/P_0 = 0.6-0.7$, a single continuous water layer is formed on the pm. Above this relative humidity value a rapid water uptake occurs.

Kinetic measurements

The protein electric response signal (PERS) evoked by flash excitation of bR in a dried oriented sample has three main components (Fig. 3). The first and second components have features similar to PERS in pm suspension (3) but with different lifetimes. The third component differs both in sign and lifetime. The first component of PERS is negative with respect to the direction of the proton pump. The fast signal cannot be further resolved into components at all humidities because of the $1 \mu\text{s}$ laser pulse and the time resolution of the electronics ($\sim 0.5 \mu\text{s}$). This signal is the electric counter part of all transitions before L formation. The second component is positive and may be resolved into two exponentials with lifetimes $\tau_1 = 6 \mu\text{s}$ and $\tau_2 = 40 \mu\text{s}$ at a relative humidity $P/P_0 = 0.5$. The same lifetimes were found in light absorption measurements at $\lambda = 400 \text{ nm}$ and 525 nm corresponding to the L-M transition. Therefore this

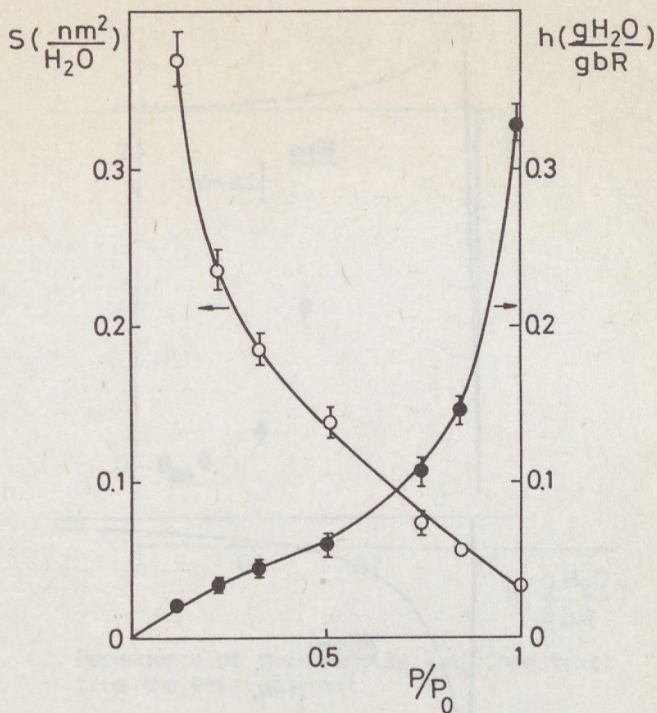


Fig. 2. The weight of water molecules per weight of bacteriorhodopsin molecule (•) and the average area per H_2O on the surface of the purple membrane (o) at different humidities.

PERS component is assigned to the M formation. The third component is negative and can also be decomposed into two exponentials: $\tau_3 \approx 40$ ms and $\tau_4 = 800$ ms (at $P/P_0 = 0.5$). These lifetimes were also found in light absorption measurement at $\lambda = 400$ nm.

The PERS and light absorption signals were measured on samples of different water content and the lifetimes were compared. All decay curves could be fitted by two components of different timeconstants τ_1, τ_2 and τ_3, τ_4 , respectively. The data show excellent agreement between the PERS and absorption time constants.

With increasing water content, τ_1 and τ_2 get longer, τ_2 approaches the lifetime of the L-M transition measured in pm suspension (3), and the amplitude of the τ_1 component is reduced.

While the photocycle in the dried samples is similar to the photocycle in suspension until the M state is formed, during the

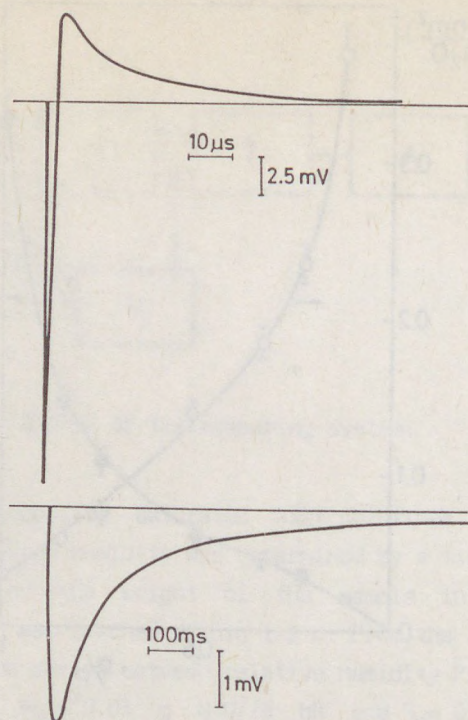


Fig 3. Protein electric response signal (PERS) measured in dried samples. The values of the measuring resistance were $1\text{ k}\Omega$ for the fast signal and $5\text{ M}\Omega$ for the slow one. $P/P_0 = 0.5$

subsequent transitions both the PERS and optical signals show different behavior. The most striking difference is the slow negative PERS in contrast to the positive components in pm suspension (3) and the lack of the characteristic 0 absorption peak at 640 nm (9). The changes of the τ_3 and τ_4 lifetimes with the water content show a good correlation of the values from electric and optical measurements for the whole humidity range.

The negative sign means a backflow of protons in our interpretation of PERS (3,10). If the protons move forward in the L-M transition and flow back from this position, then the positive and negative areas of the signals representing forward (A_2) and backward (A_1, A_3) motions should reflect this. In Fig. 4 the sum of the areas ΣA_i is given depending on water content. It is clear that water molecules are very important in the mechanism of proton translocation.

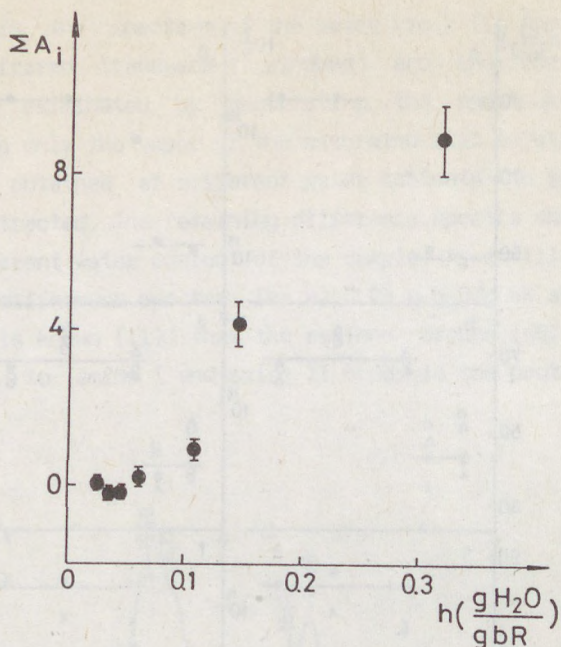


Fig. 4. Dependence of the quantity ΣA_i (see text) from the water content.

In a following series of measurements the temperature dependence of the time constants of the different transitions of electric and light absorption signals were measured (7). The aim of these experiments was to determine the Arrhenius parameters from equation:

$$\tau = \frac{1}{f} e^{\nabla H/RT},$$

where f is the preexponential factor, H the activation enthalpy, R the universal gas constant and T the absolute temperature. In Fig. 5 the data on the different transitions are reproduced. One sees clearly that the Arrhenius parameters ∇H and f have a jump at around $W_S = 0.05 \text{ g H}_2\text{O} / \text{g bR}$ water content. The values of the parameters coincide with the ones in suspension above the transition value W_S .

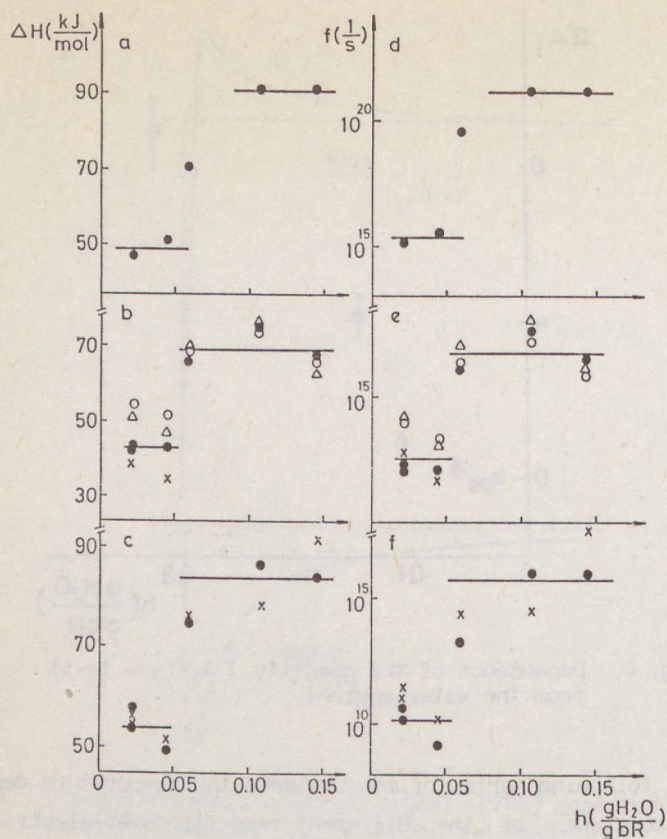


Fig 5. Dependence of the activation enthalpy (a,b,c) and the preexponential factor (d,e,f) from the water content. (a,d) K-L; (b,e) L-M; (c,f) M-bR transitions. The different symbols in b,c,e,f relate to repeated measurements

Infrared spectroscopy

The data from kinetic measurements suggest that bR may undergo a conformational change at a water content of W_5 . An infrared study offers details on these conformational changes and at the same time it may give a better understanding of water absorption by purple membrane.

The data were collected on $\sim 10 \mu m$ thick bR films incubated as previously and their analysis were performed using a Nicolet Fourier Transform Infrared Spectrometer (7199) with a detector sensitive above $800 cm^{-1}$. The spectra were taken with a resolution of $2 cm^{-1}$. In the process

of data analysis the spectrum of the water vapor (in the closed sample holder with infrared transparent windows) and baseline shifts of the instrument were eliminated by subtracting the spectrum of the sample holder containing only the vapor of the saturated salt solutions. From the spectra of bR obtained at different water contents the spectrum of the dried bR was subtracted. The remaining difference spectra show the changes due to the different water content of the sample. As an illustration Fig. 6 shows the difference spectra for a) 0.05 g H₂O/g bR and b) 0.06 g H₂O/g bR. It is known (11) that the regions around 1687 cm⁻¹ and 1505 cm⁻¹ correspond to amide I and amide II bonds in the proteins which are

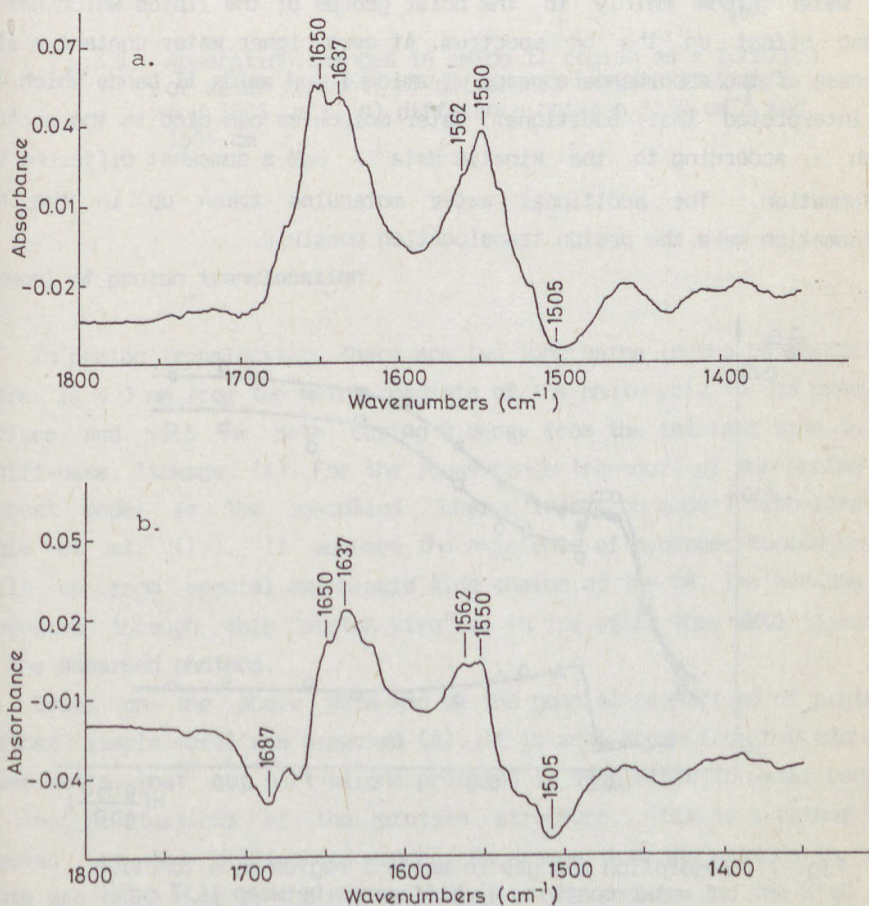


Fig. 6. Difference IR spectra of bR. Water content
a) 0.05 g H₂O/g bR; b) 0.06 g H₂O/g bR

very sensitive to bR conformation because they contain the vibrations of the atoms from the protein backbone.

By changing the water content of the sample the positions of the peaks inside the amide bands do not shift; only their relative amplitudes change as it is shown in Fig. 7 and Fig. 8.

The appearance of a hydrogen bond between the water molecule and protein seemingly does not introduce new types of vibrations, it only changes their relative probability. The saturation effect of the absorption changes shown at around $W_s \approx 0.05$ g H₂O/g bR can be explained by the fact that the highest affinity binding sites of the pm complex (proteins and lipids) are on the protein. At low water content the H₂ molecule binds preferentially to these sites on the protein and they get saturated.

The water binds mainly to the polar groups of the lipids which has no strong effect on the bR spectrum. At even higher water content a slow increase of the absorbance appears at amide I and amide II bands which may be interpreted that additional water molecules can bind to the protein which - according to the kinetic data - has a somewhat different new conformation. The additional water molecules taken up in the new conformation make the proton translocation possible.

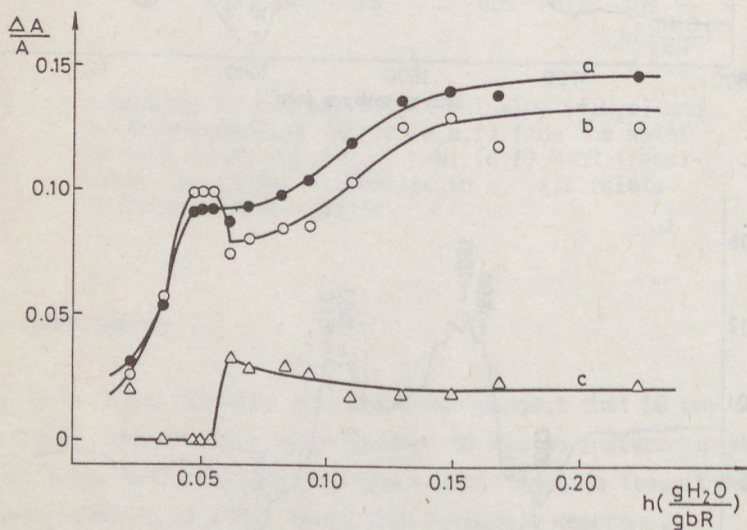


Fig. 7. Absorption changes in amide I region as a function of water content: a) difference between 1637 cm^{-1} and 1687 cm^{-1} ; b) difference between 1650 cm^{-1} and 1687 cm^{-1} ; c) difference between the baseline around 1750 cm^{-1} and 1687 cm^{-1} .

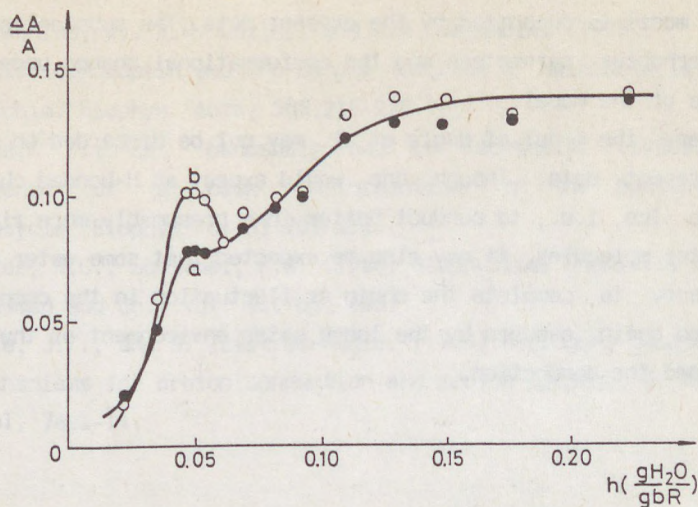


Fig. 8. Absorption changes in amide II region as a function of water content: a) difference between 1562 cm^{-1} and 1505 cm^{-1} ; b) difference between 1550 cm^{-1} and 1505 cm^{-1} .

A model of proton translocation

In proton translocation there are two long paths in the bR molecules: there is $\sim 3\text{ nm}$ from the M-intermediate of the photocycle to the external surface and $\sim 1.5\text{ nm}$ path during O decay from the internal side to the Schiff-base linkage (4). For the long-range transport of the proton the current model is the so-called "charge injection model" elaborated by Nagle et al. (12). It assumes the existence of hydrogen-bonded chains built up from special amino-acid side chains of the bR. The protons are conducted through this "proton wire" as in ice after they are "injected" by the absorbed photons.

Based on the above data and on the general properties of proteins another simple model was proposed (4). It is well known from H-D exchange experiments that any part of the proteins is accessible to water because of the fluctuations of the protein structure. This is a rather fast process (in the millisecond range). We assume that the protons in the M state are taken over by the H_2O molecules in the protein and the H^+_3O ions are driven out to the external side and taken up from the internal side by the existing internal electric field due to the primary charge separation.

This model is supported by the present data. The substantial changes of the Arrhenius parameters and the conformational change underline the correctness of the model.

However, the model of Nagle et al. may not be discarded on the basis of the present data. Though one would expect an H-bonded chain to be similar to ice, i.e., to conduct better in a presumably more rigid state without water molecules, it may also be expected that some water molecules are necessary to complete the chain or fluctuation in the components of the H-bonded chain, assured by the loose water environment of the protein, may be needed for conduction.

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DISCUSSION

Clegg: The magic number is 0.05 (gram H_2O /gram bR). It's an interesting number because presumably there is not enough water there to provide a monolayer, at that hydration, right?

Keszthelyi: It is ~ 70 H_2O molecules/bR. We calculated that it would be enough to cover one surface of the purple membranes by a monolayer.

Clegg: I thought it would be below a monolayer coverage for that kind of hydration. It almost seems like you are hydrating specifically the part of the molecule that carries the protons.

Keszthelyi: I do not know, it is sure that this quantity of water is needed to reach the conformation needed for pumping.

Clegg: Yes, and it has very little effect on proton transfer. Once you reach this threshold. So it is a monolayer!

Keszthelyi: We see from the infrared measurements that the proteins are somehow "filled up" by water and the proton transfer starts only when additional water molecules are available.

Clegg: So, what happens if you put an organic solvent, which doesn't denature the protein, but I understand, this is pretty tough for the protein. What happens if you give only a little bit of water, below 0.05, and give something that will let it jiggle. In other words my question is, what is the water doing: do you think the water is allowing flexibility in the protein to generate proton transfer?

Keszthelyi: Yes, this probably develops flexibility further. But for the pumping, I hypothesise, that the extra water goes into bR, makes a temporary water channel in which the protons are conducted as in water. To answer your question related to the organic solvent I cite some Russian colleagues who made measurements in nonwater solvent, and they found a very low proton transfer.

Cameron: Within the definition of bound water, we included water that's hydrogen bonded. The number of hydrogen bonds could be different. In a protein one can conceive of having multiple hydrogen bonds or ionic bonds that would be slightly higher than single hydrogen bond. We've recently done some studies with lysozyme and come to something around 0.4 or 0.5 g H_2O /g protein when we filled those sites. I'm wondering if you are talking about this as being multiple hydrogen bonded or ionically bonded.

Keszthelyi: The water soluble proteins like lysozymes show a stepwise

hydration process. For example at 0.07 g H₂O/g protein the hydration process is dominated by interaction with charged groups. At higher water content clusters of water molecules form and grow until most of the surface is covered. The process is finished at 0.4-0.5 g/g. The interesting events happened in the case of bR at a value of 0.05-0.06 g/g which is in good accord with the first step of hydration lysozyme.

A BIOPHYSICAL APPROACH OF CELL SURFACE PHENOMENA AND TRANSMEMBRANE SIGNALING

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The cytoplasmic membrane, the cell surface can be regarded as a mechanical barrier isolating the interior of the cell from the environment. The cell machinery is, however, completely dependent on a dynamic interaction between the outside and the inside of the cell. This interaction involves the interchange of signals, chemicals and nutrients between the environment and the interior of the cell. Cells are capable to respond very specifically to various signals. It is customary to distinguish early and late events in some specific cellular responses.

Among these elementary steps association phenomena, redistribution of receptors or other membrane constituents occur frequently inside the cytoplasmic membrane, the very place of the signal transmission. Proteins, hormones and various ligands having bound to their respective receptors might trigger local aggregation, cause the formation of molecular products, activate various transport systems. The change in the intracellular concentration of certain ions and nutrients could in turn activate intracellular biochemical processes, stimulate protein and nucleic acid synthesis. Every major element of the cell membrane has mobility with different degree of freedom in the plane of the membrane as well as perpendicular to it (1, 2). Biophysical approach to study these complicated two-three dimensional movements of the cell surface elements has been hindered by the lack of suitable methodology. The advent of new techniques like Fluorescence Recovery After Photobleaching (FRAP), Flow Cytometric Energy Transfer (FCET), measurement of transmembrane potential of living cells, determination of rotational parameters of lipid and protein elements of cell membrane, and many other methods brought about new possibilities to study dynamic properties of the membrane (1-10).

FRAP, introduced originally by Cone (11), Poo and Cone (12) and Peters et al. (13), and further developed by Axelrod, Web, Elson, Koppel and Schlessinger (see the review by Axelrod (14)), has recently been used even for studying the nuclear membranes of eukaryotic cells (Schindler et al., 1984; (15)). Another approach opened the possibility of studying proximity relationships of cell surface elements: fluorescence energy transfer measurements were successfully applied to the cell surface in order to determine distance relationships between Con A receptors by Fernandez and Berlin (16). Chan et al. (17) used energy transfer measurements in a fluorescence-activated cell sorter and extended the measurements over cell populations. The algorithm to calculate the corrected true energy transfer in a cell sorter has been developed by Trón et al. (8) and applied to studying the proximity of H-2K^k antigens and Con A receptors (9, 18).

Information on the aggregation state of the proteins, glyco- and lipoproteins of the cytoplasm can be gained, among others, by means of methods enabling us to measure average separation distances between the appropriate membrane components. According to the theory of the dipole-dipole energy transfer in the case of suitable donor-acceptor pair the transfer efficiency, E , is a function of spectral parameters and donor-acceptor separation distance

$$E = R_0^6 / (R^6 + R_0^6) \quad (1)$$

where R_0 means the characteristic separation distance at which energy transfer efficiency between the two fluorophors is 50 percent. The numerical value of R_0 can be calculated from spectral and structural data according to (e.g. ref. 19):

$$R_0^6 = 8.8 \cdot 10^{-25} n^{-4} \kappa^2 \Phi_D J \quad (2)$$

where n is the refractive index of the medium in which the energy transfer takes place, κ^2 is the orientation factor for the transition dipole moments of the donor and acceptor, Φ_D is the quantum yield of the donor in the absence of acceptor, J is the spectral overlap integral.

There exist several ways for the experimental determination of the transfer efficiency. The numerical value of E can be calculated:

- a. from the decrease of the life time of the donor in the presence of the acceptor;
- b. from the decrease of the intensity of the emission of the donor because of the energy transfer;
- c. from the sensitized fluorescence of the acceptor (i.e. from the increased acceptor fluorescence due to the energy transfer, excited in the donor absorption band).

The previous methods are routinely used for the determination of intra- and intermolecular distances between specific sites in molecular systems (20). Each of these methods requires the measurement of fluorescent parameters of a double (with both donor and acceptor) labeled and a single (either with donor or acceptor alone) labeled sample.

Any of the listed means for determination of E is principally applicable for average distance measurements on cell surfaces. The specificity of the donor and acceptor labeling in cellular systems can be assured by the incubation of the cells with donor and/or acceptor conjugated ligands. These conjugated ligands bind to the receptors (or specific binding sites) thus allowing to carry out distance measurements (16, 21). Numerous publications are reported on E measurements of that type. These measurements, however, yield transfer efficiencies averaged over the entire cell populations. It seems quite reasonable that one is anxious to carry out similar measurements on single cells in order to be able to detect differences between subpopulations of a heterogeneous sample. The heterogeneity can be connected either with the many kinds of the cells in their different functional states. For this purpose we have developed a flow cytometric method for energy transfer measurements (FCET) being capable of determining the absolute transfer efficiency on single cells.

The determination of the numerical value of E on a single, double labeled cell generally demands several measurements. First of all, the emission intensity of the acceptor has to be measured excited in the donor absorption band. In our experiment fluorescein (fluorescein isothiocyanate - FITC-conjugated ligands) served as a donor and rhodamine (tetramethylrhodamine-isothiocyanate - TRITC-conjugated ligands) as an acceptor (Fig. 1).

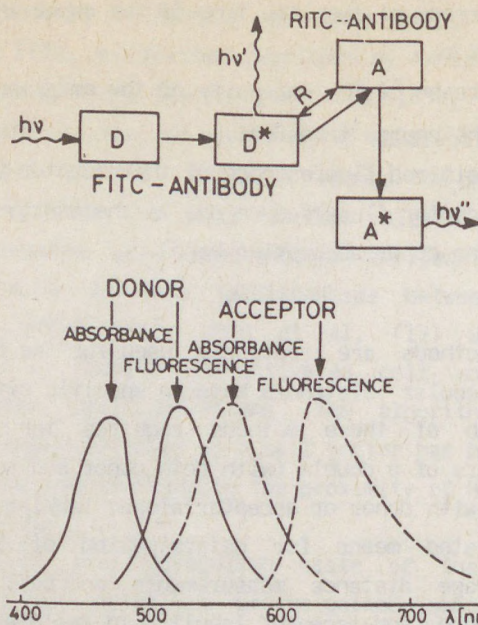


Fig. 1. Fluorescence excitation and emission spectra of FITC and TRITC conjugated ligands.

In the case of this transfer pair, however, (and very often also of other transfer pairs appropriate to measure energy transfer) only a fraction of the detected intensity is related to energy transfer processes because rhodamine emission can be excited in the donor absorption band, too. In order to correct for the directly excited acceptor fluorescence a second intensity determination has to be completed. It is advisable to carry out this second fluorescence intensity measurement on the double labeled sample with excitation at the wavelength where the donor displays a negligible absorbance (Fig. 2)..

This second intensity reading and the measured excitation spectrum of the acceptor fluorescence enable us to perform the mentioned correction. In order to express the probability of the energy transfer in percents, the corrected transfer signal has to be normalized also to the number of the donor excitation events. For this purpose the donor emission (excited in the donor absorption band and detected in its emission band) is also to be measured.

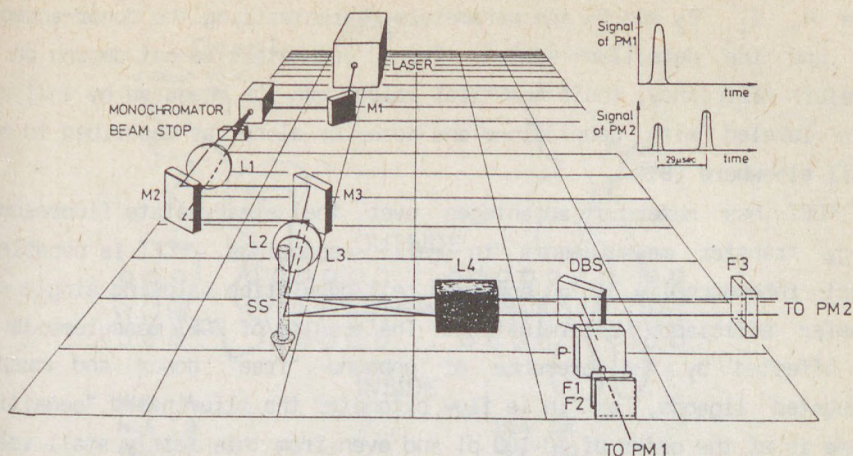


Fig. 2. The optical arrangement of the flow cytometer. The 488 nm lines of 15 W argon ion laser working in "all line" mode were selected and focused to the sample stream, SS, with the aid of mirrors (M) and lenses (L_1 - L_3). The cells carried by SS intersected the 488 nm and 514 nm lines at different heights so that the excitation at these wavelengths were separated both spatially (by 0.1 mm) and temporally (by 29 μ sec). Using a dichroic beam splitter (DBS) a P pinhole and appropriate filters (F_1 - F_3). PM1 detected the fluorescence intensity in the fluorescein emission band and PM2 in the rhodamine emission band.

Because of the reasons outlined above in the experiments carried out on a fluorescence flow cytometer we detected three fluorescence intensities from each single cell. We focused two light beams of an argon ion laser (with wavelengths of 488 nm and 514 nm, respectively) on the sample stream carrying the cells in such a way that the cells intersected the two focused beams with a time difference of 30 μ s. Thus, the excitations of the same cell at these wavelengths were separated in time. This fact and the detection of the emission in the donor and acceptor emission band separately (using on appropriate optical system) made it possible to measure three independent fluorescence intensities from each analyzed cell. From these I_1 , I_2 and I_3 intensities the efficiency of the energy transfer can be calculated as follows:

$$\frac{E}{1-E} = \left\{ \frac{I_2 - S_3 I_3}{\left(1 - \frac{S_3}{S_1} S_2\right) I_1} - S_1 \right\} \frac{1}{\alpha} \quad (3)$$

$$E = \frac{A}{1-A} \quad (4)$$

where α , S_1 , S_2 and S_3 are parameters characterizing the donor-acceptor pair and the detection system. These parameters do not depend on the transfer efficiency, their numerical values can be measured by analyzing cells labeled with donor alone and acceptor alone, as described in more detail elsewhere (8).

FCET has numerous advantages over the steady-state fluorescence energy transfer measurements in cell suspensions. FCET is capable to detect inhomogeneity in an analyzed cell population allowing single cell transfer efficiency determinations. The results of FCET measurements are not affected by the presence of unbound "free" donor and acceptor conjugated ligands, as in a flow cytometer the illuminated "sensitive" volume is of the order of 10-100 pL and even from this fairly small volume a substantial part is given by the nonfluorescent sheet fluid.

Thus, using FCET, it is possible to analyze a cell, without any washing, immediately after the incubation with the labeled ligands is completed. This advantageous feature is of great importance in cases when the ligand off-rate is high and any working step in the protocol would significantly perturb the binding equilibrium.

Simultaneously with the measurement of the three fluorescence intensities required for an E determination FCET makes it possible to detect additional parameters, too. As a result of such combined measurements the correlation of energy transfer efficiency with other cellular parameters can be investigated. The accuracy of the E data is not influenced by cell debris since data collection can be triggered by light scattering signal (indicating the size of the analyzed objects) above a set threshold.

Herewith we summarize some of our recent data obtained with FCET method. In the immune system, major histocompatibility complex (MHC) antigens are co-recognized with foreign antigens by T lymphocytes (22). The MHC Class I and Class II antigens are biochemically as well as functionally distinct molecules (Fig. 3).

Though the Class I and Class II molecules have quite different tasks in the immunological processes our results indicated that the MHC Class I and Class II antigens may more intricately interrelated than it was previously thought. Neppert and Müller-Eckhardt (23) have shown by capping experiments that there is an unexpected interdependence between Class I and Class II antigens. The cocapping phenomenon indicates that intermolecular associations exist between these antigens.

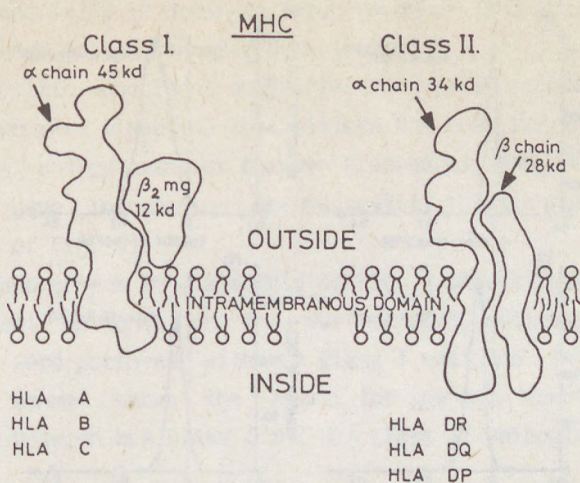


Fig. 3. Schematic drawing of Class I and Class II MHC glycoprotein molecule.

Our questions to be addressed here were the followings:

1. Can molecular association be detected between these antigens applying fluorescence resonance energy transfer techniques?
2. Does this association exist prior to cocapping as well, or is it induced only by the cocapping process?

In our experiments a human cell line was used designated as PGF cell, which has an abundance of HLA antigens.

In order to answer the above mentioned questions, we labeled the antigens with different monoclonal antibodies:

<u>Monoclonal antibodies</u>	<u>Specificity</u>
L 368	β_2 -microglobulin
W6/32 monomorphic	HLA - A, B, C
Genox 3.53 polymorphic	DQ ($\alpha_1\beta_1$)
anti-Leu 10 polymorphic	DQ ($\alpha_1\beta_1$, $\alpha_2\beta_2$)

The first two antibodies bind to the Class I antigens. The L 368 is specific to β_2 microglobulin, which is a common part of Class I antigens. W6/32 is monomorphic, thus it binds to all HLA - A,B,C molecules.

The other two recognize only the DQ molecule of the Class II antigens. While the anti-Leu 10 binds to all DQ molecules, the Genox 3.53 recognizes only a subpopulation of those. These antibodies were conjugated

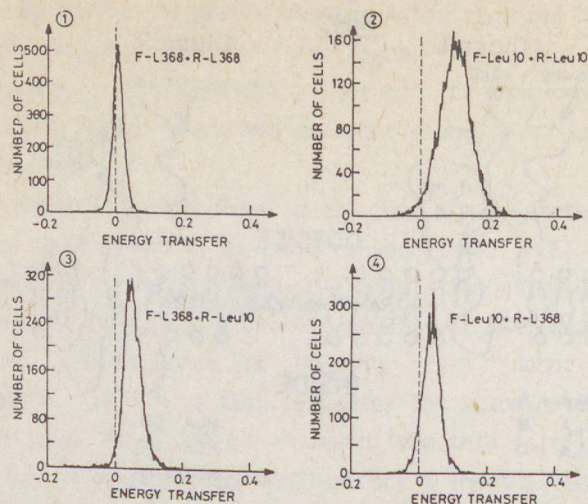


Fig. 5. Energy transfer histograms of PGF cells labeled with rhodaminated and fluoresceinated monoclonal antibodies: L368 was raised against β_2 microglobulin; Leu 10 against DQ antigens. The transfer efficiency was measured between HLA Class I and Class I (1); between HLA Class II (DQ) and Class II (DQ) (2); and between Class I and Class II (DQ) antigens (3,4).

Significant energy transfer was observed between antibodies binding to identical epitopes of DQ antigens suggesting that DQ molecules form dimers on the cell surface. The third and the fourth histograms demonstrate the association between HLA Class I and HLA Class II molecules.

Mean value of energy transfer histograms are summarized in Table 1. In order to interpret these transfer efficiency data we have introduced two models. In both models the HLA Class I antigens are assumed to be monomers because low ET efficiency was measured between Class I molecules (first line in Table 1). According to the data in the first line of the third section of Table 1, the DQ antigen is likely to form a dimer. Another possibility may exist that two Leu 10 antibodies can bind to the same DQ antigen. The middle section of the table demonstrates the association between HLA Class I and Class II antigens.

In summary, on the basis of experimental results two models can be set up, as shown in Fig. 6. In the model A one HLA - A, B, C monomer form a complex with a DQ dimer. In the model B one HLA - A, B, C monomer forms a complex with a DQ monomer (Fig. 6). The complex formation between Class I and Class II molecules seems to be proved, so we can conclude that these

with FITC and TRITC in order to serve as donors and acceptors, to be used in fluorescence energy transfer experiments.

DQ specific monoclonal antibodies conjugated either with fluorescein or rhodamine were mixed and this mixture was used for labeling the cells. Fluorescence energy transfer between fluorescein and rhodamine conjugated antibodies gave information on the spatial relationships of DQ antigens (upper part of Fig. 4).

The data and scheme in the middle of Fig. 4 demonstrate the existence of two different epitopes on the surface of DQ antigen. The same kind of experiments were performed with HLA Class I antigens. In the lower part of Fig. 4 a scheme shows the answer for the main question: Is there any association between HLA Class I and HLA Class II molecules?

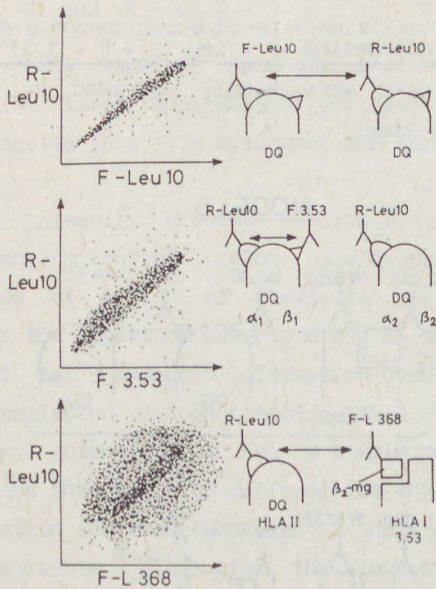


Fig. 4. Two-parameter cytogram of red fluorescence (ordinate) against green fluorescence (abscissa). Human PGF cells were labeled with the mixture of rhodaminated and fluoresceinated monoclonal antibodies.

The frequency distribution histograms of the energy transfer (ET) are shown in Fig. 5. The cells were labeled with various combinations of fluorescently conjugated monoclonal antibodies. In the case of monoclonal antibodies binding to HLA Class I antigens the mean value of ET histogram is around zero, indicating that the HLA Class I molecules exist mainly in monomeric form on the cell surface.

Table 1.

FLUORESCENCE ENERGY TRANSFER DATA ARE MEAN VALUES OF ENERGY TRANSFER
HISTOGRAMS

ANTIGENS	MONOCLONAL ANTIBODIES	ET
Class I	competing F - L 368 + R - L 368	2 %
	F - W6/32 + R - L 368	8 %
	noncompeting F - L 368 + R - W6/32	4 %
Class I and Class II	F - L 368 + R - Leu 10	6 %
	F - W6/32 + R - Leu 10	5 %
	F - Leu 10 + R - L 368	7 %
	F - 3.53 + R - L 368	5 %
Class I	competing F - Leu 10 + R - Leu 10	8 %
	F - 3.53 + R - Leu 10	12 %
	noncompeting F - Leu 10 + R - 3.53	5 %

(The monoclonal antibodies were kindly provided by Frances Brodfky, Becton-Dickinson, Montan-View.)

MODELS

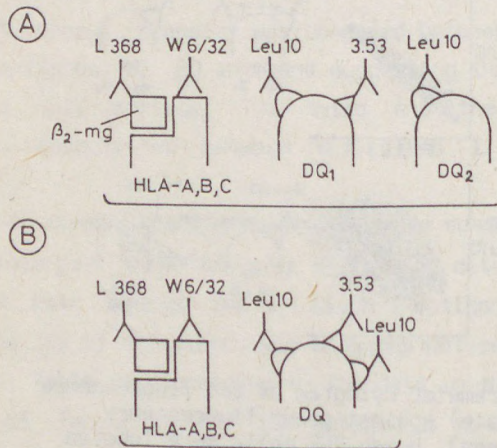


Fig. 6. Models describing associations of HLA Class I and HLA Class II antigens. The models are based on fluorescence energy transfer data.

antigens are associated on the cell surface not only during the co-capping but prior to cocapping as well.

One of the most promising medical/biological fields of flow cytometry, besides clinical diagnostics, is pharmacology. The goal of

modern pharmacological testing is to reveal the mode of action of drugs at cellular and subcellular levels.

Before the final approval of a drug for human use, much information is necessary (gained under conditions as close to physiological as possible) on the drug/cell interaction with living target cells. Thus, the most sensitive and fastest methods (with the least possible harm to cells) to monitor early drug-cell interactions with living cells have growing importance. The first stage of a drug/cell encounter is the cytoplasmic membrane. Some drugs, hormones, and ligands will penetrate this membrane either in a direct or indirect way (an example of the latter is endocytosis). One of the earliest possible observable changes in membrane properties is membrane transport, highly regulated by both the lipid and protein elements of cytoplasmic membranes. A logical and immediate consequence of such a change could be an alteration in membrane potential. In recent years, many membrane potential-indicating dyes have been developed (24). The first application for studying membrane potential changes in lymphocytes in a flow cytometer was carried out by Shapiro et al. in 1979 (25).

Despite the steadily growing availability of flow cytometric techniques, membrane potential probes have rarely been applied for the study of the mode of action of drugs (26). Although flow cytometric measurements offer the important advantage of the ability to collect data on a cell-by-cell basis and gain information over either the whole cell population or preselected subpopulations within a few minutes, the flow cytometric membrane potential is not a simple measurement. This paper describes some of the characteristic difficulties and the possible means to avoid misinterpretation for those who wish to apply these methods.

The most suitable dyes for the investigation of drug/cell interactions via membrane potential changes are the carbocyanine and oxonol dyes (27, 28). Since these dyes easily permeate lipids due to their strong hydrophobic character, they are well-suited for such investigations. Since the carbocyanine dyes are cationic, and the oxonols are anionic at physiological pH, the carbocyanines go from extra-cellular space into the cells, while the oxonols have an opposite translocation when the cell interior becomes more negative. One of the most frequently used sensitive membrane potential probes is dihexyloxacarbocyanine iodide ($\text{DiOC}_6(3)$), a cationic dye with useful fluorescence properties. The dye has good excitability at visible spectral regions and emits at wavelengths

convenient for photomultiplier detection. The dye signal changes in membrane potential by distribution in the extra- and intracellular space and the cell membrane. Although their distribution is governed mainly by electrochemical gradients, the solubility of the dye in lipids may change the distribution independent of the membrane potential.

It is imperative to scale the upper and lower limits of the fluorescence signals under conditions in which the membrane potential of intact living cells, such as lymphocytes, is at maximum and minimum. Such conditions can be generated by changing the extracellular potassium concentration from 1.5 to 160 mM. Since the membrane potential of resting lymphocytes is mostly due to the potassium potential (29), determined by the Nernst equation, the cytoplasmic potential will be maximum at low, and minimum at high external potassium concentration. Further orientation can be gained regarding the meaning of changes in fluorescence signals by adding ionophores with known effects on the membrane potential to the cell samples. Ionophores are specific compounds having the capacity to bind certain ions in a loose but usually very specific way. The ionophores freely diffuse in the membrane, thereby transporting ions from one side of the membrane to the other, according to the concentration gradient of the ion in question, or form a permanent channel across the membrane, thereby inducing a strong leakage of ions (30).

To obtain a fluorescence signal indicating the lower limit of membrane potential, 1-10 μ M of the channel-forming ionophore gramicidin is sufficient. This antibiotic forms a channel through the membrane that is permeable mostly for sodium and potassium ions. Valinomycin is another ionophore with very high specificity for potassium ions. This circular compound merges into the cell membrane, and by free diffusion in the lipid layer, shuttles the potassium along the concentration gradient. This ion transport usually results in depolarization of the membrane. However, low amounts of valinomycin can also hyperpolarize membranes (Fig. 7). The mechanism is relatively simple: since valinomycin will transport only potassium, if the counterions can not permeate the membrane or diffuse much slower than the potassium, the positive potassium ions will be held at the cell surface by the electrostatic forces which increase the transmembrane potential gradient. The valinomycin also affects the mitochondrial functions.

The absolute value of the membrane potential cannot be determined easily in a flow cytometer, and the measurement is usually performed in

combination with electrophysiological measurements. Simple calculation can give approximate values, but the possibility for miscalculation is very great. Fortunately, in most cases, the relative size and direction of changes are satisfactory. A possible and frequently overlooked problem with cells having plenty of mitochondria is the differentiation between cytoplasmic and mitochondrial membrane potential changes. The use of the mitochondria-specific dye, rhodamine 123, or the scaling of the upper and lower potential limits with extracellular potassium, can help to handle this problem. Resting lymphocytes have practically no functioning mitochondria; thus, most of the immunological applications of these measurements are free of the above-mentioned complications. The situation is quite different with stimulated cells, where the mitochondria can also accumulate high concentrations of dyes and thus confuse the evaluation of the measurements.

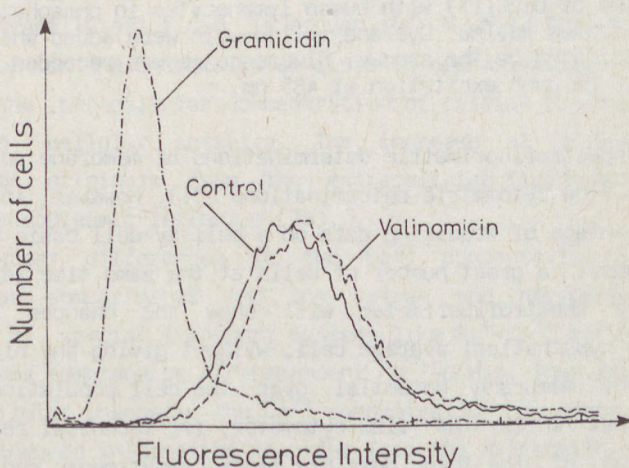


Fig. 7. Effect of ionophores on the $\text{DiOC}_6(3)$ fluorescence of human lymphocytes detected by flow cytometer. The cell were stained with a final concentration of 50 nM $\text{DiOC}_6(3)$. After being equilibrated with the dye valinomycin (2 M) and gramicidin (1 M) were added to the samples.

Steady-state fluorimetric methods can also be applied to study cell membrane potential (Fig. 8).

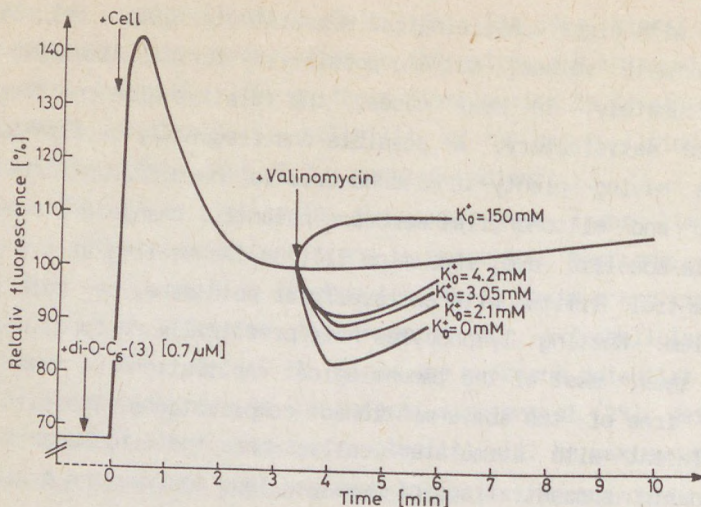


Fig 8. Characteristic changes in fluorescence intensity with time of $\text{DiOC}_6(3)$ with human lymphocytes in phosphate buffered saline. Dye and valinomycin were added when indicated by the arrows. Fluorescence was recorded at 510 nm upon excitation at 485 nm.

Actually, the spectrofluorimetric determinations of membrane potential far outnumber the flow cytometric determinations (31). However, in that case, the great advantage of acquiring data on a cell-by-cell basis and gaining information about a great number of cells at the same time is lost. Data obtained in a spectrofluorimeter will show the changes in membrane potential of a hypothetical average cell, without giving any hint about distribution of membrane potential over the cell populations. Another problem is that while the flow cytometer, for technical reasons, will "see" only two of the three possible major compartments containing the dye, that is, the membrane and the cell interior, a spectrofluorimeter will also see the third compartment - the extracellular space. This fact further complicates the evaluation of data. At higher than micromolar concentration, the accumulation of cationic dyes in the cell interior results in an aggregation. The immediate consequence of this phenomenon is a concentration quenching, which results in a decrease in fluorescence intensity.

The advantages of the flow cytometer are the following. The greater power of laser excitation makes the application of much lower concentrations of dye possible. At nanomolar dye concentration, quenching cannot occur because the concentration of the dye available in the cell

interior is not in high enough to aggregate. This low concentration also prevents toxic effects of the dye. Furthermore, since the extracellular space cannot be seen by the flow cytometer, the translocation of the dye will result in an increase in the fluorescence intensity.

Water soluble molecules including ligands like hormones, growth factors, antibodies, etc. bind to specific receptor proteins on the cell surface. These ligand molecules bind to the surface receptors with high affinity and convert this extracellular event into an intracellular signal. The cell activation generally starts with an increase of intracellular Ca^{2+} ion concentration, activating calmodulin and phosphokinase systems (32), ultimately leading to the regulation of DNA replication (33). It is proved by a series of observations that the calcium ions are involved in many cells as intracellular messengers. The basis of this control function of the Ca^{2+} ions is their quickly alternating affinity to form chelate complexes. The Ca^{2+} ion concentration in the extracellular fluid is around 10^{-3} M while the concentration of free calcium in the intracellular fluid is approximately 10^{-7} M. A small change in the intracellular concentration of calcium can trigger profound changes in cellular activity. The increase of intracellular Ca^{2+} concentration originate from the extracellular fluid, the mitochondria and/or the endoplasmic reticulum (34).

The great differences in the Ca^{2+} concentrations between the intracellular compartments and the extra- and intracellular space are maintained by special transport systems like Na^{+} - Ca^{2+} exchange mechanism of the plasma membrane or ATP-dependent Ca^{2+} -pumps. Thus the detection of any intracellular change in the Ca^{2+} concentration has great importance in the ligand induced information transfer from the extracellular environment into the cells.

In order to detect these small changes of intracellular Ca^{2+} concentration it is necessary to develop highly sensitive methods (35).

There are a number of methods which have been used to detect free Ca^{2+} changes in the intracellular fluid (36-41). These methods have provided valuable information, but each is subjected to experimental limitations. For example the photoprotein aequorin (42, 43) (the msot widely used chromophor) is not readily applicable for measuring the total amount of released Ca^{2+} , only the changes in Ca^{2+} concentration, as a result of the change in Ca^{2+} flux (44). The Ca^{2+} -sensitive microelectrodes can only be used in cells large enough to be introduced and they only

monitor Ca^{2+} concentration near the electrode tip and microelectrodes may perturb the calcium homeostasis of cells (45). One of the best methods to measure Ca^{2+} concentration with the least possible harm to cells is the application of fluorescent chelators binding Ca^{2+} with 1:1 stoichiometry (46, 47). The structural formulas of the most frequently applied dyes are shown in Fig. 9. The structure is related to that of the well-known chelator ethylene glycol bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic

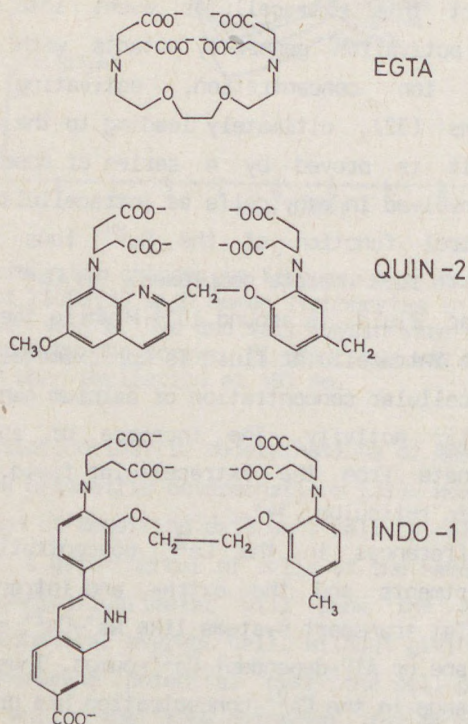


Fig. 9. The structures of EGTA and its fluorescent derivatives (quin-2 and indo-1).

acid (EGTA). The indicators themselves are impermeant and do not pass through the cell membrane. A method has been worked out by Tsien (46) for loading the chelators into living cells by forming hydrophobic membrane-permeant molecules which are digested by cytoplasmic esterases, thereby transporting the dyes into the cells (Fig. 10).

The equations (5) and (6) show, the method of calculation of the intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$:

$$\text{QUIN-2} \rightarrow |\text{Ca}^{2+}|_i = K_D \left(\frac{F - F_{\min}}{F_{\max} - F} \right) \quad (5)$$

(ref. 46)

$$\text{INDO-1} \quad |\text{Ca}^{2+}|_i = K_D \frac{\frac{F_1}{F_2} - \frac{R_{\min}}{F_1}}{R_{\max} - \frac{F_1}{F_2}} \frac{S_{f2}}{S_{b2}} \quad (6)$$

(ref. 47)

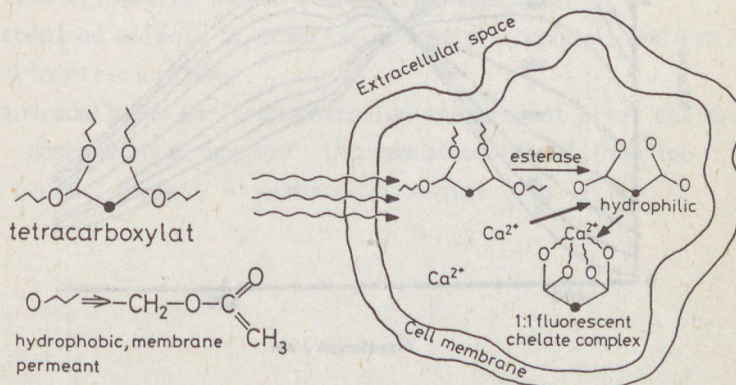


Fig 10. Schematic drawing to indicate method of loading cells with fluorescent tetraacetoxymethyl ester (like, indo-1AM, quin-2AM).

Grynkiewicz et al. (47) has reported the latest fluorescent chelators indo-1 and fura-2 which can be loaded into the cells as their membrane permeant acetoxymethyl (AM) esters. Indo-1 shares most of the advantages of fura-2 except that rather than having large changes in its absorption on calcium binding, its emission shifts from about 490 nm maximum without calcium to 400 nm with calcium (Fig. 11). The ratio of these values can also be used to determine the intracellular calcium concentration independently of dye concentration.

Many different agents, such as hormones, drugs, and cell metabolism-regulating peptides may exert their effects at the level of the cell membrane. Their earliest mode of action may be, for example, a potassium leakage that can be a trigger signal for many biological or biochemical reactions.

Some of our data are presented in the case of various kind of lymphocyte membranes (peripheral human lymphocytes and mouse spleen and thymus cells) when cyclosporin A (CsA), an immunosuppressive drug with largely unknown mode of action was applied to these cells.

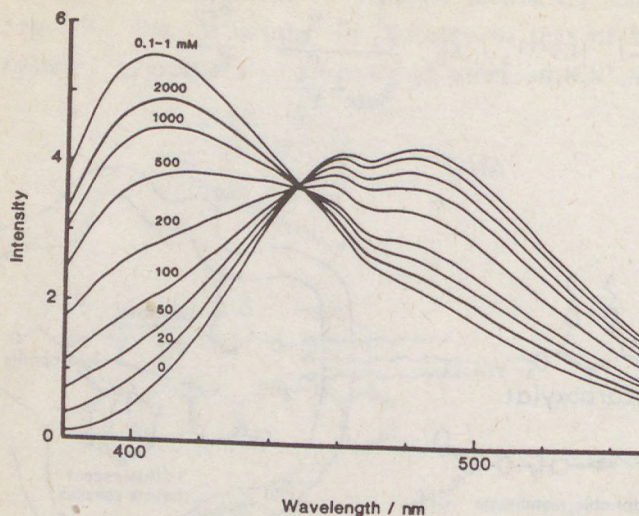


Fig. 11. Emission spectra for indo-1 as a function of free Ca^{2+} . Excitation was at 355 nm (46).

Cyclosporin A (48-50) is a cyclic endecapeptide with profound clinical and experimental immunosuppressive properties (51-53). CsA is applied in the treatment of graft-versus host disease after bone marrow transplantation, for graft rejection in solid organ transplantation and as a potential therapeutic agent in autoimmune diseases, malaria and schistosomiasis (52, 54).

The cellular and molecular mechanism of the action of CsA has not been clarified yet. It appears from in vitro studies that the lymphokine, interleukin-2 production is inhibited by CsA at the level of mRNA synthesis (55,56).

CsA does not block interleukin-2 in mouse lymphocyte cultures of Balb/C and C 57BL/67 mouse spleen cells (57).

Handschumacher et al. (58) reported on a specific intracellular protein fraction, cyclophylin, which binds CsA, and it has been found recently that the drug CsA may inhibit the function of calmodulin through binding to the protein (59).

In order to understand the mechanism of the effect of CsA on lymphocyte activation, its influence on the events triggering early cell activation must be investigated (60, 61). Since all activation is a very complex phenomenon, the selection of a sensitive early signal to monitor changes caused by cyclosporin A has great importance. In our study on human peripheral blood lymphocytes and mouse spleen and thymic cells the following techniques were applied to study the properties of the cytoplasmic membrane immediately after CsA treatment:

1. flow cytometric membrane potential measurements;
2. combined effects of insulin, specific ionophores and CsA in flow cytometric system;
3. intracellular Ca^{2+} concentration measurement after CsA treatment.

Our observations suggest incorporation of CsA into the cytoplasmic membrane causing changes in membrane potential (Fig. 12).

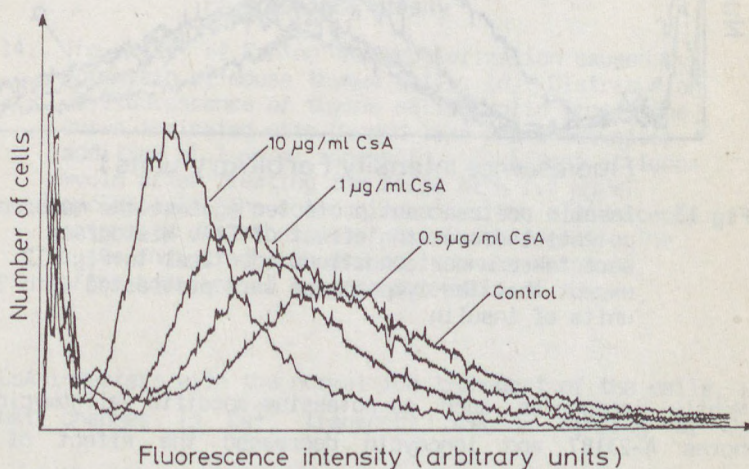


Fig. 12. Membrane potential decreasing effect of CsA on mouse spleen lymphocytes. The Y axis of the histogram shows the number of cells, measured in flow cytometer; the X axis shows the fluorescence intensity proportional to the membrane potential.

Pharmacological doses of CsA decreased the cytoplasmic membrane potential, i.e. the mean of the above histograms shifted to the left side. The data shown in Fig. 12 clearly demonstrate the dose dependence of the fluorescence distribution of spleen cells pretreated with 0.5, 1 and

10 $\mu\text{g/ml}$ CsA. Thymus cells and peripheral human lymphocytes show similar sensitivity and direction of changes in membrane potential. However pretreatment of mouse lymphocytes with insulin reduced the effect of the CsA (Fig. 13). Insulin is known to counteract the immunosuppressive effect of CsA (63). Insulin was ineffective when human cells were tested, since resting human lymphocytes practically have no insulin receptors (64).

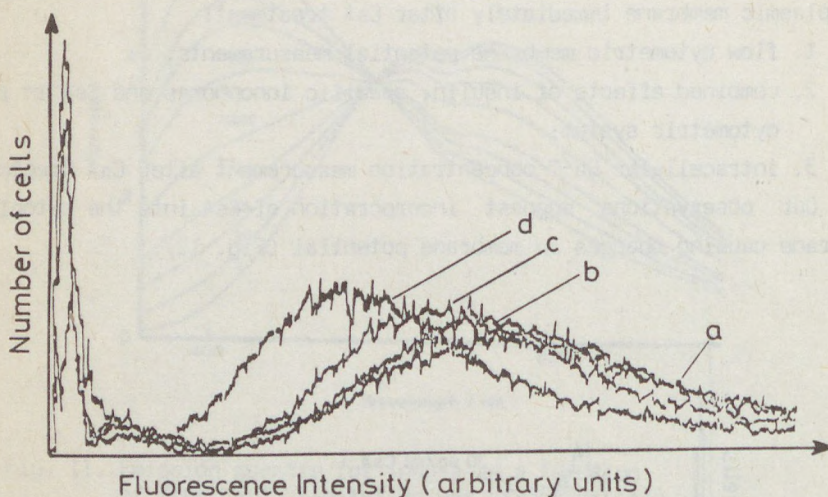


Fig 13. Insulin pretreatment protected against the membrane potential decreasing effect of CsA. Histograms were taken under conditions identical to Fig. 12 except that the lymphocytes were pretreated with 2 units of insulin

Mobile ionophores such as potassium specific valinomycin, the Ca^{2+} ionophores A-23187 and ionomycin decreased the effect of CsA on the membrane potential of mouse lymphocytes (Fig. 14). Since most of the experiments were carried out with spleen cells the question of selectivity arises as the spleen cells consist of approximately equal amounts of T and B lymphocytes. The spectrofluorimetric titration of thymus or spleen cells with CsA for changes in membrane potential did not show any selectivity. It is quite acceptable that the CsA decreased the membrane potential of both T and B lymphocytes (61). These phenomena are consistent with observation that CsA also interacts with B cells (62). This would suggest that CsA nonspecifically binds to the lipid membrane and/or perhaps to a protein in the membrane of the cell.

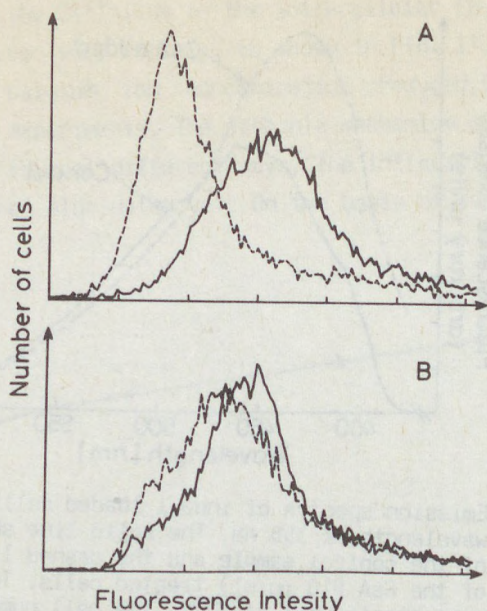


Fig. 14. The effect of CsA on the depolarization caused by ionomycin of mouse thymus cells. (A): Distribution of fluorescence of thymus cells (solid line). The curve indicated with dashed line was taken after addition of 1 $\mu\text{g/ml}$ ionomycin. (B) Effect of ionomycin after treating the cells with 1.5 $\mu\text{g/ml}$ cyclosporin. The solid line shows the fluorescence intensity distribution of CsA treated cells. The curve indicated with dashed line was obtained after addition of 1 $\mu\text{g/ml}$ ionomycin.

Since CsA interferes with the normal ion transport of the cells, it is possible that changes in Ca^{2+} transport precede binding of CsA to calmodulin.

In other type of experiments we followed the changes of intracellular Ca^{2+} concentration in Balb/C thymus cells. The cells were loaded with indo-1-AM (as described before; ref. 47).

The emission spectra of indo-1 excited at 355 nm were recorded in the presence and absence of CsA (Fig. 15). The spectral shift and the increased fluorescence intensity at 400 nm are obviously indicating the increase of Ca^{2+} level upon the treatment of the cells with CsA. In this case the extracellular Ca^{2+} concentration was negligible. CsA increased the ratio of the 400/490 nm fluorescence in a dose-dependent manner (Fig. 16) indicating an increase in the intracellular Ca^{2+} concentration (47).

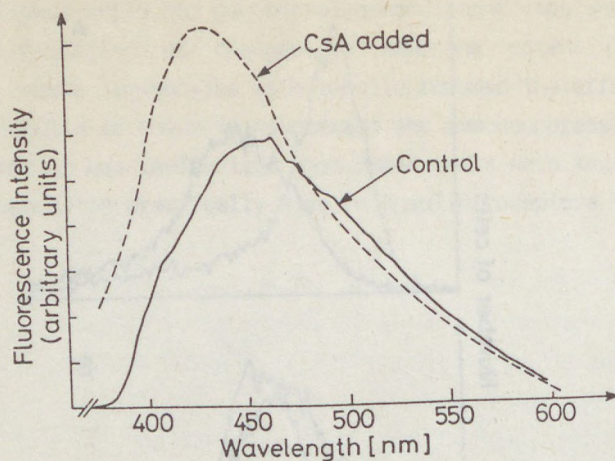


Fig. 15. Emission spectra of indo-1 loaded cells at excitation wavelength of 355 nm. The solid line shows the spectrum of the control sample and the dashed line shows that of the CsA 810 $\mu\text{g/ml}$ treated cells. The experiments were carried out at 37°C, the cell number was 1×10^6 cells/ml.

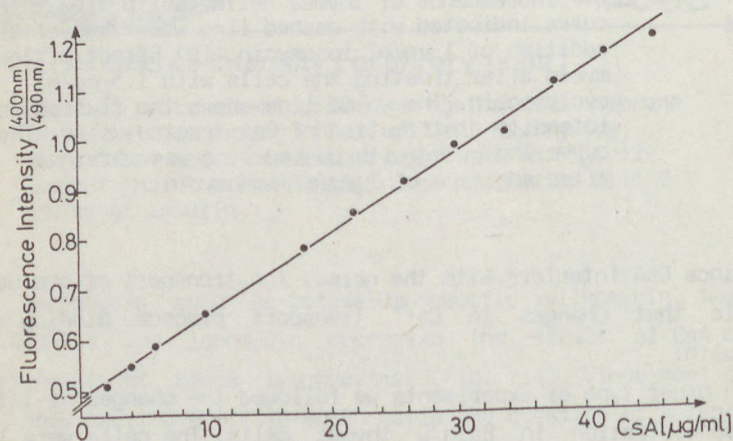


Fig. 16. Spectrofluorimetric titration of indo-1 loaded thymus cells with CsA. The Y axis shows the increase of the ratio of fluorescence intensities measured at 400 and 490 nm, respectively, (λ_{ex} : 355 nm). The X-axis shows the amount of CsA applied to the cells.

The accumulation of the Ca^{2+} ions in the intracellular fluid subsequent to CsA treatment occurs very rapidly, as shown in Fig. 17. The increase of the intracellular calcium ion concentration produced by CsA is clearly demonstrated in our experiments. The probable mechanism of this action may be interpreted in several different ways. The intracellular localization of CsA has not been elucidated yet. On the basis of experiments carried

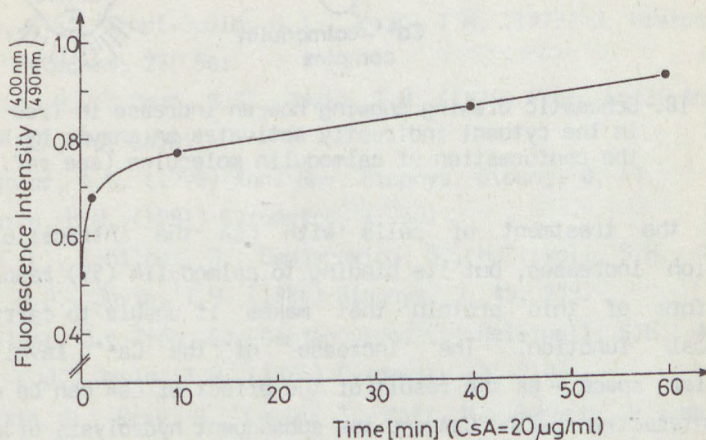


Fig. 17. Time course of the ratio of fluorescence intensities measured at 400 and 490 nm, respectively, (λ_{ex} : 355 nm) in indo-1 loaded cells after treatment with 10 $\mu\text{g/ml}$ CsA.

out with fluorescently labeled CsA derivatives is supposed to accumulate in small lipid droplets (66), according to others it binds to the hydrophobic part of proteins. Colombani et al. (59) claim that CsA binds to the Ca^{2+} binding protein, calmodulin. The calmodulin and the calcium ions play an important role in controlling several cellular functions. A well defined intracellular Ca^{2+} concentration is necessary for the calmodulin activation. The activation of the suppressor T-cells and B-lymphocytes may be independent of calmodulin or probably it may require a higher "resting" concentration of calmodulin for activation (Fig. 18; ref. 67). This fact may account for their relative resistance against the CsA stimulus.

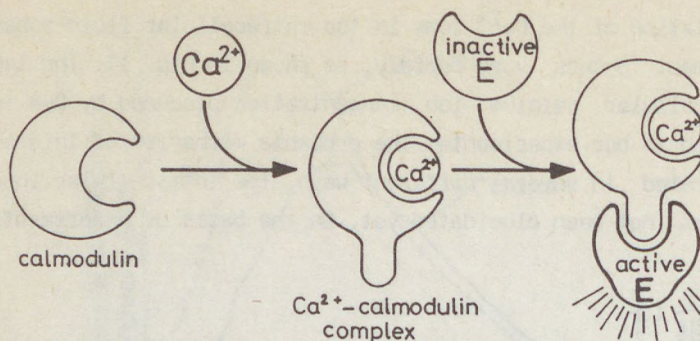


Fig. 18. Schematic drawing showing how an increase in free Ca^{2+} in the cytosol indirectly activates an enzyme by altering the conformation of calmodulin molecules (see ref. 67).

After the treatment of cells with CsA the intracellular Ca^{2+} concentration increases, but its binding to calmodulin (59) results in an inactive form of this protein that makes it unable to carry out its physiological function. The increase of the Ca^{2+} level in the intracellular space - as the result of the effect of CsA can be explained by the interaction with CsA and the subsequent hydrolysis of a special membrane lipid phosphatidil-inositol-4,-5-diphosphate (PIP_2) located in the intracellular site of the plasma membrane. The products of hydrolysis are inositoltriphosphate (IP_3) and diacylglycerole. The information pathway has two branches: the inositoltriphosphate passing through the cell membrane releases calcium from different cell stores such as mitochondria and endoplasmic reticulum (68).

The diacylglycerol remains in the cell membrane and can trigger another cell reaction by activating membrane-bound protein kinase C. CsA may possess a mutual promoting influence on both branches in the following way: it causes the release of Ca^{2+} ions in the IP_3 branch and, at the same time, in the diacylglycerol branch while CsA is bound to an intracellular protein that protein may be the calmodulin. The CsA-calmodulin complex may inhibit the cell reaction regulated by the calmodulin hereby inhibiting the synthesis of RNA and DNA (59).

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DISCUSSION

Ling: First I want to say that I really appreciate your presentation. But during it, I was undergoing an internal debate. Should I come up with something that would probably slightly disturb you. Yet, I ask myself why am I here, and I have decided therefore to come forth and point out differences of opinion that are on an issue that is not your primary concern. First, the word "membrane potential". This was a classic idea that Oswald first experimentally demonstrated, then Bernstein developed it, and it is classical neurophysiology. It is that on which I was brought up. I was a neurophysiologist to begin with. Now, after this many years I strongly suspect that this is a wrong name. That all models examined, too long to innumerate, described in my rather big book published recently, show that the cellular potential is not a membrane potential. It's primarily a surface potential. Just to demonstrate this point: valinomycin, for example, in the presence of potassium, changes the potential, there is no question about that, and yet every time it was measured, there was no change in potassium conductance. In squid axon, in frog muscle, in frog ovarian eggs, in the inner membrane liver mitochondria, no changed the potassium permeability. The only thing that had a slight change, about 40%, is the potassium conductance of red blood cells. In other words, your dye measurements actually are much closer to the event, to the theory that we are talking about. Primary it's a surface phenomenon. The membrane potential happened to be the two surface potentials backed against each other. So, formally you cannot tell the difference. What I'm hopeful for is to get you interested in looking a little bit into this view. This view is exactly as old as Hodgkin-Huxley's theory dating from 1952. And it has broad acceptance by surface chemistry and electrochemistry. That's why our articles always come out in monographs of that sort. But it has a great deal of relevance to what you are talking about. Because you are essentially dealing with surface phenomena. There is no permeability change. The permeability actually measured with isotope or electroactivity does not respond to additional valinomycin which increases the potassium conductance in the artificial bilayer membranes.

Damjanovich: Thank you very much for your comment, and let me answer the following. Valinomycin can hyperpolarize, hypopolarize, depolarize or not

change anything. That's correct. But valinomycin was proven to be a very specific potassium ionophore that can accommodate a single potassium ion, as shown by X-ray microanalysis. Now, I believe that valinomycin goes into the lipid phase of the membrane because it's a hydrophobic compound. It shuttles there, and it will transfer potassium alongside the chemical gradient, or rather electrochemical gradient. Let me just tell you, when you mention surface potential, I agree with you in the sense, that a 6 Å layer of the intracellular fluid is enough to provide the amount of the potassium to be "nailed" to the surface of the membrane, because the nondiffusible, nonpenetrable big anions will prevent its diffusion. In that sense, I agree with you that there is a surface potential, and that is what you mentioned, but the membrane has a thickness and it also has an inside deepness, and in this deepness, there is a gradient. It is my belief. I agree with you that it is very hard to prove directly.

Ling: I think I want to continue this argument because what you see through a small hole is actually another space. And it is filled with enormous amount of work, enormous amount of literature. I give you an example. The membrane theory has dominated the thinking of cell physiologists for a whole century. Right now it's just about a century. And model after model have been constructed to testify to the validity of these membrane phenomena: membrane potential, membrane permeability. Model after model, which include the oil membrane, glass membrane, collodion membrane, and phospholipid bilayer, if you follow the literature patiently through time, all discover they have nothing to do with permeability but have everything to do with two surfaces.

For example if you take a lipid layer and you put liquid on both sides, and you introduce a substance like SDS, which has a long hydrocarbon chain, that goes into the lipid phase, that surface will develop a potential that is cation sensitive. Now, on the other hand, if you introduce another compound, like dodecyl-trimethyl-ammonium-bromide, which carries a positive charge, then it would become a chloride-sensitive electrode. You can do this either on one surface or on to the other surface. In other words, you can create opposite potential differences. And this applies to all the surfaces, they are innumerable. Glass membrane, collodion membrane, neutral lipid membrane and phospholipid bilayer membrane. Now, go to a determination of permeability. We have a paper just ready to come out. The effect of valinomycin is 10^{-7} . In the

presence of potassium, say, from 10 to 100 mM in the outside, increased the potassium permeability of the lipid bilayer by three orders of magnitude. It has absolutely no effect on the resting potential of frog muscle. And absolutely no effect on the potassium permeability of frog muscle. The same has been confirmed by Steelen and others in squid axon and by still others in the mitochondrial inner membrane. By the way, I would like to further point out that the inner membrane of liver mitochondria contains only 20% lipid which creates a strong argument that lipid does not form a continuous layer in the membrane. It only forms pockets. Because we have discounted water in this calculation, if you take all of this in account, the idea that you have a ferryboat is becoming remote because in order for a ferryboat to work you have to have a continuous phase a few water puddles are not going to be effective.

Damjanovich: Do you believe in voltage gated potassium channels?

Ling: Oh, no conflict with that at all! I'm talking about what you called a membrane potential. I'm not talking about the action potential, where there is a transient. I'm talking about the resting potential. The resting potential is not a membrane-determined phenomenon. Membrane-determined in terms of permeability. All the evidences indicate that it is a surface potential. When you have a change in the cell membrane, then you have a temporary change in the permeability, then you have a dynamic phenomenon that initiates the action potential.

Damjanovich: Dr. Ling, I think our discussion is a bit misdirected, because I'm not very much interested in membrane potential, but I am interested in the parameters that might reflect some dynamic changes in the cell membrane. And I don't care if the transmembrane gradient potential will be changed, and that will be reflected with my dyes, or the cell surface potential will be changed and that will be the monitored effect. But what I'm looking for is that I try to collect parameters that will reflect specific changes in the membrane's dynamic parameters. Because my principal aim is to find an interface between the physical parameters of membrane elements, and the biochemical events that take place during for example transmembrane signaling. And if I look from this aspect, I don't think that we have too much difference.

Ling: I don't think we have, I completely agree with you!

Hazlewood: I am concerned about the picture that the membrane being a sea of lipid in which a little protein is found. The data in the literature

indicates that, for the most part, the major component of the cell surface or the structures that have been isolated and are said to be membranes is protein. Therefore, if the membrane is a sea of protein in which a little lipid is found, what effect, if any, would this have on your thinking, and your position.

Damjanovich: Actually I don't see much difference between my explanation and your question, because you asked me whether the ratio of the lipid and protein will change anything of my philosophy. Not at all! I started working with the fluctuations of proteins. And we try to combine the fluctuation pattern and the functional properties of different proteins. And now we try to do the same thing in the membrane. My aim is to study transmembrane signaling, the information transfer through the membrane. That is a question of a second messenger, that is a question of inositol phosphate cycle, calmodulin, phosphokinase C, or phosphorylated proteins. My philosophy is that beside these things there must be, and there are quite a few physical changes regardless, a transmembrane potential change, or a cell surface potential change.

THE ASSOCIATION-INDUCTION HYPOTHESIS AND LIFE

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The basic unit of all life is the living cell. To understand the living phenomenon in general and to build a solid foundation for future biomedical research, it is essential to know "What are the fundamental distinctions between a living cell and a cell that is no longer living?"

A clue may be found in a familiar phenomenon: exposure to dyes like erythrocin, nigrosin, and trypan blue deeply stains dead cells but not living ones. Indeed the different responses of living and dead cells to dyes reflect the fundamental attributes of the living cells to maintain their integrity and hence existence in environments which in chemical composition are different from those within the living cells. Thus the living cells contain concentrations of Na^+ only a fraction of that in the aqueous environment; when killed, the cell Na^+ concentration dramatically increases. However, the living cells do not hold all their components at a lower concentration. K^+ is held at a much higher concentration in the cells than found in the environment. Thus, at the cell level, the transition from life to death is intrinsically related to a dramatic loss of the ability of the cell to maintain its unique composition.

In general principles, there are three ways - and three ways only - whereby the concentrations of a substance can be sustained indefinitely at different levels in two contiguous spaces. Consider the case of a camper at a mosquito-infested site. The camper can keep the mosquitos away (1) by using a mosquito net, (2) by detecting and catching all mosquitos that come near and releasing them a far distance away, or (3) by spraying the site with an insect repellent. The molecular equivalent of each of the three basic mechanisms has been invoked to explain the asymmetric distribution of K^+ and Na^+ between the living cells and their

environments: (1) in the original membrane theory, a net-like cell membrane permeable to water but not to ions, permanently keeps K^+ inside and Na^+ outside the cells; (2) in the membrane-pump theory, membrane pumps, like the diligent camper, continually capture and transport Na^+ out of the cells and K^+ into the cells; (3) in the protoplasmic theories, distinctive physico-chemical environments within the cells attract K^+ and repel Na^+ .

The original membrane theory was disproved in the late 30's and early 40's when radioactive-tracer studies revealed that the cell membrane is permeable to both Na^+ and K^+ . After that, only two types of theories have survived: the membrane-pump theory is by far the better known and often taught as fact in popular textbooks written by poorly informed authors (see below). There are two versions of the protoplasmic theories: the sorption theory of A.S. Troshin of the Soviet Union (Troshin, 1966) and the association-induction (AI) hypothesis, which I first introduced formally in 1962 and in 1965 (Ling, 1962, 1965, 1984). Troshin's sorption theory and the AI hypothesis are in general harmony with each other. However since the 60's, the only protoplasmic theory that has been continually developed and tested is the AI hypothesis. Professor Troshin died in December 1985.

1. The Membrane-Pump Theory

The membrane-pump theory was not articulated and defended by one (or more) advocates and in this respect, quite different from most other serious theories. The scientists whose names have been sometimes cited as the originators of the membrane-pump theory like A. Krogh (1946) and R. Dean (1941) could not be construed as the theory's originators because they only reiterated what had been said long before (see Lillie, 1923) and had added little that was new.

By definition, physiology concerns itself with mechanisms of living phenomena. A bona fide theory of the Na pump must offer a mechanism for the postulated pump. Yet, in the first-of-its-kind review on the subject of the Na pump, Glynn and Karlish apologized for not being able to compare the "great mass of work" with "a hypothesis accounting for the working of the pumps" because "no such hypothesis exists." (Glynn and Karlish, 1975, p. 13)

1.1. The Excessive Energy Need of the Na Pump

In the membrane-pump theory, intracellular K^+ , Na^+ as well as the bulk of cell water are free. The orientation of the Na^+ concentration gradients and the polarity of the resting potential are such that virtually all the Na^+ ion leaving the cell must be by pumping (Ling, 1965). In the late 40's I discovered that frog muscles could maintain their normal K^+ and Na^+ contents at $0^{\circ}C$. for hours in the absence of respiration and glycolysis when they were exposed to anoxia which blocks respiration and iodoacetate which blocks glycolysis (Ling, 1952, p. 765). Furthermore, the Na^+ efflux rate from frog muscle was also indifferent to the combined action of anoxia and glycolysis (Ling, 1952), a finding later fully confirmed by Keynes and Maisel (1954) and by Conway et al. (Conway, et al., 1961; for details, see Ling, 1984, pgs. 91-92). A possible explanation for this unexpected indifference of frog muscle to poison was the cell's reliance on their store of ATP and creatine phosphate (CrP), both present at high concentrations in frog muscle.

I spent a great deal of time in the early fifties, developing suitable methods accurately to assay the ATP and CrP contents of muscle immediately after complete suppression of both respiration and glycolysis and some hours later, being kept at $0^{\circ}C$ at all times. In the meantime, the Na^+ pumping rate as well as the resting potential were continually monitored. Having shown that under normal conditions, respiration, glycolysis and the store of ATP and CrP were the only energy sources available to the muscle cells (Ling, 1984, p. 125) and that the muscle spent its energy only on the outward pumping of Na^+ (an assumption, obviously untrue) I found, from a total of 28 sets of experiments, that the cell did not have enough energy to operate the Na pump. Indeed the results of the last three sets of data completed in 1956, showed that minimal energy need of the Na pump is from 15 to 30 times higher than the maximally available energy (Ling, 1962; 1984, p. 124) (Table 1).

This data has been part of the literature for nearly a quarter of a century; it has never been challenged in print. Three remedial postulations aimed at keeping the Na pump concept afloat (Ussing's exchange diffusion, sequestration of cell Na^+ in the sarcoplasmic reticulum, and the nonenergy consuming Na^+ pump) have all been experimentally disproven (see Ling, et al., 1979, p. 274).

TABLE 1.
Energy Balance Sheet for the Na^+ Pump in Frog Sartorius Muscles (0°C)^{a,b}

Date	Duration (hr)	Rate of Na^+ exchange integrated average (moles/kg per hr)	$\psi + E_{\text{Na}}/\mathcal{F}$ integrated average (mV)	Minimum rate of energy required for Na^+ pump (cal/kg per hr)	Maximum rate of energy delivery (cal/kg per hr)	Minimum required energy Maximum available energy
9-12-56	10	0.138	111	353	11.57 (highest value, 22.19)	3060%
9-20-56	4	0.121	123	343	22.25 (highest value, 33.71)	1542%
9-26-56	4.5	0.131	122	368	20.47 (highest value, 26.10)	1800%

a - The minimum rate of energy delivery required to operate a Na^+ pump according to the membrane pump theory was calculated from integrated values of the measured rates of Na^+ ion out against the measured electrical and concentration gradients. The maximum energy delivery rate was calculated from the measured hydrolysis of CrP, ATP, and ADP, the only effective energy sources available to the muscles, which had been poisoned with IAA and nitrogen.

b - From Ling, 1962

However, the energy for the sodium pump described in Table 1 was by no means the only energy need of the living cells for pumping. As years went by it became increasingly clear that pumps are needed for virtually all the solutes found in the living cells as well as for man-made solutes like phenol red which made its first appearance through the past efforts of organic chemists and has been shown also to require pumps (see Troshin, 1966, p. 206); so do other pump-requiring solutes yet to be synthesized!

Furthermore, many subcellular particles require their own pumps at their covering membranes because the solute levels in them may differ considerably from those in the cytoplasm. It has been estimated that the liver mitochondrial membrane may be as much as 20 times larger than the liver cell plasma membrane (Lehninger, 1964, p. 30) and that the membranes of the sarcoplasmic reticulum of muscle cells may be as much as 50 times that of the muscle plasma membrane (Peachey, 1965). Since energy need for pumping varies directly with the membrane area, the energy need of the pumps of these subcellular particles may exceed by far those of the plasma membrane pumps.

1.2. Other Evidence For and Against the Membrane-Pump Hypothesis

Much change has occurred in the last 30 years. As of this day, and to the best of my knowledge, there is no longer any compelling evidence unequivocally in favor of the membrane-pump theory. For a detailed review, the reader must consult my recent monograph, In Search of the Physical Basis of Life (Ling, 1984). Some of the earlier evidences once widely regarded as unequivocally supporting the membrane-pump theory (e.g., K^+ mobility in squid axon, K^+ activity in squid axon and other cells measured with K^+ specific intracellular electrodes) have been shown to be erroneous or equivocal (Ling and Negendank, 1980; Ling, 1984, p. 245 and p. 250; Ling, 1984a). Neither squid-axon-membrane sacs freed of axoplasm, nor intact erythrocyte ghost membranes freed of hemoglobin achieve net transport of K^+ or Na^+ against concentration gradients (Ling and Tucker, 1983; Ling, 1984, p. 129; Ling et al., 1984), nor do man-made phospholipid membrane vesicles incorporating Na, K activated ATPase (Ling and Negendank, 1980). In contrast, muscle cells without a functional cell membrane and postulated pumps continue to accumulate K^+ and exclude Na^+ much as intact muscle cells do (Ling, 1978; Ling, 1984, p. 133; see also Kellermayer, et al., 1986). Still other evidence demonstrating the adsorbed state of cell K^+ and the bulk of cell water to be reviewed below also refute the basic free- K^+ and free- H_2O tenets of the membrane-pump theory.

This then is what I know, as of July 30, 1986, of the true status of the most basic theory of cell physiology and biomedical research. I pause to ask the question, "Why is this disproven theory continued to be taught as unqualified fact in virtually all textbooks and serve as the foundation of the great majority of cell physiological and biomedical research?"

Sociologist Bernard Barber (1961) captioned his article published in Science entitled "Resistance by Scientists to Scientific Discovery" in these words: "This source of resistance has yet to be given the scrutiny accorded religious and ideological sources" (underline mine).

Barber then cited Hans Zinser (1940): "The academies and learned societies - commonly dominated by the older foofoos of any profession - are slow to react to new ideas is in the nature of things. For, as Bacon says, scientia inflat, and the dignitaries who hold high honors for past accomplishments do not usually like to see the current of progress rush

too rapidly out of their reach." Only in the intervening years since Zinser, the power of the "dignitaries" has been vastly expanded and made virtually unchallengeable, through the near universal adoption of the peer review system in evaluating science and scientists. The peer review gives the dignitaries the power to decide who get research grants, who get academic appointments, who receive promotion to tenure, whose work is published in widely-read journals...

As pointed out by Prof. Catchpole, Glynn and Karlsh in their review cited above on the "Na Pump", listed 245 articles in support of the sodium pump and none opposed. Yet Ling's idea had been around for 25 years, so had ours, so had Troshins..." (Catchpole, 1981). An individual scientist, who deliberately cites only favorable evidence for and ignores negative evidence against a specific theory, commits what 19th century English mathematician, Charles Babbage (also from Cambridge University) called "cooking", a form of scientific fraud (Babbage, 1830; see also Honor in Science, 1986). In my view, that Glynn and Karlsh should nevertheless choose this course of action (by no means uncommon) attests to the intensity of fear for reprisals they must have experienced. Unfortunately scientific wrong doing begets more wrong doing. One of mankind's noblest activities is in jeopardy when those with new ideas are thus ignored and ostracized.

Science is a new human experience; only very recently have historians come to agree that past progress in science has been achieved by a dual mechanisms: drastic directional changes (called revolutions if eventually proven valid) and fruitful exploration of the new directions thus opened up (Kuhn, 1962; Cohen, 1985, p. 389). Louis Pasteur's work was of the first kind; Jonas Salk's was of the second kind.

The peer review system was adopted before the importance of drastic directional change was recognized and was based on the erroneous idea that science progressed only by the smooth accumulation of knowledge; hence the belief that peers cannot be far wrong. The peer system thus conceived has provided an effective way to promote research that happened to be in the right direction at the start (e.g., genetics). By making it virtually impossible to pursue major directional changes, the peer review system has also become the major road block to further progress in areas of science that are still young and therefore offer the great promise for the future (e.g., cell physiology).

It is high time that scientists, concerned about the future of science in general and biomedical science in particular, rise to the challenge and lend support to scientists and government officials trying to improve the peer review system so that it will foster both modes of progress and not limiting its support only to currently popular directions. It is not going to be an easy task but one may take heart in remembering that others have already so successfully dealt with even more forbidding and more deeply rooted sexual, racial, religious, ideological bias and abuse of power in efforts that began not so long ago.

2. The Association-Induction (AI) Hypothesis

In the AI hypothesis, the molecules and ions making up a living cell stay together by a mechanism not totally unlike that keeping together a school of free-swimming fish in the open sea. "Near neighbor interactions" among closeby fish not only hold the school together; it can, at a signal, also sharply change the direction of swimming of all the fish in an all-or-none manner.

A simple, more cogent model is a chain of loosely tethered nails and iron filings scattered among the nail chain. If a magnet is brought into contact with one terminal nail, the nails will be one by one magnetized. On the magnetized nails, the iron filings are then attracted and organized according to a fixed pattern. Thus interaction with the magnet has brought the whole assembly from a low energy, high entropy state to a high energy-low entropy state. Like the school of fish, it is primarily short-range interactions between immediately neighboring nails and iron filings that provide the coherence and put the nails and iron filings under the controlling influence of the magnet. Of course, in the nail model, the interaction is magnetic polarization. In the living cells, the polarization is electronic - or induction. In this case, it is the polypeptide chains of certain cell proteins that function as the equivalents of the nail chain; ions and water molecules then behave like the equivalent of iron filings. The equivalent of the controlling magnet is a class of biologically potent agents, collectively referred to as "cardinal adsorbents". A highly important cardinal adsorbent is ATP.

The high energy-low entropy state of the cell is called the resting living state. It is separated from active living state (e.g., contracted

muscle) by a small energy barrier. Both resting and active living states are separated by a higher energy barrier from the death state of still lower energy and high entropy. A still higher energy barrier separates the death state from the ultimate random state.

Figure 1 present a diagrammatic illustration of a living cell. Note that the cell membrane, mitochondria, and other subcellular structures are not shown. Rather the figure illustrates the basic components of all these structures as well as the cytoplasm. Protein-protein interaction, protein-ion interaction, and protein-water interaction link the cell into a coherent unit like the school of fish or the nail-filing model.

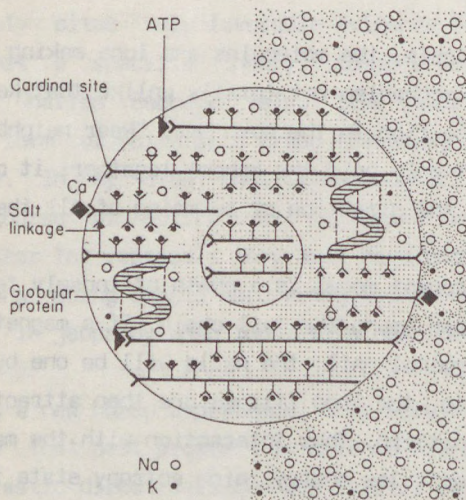


Fig. 1. Diagrammatic illustration of the substance of a living cell. Ordered dots represent water existing in the state of polarized multilayers due to interaction of certain pervasive cell proteins existing in the fully extended conformation. Reduced solubility of polarized multilayered water for large and complex molecules and hydrated Na⁺ (open circles) (and K⁺) and inability to compete for β - and γ -carboxyl groups preferentially adsorbing K⁺ (solid circles) keep intracellular Na⁺ concentration low. Selective adsorption of K⁺ and multilayer polarization of cell water depends on the interaction of the involved proteins with ATP and other cardinal adsorbents. Salt linkages among different proteins maintain cell structure.

In the highly associated state, the cellular proteins as a rule do not assume the same conformation they assume after their isolation from the living cells. Indeed, in the AI hypothesis, the "native" state of some of the cellular proteins corresponds to the dead state. In the resting living state, the conformation of the proteins is different both in terms of steric conformation and electronic conformation from the so called "native" state.

An expression of the high energy electronic conformation is that the anionic β^- and γ -carboxyl groups carried on aspartic and glutamic acid residues of the cell proteins assume such electron density (expressed as the c-value in the AI hypothesis, see Ling, 1984, p. 156) that they adsorb preferentially K^+ over Na^+ by a margin large enough to overcome the concentration bias in favor of Na^+ (100 mM) over K^+ (2.5 mM) in the external medium. As a result, these fixed anionic sites predominantly adsorb K^+ . Since the cell proteins contain a high concentration of these β^- and γ -carboxyl groups the theory offers an explanation why K^+ concentration in the cell is high while K^+ concentration in the external medium is low.

Unable to compete against K^+ for the fixed anionic sites in the cells, a major share of the intracellular Na^+ exists as free ions in the cell water. The concentration of Na^+ in the cell water is much lower than that in the surrounding medium, because the cell water exists in a physical state different from that of normal liquid water (i.e., the state of polarized multilayers, see below) and in this state, water has reduced solubility for hydrated ions like Na^+ , as well as K^+ .

In summary, through its association with other proteins and with cardinal adsorbents, especially ATP, certain proteins in the living cell offer their β^- and γ -carboxyl groups for the preferential adsorption of K^+ . The same or other proteins, also under the control of ATP, assume the fully extended conformation with their polypeptide $NHCO$ groups directly exposed to the bulk phase water, polarizing the water molecules in multilayers. The exclusion of Na^+ from adsorption onto the β^- and γ -carboxyl groups and the reduced solubility of water in this state for Na^+ (and K^+) account for the low concentration of Na^+ in the resting living cells. In this model, the physical basis of life, is not merely the presence of a suitable concentration of each essential chemical component occupying locations specific to the cell type. It is that, but more: The operation

of short-range electronic polarization or "induction", mediated through the partially resonating and hence highly polarizable polypeptide chains, enables the closely associated protein-water-ion systems to assume the high energy living state and to behave as a coherent system.

2.1. Selective K^+ Accumulation

The idea that K^+ accumulation might be due to its adsorption on cell proteins in a way similar to oxygen accumulation in red blood cells was suggested by Moore and Roaf long ago (Moore and Roaf, 1908). However attempts do demonstrate in vitro similar selective K^+ adsorption by isolated proteins in a scale large enough to explain K^+ association in cells had ended in failure (see Lillie, 1923). In 1952 I suggested that this failure to demonstrate in vitro alkali-metal ion adsorption might reflect the assumption of different conformations of the proteins in vitro and in living cells (Ling, 1952). More specifically, in native proteins, the anionic β - and γ -carboxyl groups might be locked in "salt linkages" with cationic ϵ -amino groups and guanidyl groups and therefore not available for K^+ or Na^+ adsorption. Very recently Ling and Zhang (1984) had taken advantage of the good Na^+ -selective electrodes now commercially available and had fully confirmed this "salt-linkage hypothesis". It was found when ϵ -amino groups, guanidyl groups, etc. of bovine hemoglobin were neutralized with NaOH; for each fixed cationic group discharged, one Na^+ is adsorbed. Furthermore, this stoichiometric binding is ion-specific and exhibits autocoperativity much as the binding of oxygen on the same protein shows autocoperativity (i.e., $n > 1$). These findings on one hand, removed one of the major objections against selective adsorption as the basic mechanism for the selective accumulation of K^+ in living cells and on the other hand, provided a solid base for the selective adsorption of alkali metal ions as a cooperative phenomenon, a basic concept also introduced in the AI hypothesis (Ling, 1962, 1984).

The hypothesis that it is the β - and γ -carboxyl groups that specifically adsorb K^+ in living cells leads to two predictions: (1) since in striated muscle over 60% of the β - and γ -carboxyl groups is found in myosin and myosin is found only in the A band, the first prediction is that most of the K^+ in resting skeletal muscle should be localized in the A band; (2) since there is evidence that the β - and γ -carboxyl groups

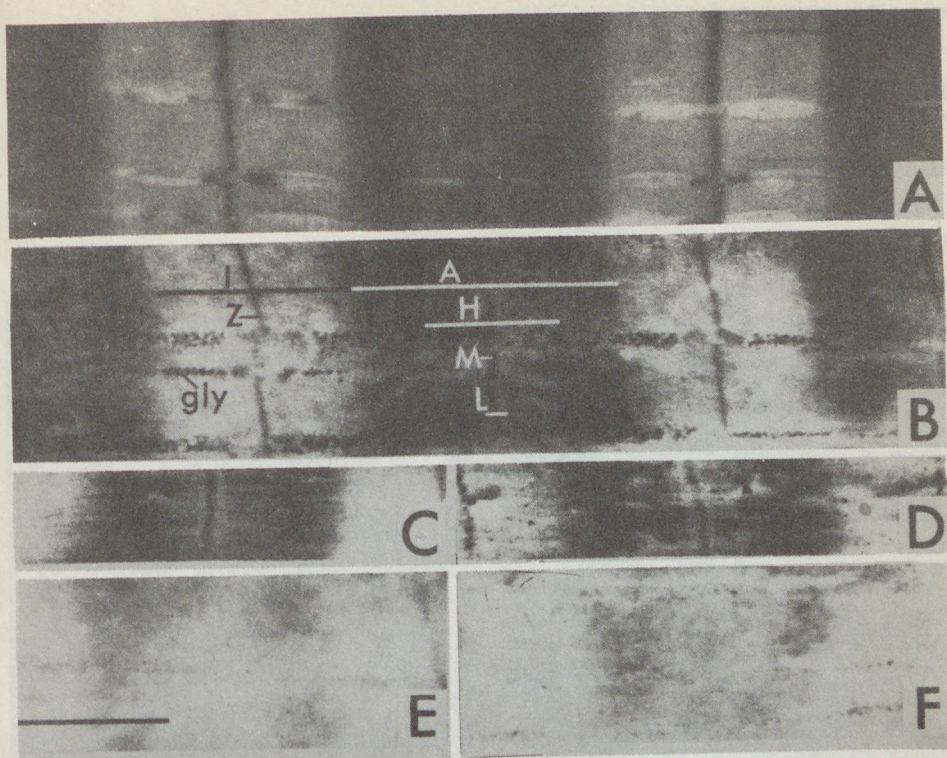


Fig. 2 - Electron micrographs of frog sartorius muscle. (A) Muscle fixed in glutaraldehyde only and stained only with uranium by conventional procedure. (B) EM of section of freeze-dried Cs^+ -loaded muscle, without chemical fixation or staining. (C) Tl^+ -loaded muscle without chemical fixation or staining. (D) Same as (C) after exposure of section to moist air, which causes the hitherto even distribution of thallium to form granular deposits in the A band and Z line. (E) Section of central portion of (B) after leaching in distilled water. (F) Normal " K^+ -loaded" muscle. Scale bare: $1 \mu\text{m}$. (A) from Edelmann, unpublished. (b-F) from Edelmann (1977), by permission of Physiol. Chem. Phys.

are also the protein sites binding uranium (Hodge and Schmidt, 1960) one expects to find K^+ in resting cells at subcellular loci stained dark in the EM plates fixed and stained with uranium by the conventional manner.

Both predictions have been confirmed (Figure 2). When K^+ in frog muscle cells has been stoichiometrically (and reversibly) replaced by the electron dense Cs^+ and Tl^+ and these heavier ions visualized directly in freeze dried and imbedded, unfixed and unstained muscle cell sections, Cs^+ and Tl^+ are found primarily at the two edges of the A bands and the Z-line (B, C), looking just like the uranium-stained controls (A).

Other experiments, utilizing the technique of effectively membrane(pump) less open ended cell (EMOC) preparation, established that the localization demonstrated is due to one site-one ion close contact adsorption (Ling, 1977, 1984, p. 240). (Figure 3)

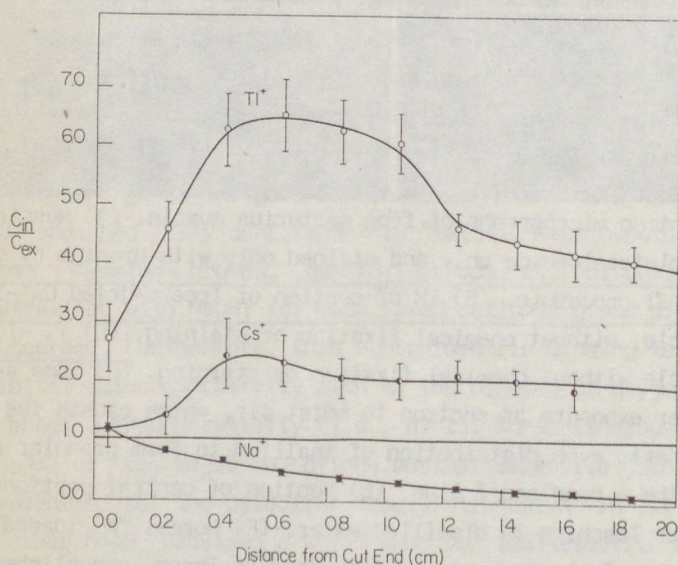


Fig. 3. Accumulation of labeled Tl^+ , Cs^+ , and Na^+ in frog sartorius EMOC preparation. Source solution contained 1 mM ^{204}Tl -labeled Tl^+ , 1 mM ^{134}Cs -labeled Cs^+ , and 100 mM ^{24}Na -labeled Na^+ . Ordinate represents ionic concentration in cell water divided by the final concentration of the same ion in the source solution bathing the cut end and the conclusion of the experiment. Incubation was for 3 days at $25^{\circ}C$. (From Ling, 1977)

Within the last nine years, extensive evidence has collected demonstrating the adsorbed state of K^+ in muscle cells using no less than four independent methods from three laboratories across the world (for review, see Ling, 1984; Edelmann, 1984) including dispersive x-ray microanalysis of Edelmann (1978), Trombitás and Tigyi-Sebes (1979) (Figure 4). The conclusion was unanimous except for a single report from one

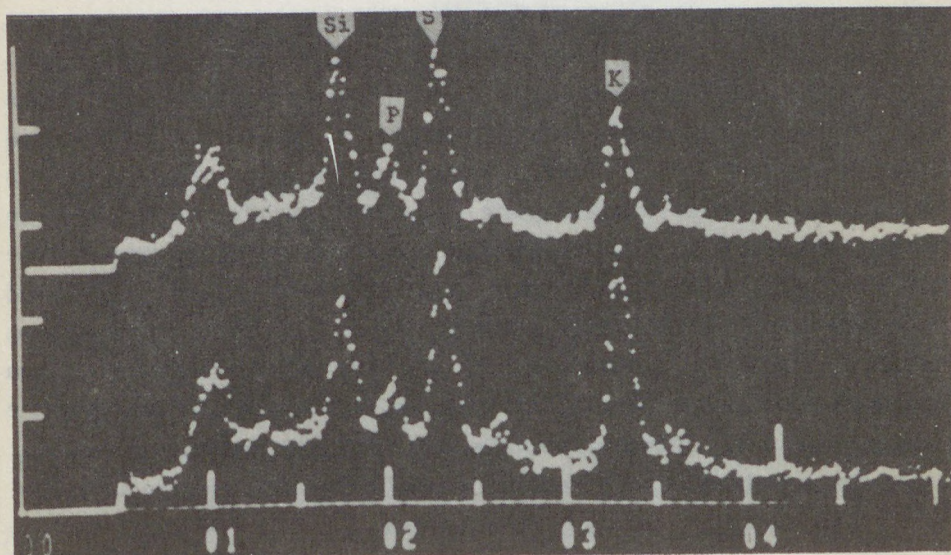


Fig. 4 - X-ray microanalytic curves. A bands (lower ones) and I bands (upper curve) of single isolated myofibril from honeybee muscle. (From Trombitás and Tigyi-Sebes, 1979)

laboratory (Somlyó, et al, 1981). The cause of this difference was found and the differences resolved in favor of the earlier conclusions of Edelmann, Ling, and Trombitás (Edelmann, 1983, 84).

2.2. Exclusion of Na^+

According to the AI hypothesis, the low concentration of Na^+ maintained in living cells reflects in part the inability of Na^+ to compete successfully against K^+ for the β - and γ -carboxyl groups and in part reflects the physical state of cell water, which in turn is the consequence of the existence of certain intracellular proteins in the fully extended state with their negatively charged CO (or N sites) and positively charged NH (P sites) directly exposed to and polarizing the

bulk of cell water. A matrix of such fully extended protein chains provides what has been called an NP-NP-NP system. In this system the alternately positive P sites and negative sites separated by distances of approximately the distance of one diameter of a water molecule, anchor and polarize rows of water molecules which are further stabilized by the cohesive dipole-dipole interaction between immediately neighboring water molecules oriented in opposite directions. A variant is an NO-No-No system where P sites are replaced by neutral (O) sites.

For both enthalpic and entropic reasons, water existing in the state of polarized multilayers has reduced solubility for large and complex molecules (e.g., sucrose) and hydrated ions (e.g., Na^+). The enthalpic reason arises from the fact that it takes more energy to excavate a hole in the polarized water to accommodate the Na^+ than the energy gained in filling the hole in the normal water from where the Na^+ is removed. The entropic reason is due to the greater motional restriction a hydrated Na^+ encounters in the polarized water than in normal liquid water, much as butterfly caught in a spider web suffers more motional restriction than flying in air. Among the different degrees of motional freedom, both the translational and rotational motion are likely to be reduced. However, since the rotational entropy contributes to the total entropy increases with the size and complexity of the molecules, the reduction of entropy in the polarized water increases with the size and complexity of the molecules involved. Thus both the enthalpic reason and entropic reasons favor decreased solubility for large and complex molecules.

2.2.1. q-Value and the Size Rule

The true equilibrium distribution coefficient or q-value is the ratio of the concentration of a solute (e.g., Na^+ or sucrose) in the water under study to that in a reference normal liquid water at equilibrium. The apparent equilibrium distribution coefficient, or ρ -value, is at best equal to the q-value but often exceeds it due to the inclusion of unrecognized adsorbed or complexed solute.

In those cases where low q-value for a solute could be clearly separated from adsorbed solute and studied at more than one temperature, we found that the major cause of the low q-value for ions in frog muscle cell was primarily due to entropic causes (Ling, et al., 1979).

Small molecules and molecules of such a chemical structure that can fit into the dynamic water structure, may exhibit a q-value of unity or

even higher than unity. With increasing size and complexity of the solute, the q -value falls. This relation between molecular sizes and q -values has been called the "size rule".

2.2.2. The ρ -value of Solutions of Native Proteins, Gelatin and Urea-denatured Proteins

According to the polarized multilayer theory of cell water, only cytoplasmic proteins with their polypeptide chains in the fully extended state polarize water. With their backbone NHCO groups locked in α -helical or β -pleated sheet conformation, native proteins are not expected to polarize water to a level required in living cells. The small amount of hydration water usually in the range of 0.2 to 0.3 gm/gm of proteins are apparently due to polar side chains (Ling, 1972). To test the predictions that native proteins exercise only minor effect on the solvency of the bulk phase water, we studied the distribution of ^{22}Na -labelled Na^+ in solutions of 12 native proteins and one polysaccharide initially at 20% concentration (w/v). They were placed in dialysis sacs and equilibrated in 1.5 M Na_2SO_4 . The ρ -values of Na^+ in the water of these thirteen solutions were uniformly close to unity confirming the prediction (Table 2A).

In contrast, solution of gelatin behaved quite differently (Table 2B). A 16% gelatin solution shows a ρ -value of 0.89 ± 0.002 ; while a 43% gelatin solution shows a ρ -value of 0.537 ± 0.013 . This ability of gelatin to reduce the solvency of water for Na_2SO_4 is not new (see Holleman, et al, 1934; Troshin, 1966); however our interpretation is. We attributed the different behavior of gelatin to its possession of the amino acid residues: glycine, proline, and hydroxyproline. Glycine is a helix breaker (Chou and Fasman, 1974), proline and hydroxyproline lack a proton on its backbone N atom to form H-bonds. Together, they prevent the gelatin molecule to exist in the α -helical conformation. Being a denatured collagen, the chain-to-chain H-bonds are also largely broken during the denaturation process even though undoubtedly part of the gelatin molecule does form "collagen-folds" (Veis, 1964) and is not able to react with the bulk phase water. Nevertheless, the difference between gelatin and the native proteins is striking.

Since part of the polypeptide chain of gelatin lacks the positively charged H on its backbone N atom, a solution of gelatin is only partly an NP-NP-NP system and partly what is called an NO-NO-NO system, where the P

TABLE 2

ρ -Values of Na^+ in Water Containing Native Globular Proteins, Gelatin, Polyvinylpyrrolidone, Polyethylene Oxide, and Methylcellulose^{a,b}

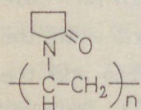
Group	Polymer	Concentration of medium (M)	Number of assays	Water content (%) (mean $\pm$ SE)	ρ -Value (mean $\pm$ SE)
A	Albumin				
	Bovine serum	1.5 a	4	81.9 $\pm$ 0.063	0.973 $\pm$ 0.005
	Egg	1.5 a	4	82.1 $\pm$ 0.058	1.000 $\pm$ 0.016
	Chondroitin sulfate	1.5 a	4	84.2 $\pm$ 0.061	1.009 $\pm$ 0.003
	α -Chymotrypsinogen	1.5 a	4	82.7 $\pm$ 0.089	1.004 $\pm$ 0.009
	Fibrinogen	1.5 a	4	82.8 $\pm$ 0.12	1.004 $\pm$ 0.002
	γ -Globulin				
	Bovine	1.5 a	4	82.0 $\pm$ 0.16	1.004 $\pm$ 0.004
	Human	1.5 a	4	83.5 $\pm$ 0.16	1.016 $\pm$ 0.005
	Hemoglobin	1.5 a	4	73.7 $\pm$ 0.073	0.923 $\pm$ 0.006
	β -Lactoglobulin	1.5 a	4	82.6 $\pm$ 0.029	0.991 $\pm$ 0.005
	Lysozyme	1.5 a	4	82.0 $\pm$ 0.085	1.009 $\pm$ 0.005
	Pepsin	1.5 a	4	83.4 $\pm$ 0.11	1.031 $\pm$ 0.006
	Protamine	1.5 a	4	83.9 $\pm$ 0.10	0.990 $\pm$ 0.020
	Ribonuclease	1.5 a	4	79.9 $\pm$ 0.19	0.984 $\pm$ 0.006
B	Gelatin	0.1 b	4	84.0 $\pm$ 0.78 ^c	0.89 $\pm$ 0.002
		1.5 a	37	57.0 $\pm$ 1.1	0.537 $\pm$ 0.013
C	Polyvinylpyrrolidone (PVP)	1.5 a	8	61.0 $\pm$ 0.30	0.239 $\pm$ 0.005
D	Polyethylene oxide (PEO)	0.75 a	5	81.1 $\pm$ 0.34	0.475 $\pm$ 0.009
		0.5 a	5	89.2 $\pm$ 0.06	0.623 $\pm$ 0.011
		0.1 a	5	91.1 $\pm$ 0.162	0.754 $\pm$ 0.015

a - Temperature was $25 \pm 1^\circ \text{C}$ and test tubes were agitated. The symbols a and b indicate that the media contained initially 1.5 M Na_2SO_4 and 0.5 M sodium citrate, respectively. In (D), PED (molecular weight 600,000) was dissolved as a 10% (w/w) solution, and the viscous solution was vigorously stirred before being introduced into dialysis tubing. Na^+ was labeled with $^{22}\text{Na}^+$ and assayed with a gamma counter.

b - from Ling and Ochsenfeld, 1983

c - temperature was 37°C .

sites are replaced by vacant O sites. Polyvinylpyrrolidone (PVP)



and polyethylene oxide (PEO) $(-\text{CH}_2-\text{O}-\text{CH}_2-)_n$ are examples of

pure NO-NO-NO systems. Like gelatin, solution of these polymers exclude Na_2SO_4 (Table 2C, D). Since these polymers do not form gel, nor do they form "coacervates" (a colloidal phase that remains immiscible with water), clearly the ability of solutions of gelatin, PVP, and PEO of excluding

solutes like Na_2SO_4) does not directly depend on the assumption of the gel or coacervate state, as it is the specific case of gelatin.

Next we studied the effect of four protein denaturants on the effect of proteins on water solvency: urea, guanidine HCl, sodium dodecyl sulfate (SDS) and n-propanol. The first two denaturants are secondary-structure breakers. On the basis of the polarized multilayer theory of cell water, one expects that native proteins, which by themselves have little effect on water solvency, should acquire such an ability after its intramacromolecular H-bonds have been broken and kept in the fully extended state by urea and guanidine HCl. As a result, the water of a solution of these denatured proteins should have reduced ρ -value for the probe molecule like sucrose. This expectation was also confirmed (Ling, 1984, p. 177).

On the other hand, SDS and n-propanol are tertiary structure breakers with no or even enhancing effects on secondary structures. Therefore they should have little or no effect on the solvency of the water in SDS and n-propanol denatured proteins. None was observed (Ling, 1984, p. 177).

Of considerable significance was the finding that the ρ -value of urea itself (labelled with ^{14}C urea) in the solution of urea-denatured protein was very close to unity even though that of sucrose in the same solution was much lower. These findings affirm the general principle described as the "size rule". Urea has a ρ -value of unity because it is small and can apparently fit well into the dynamic structure of water in the state of polarized multilayers.

That urea has a ρ -value of unity in water that effectively excludes Na^+ salt, proves that A.V. Hill's conclusion that muscle cell water is normal liquid water based on urea distribution is unwarranted (Hill, 1930; Hill and Kupalov, 1930). Yet as so vividly chronicled by Ernst on p. 112 of his monograph, The Biophysics of the Striated Muscle, it was this incorrect conclusion of Hill that suddenly turned the tide of public opinion toward the membrane theory, which another fifty years later is now shown to be untenable with or without the pumps added.

It gave me great pleasure and honor to see the colloidal view of the living cell so valiantly and unwaveringly defended by Martin Fisher, E. Ernst, R. Gortner, D. Nasanov, and A.S. Troshin and their coworkers has been finally vindicated (Ling, 1984). For the hardship they endured and the honor they so richly deserved but denied in their lifetimes, mankind owes a greater debt of gratitude to them than to others who despite countless prizes and awards they received, turned out to be not so right after all.

2.3. Comparative Studies of the Model Systems and Living Cells

In vitro experimental testing of the predictions of the polarized multilayer theory on model systems have verified some basic predictions of the theory. In this section I shall review results of experimental studies that offer further evidence of harmony between theory and model behaviors and between theory and behaviors of living cells. However, to keep the length of this paper within bounds, only part of the existing evidence can be reviewed here and then only very briefly.

2.3.1. Breadley's Multilayer Adsorption Isotherm

In 1936 Bradley derived a (polarized) multilayer adsorption isotherm:

$$\log (p_0/p) = K_1 K_3^a + K_4, \quad (1)$$

where p_0/p is the reciprocal of the partial vapor pressure, a is the amount of water sorbed. Under specified conditions, K_1 , K_3 , and K_4 are all constants. Equation 1 can be written in the double-log form:

$$\log (\log (p_0/p) - K_4) = a \log K_3 + \log K_1, \quad (2)$$

which predicts a rectilinear relation between the term on the left and water sorbed, a , obtained in an environment containing water vapor at a specific partial vapor pressure p/p_0 .

The theoretical expectations between a and p/p_0 were confirmed for both gelatin where the data were those of J.R. Katz published in 1919 (for details, see Ling, 1984, p. 288) and for frog muscle, where 95% of the cell follows Equation 2 rigorously, the remaining 5% is very tightly bound and exists apparently as an adsorbed monolayer (Ling and Negendank, 1970; Ling, 1984, p. 289).

2.3.2. The Size Rule in Solute Distribution

In the model system, cross-linked polystyrene sulfonate, the equilibrium distribution of alcohol and sugars were studied. The results showed obedience to the size rule: the p -values for methanol (m.w. 32.04), glycerol (m.w. 92.09), D-glucose (m.w. 180.16), and sucrose (m.w. 342.30) were respectively 0.97, 0.464, 0.309, and 0.153. In frog muscle, the

equilibrium distribution of methanol (m.w. 32.04), ethylene glycol (m.w. 62.07), glycerol (m.w. 92.09), xylose (m.w. 150.13), D-glucose (m.w. 180.16), α -methyl glucoside (m.w. 194.18), and sucrose (m.w. 342.30) were also studied. The results also showed obedience to the size rule. Thus the p-value for each of the solutions in the order given above are respectively 1.1, 0.99, 0.71, 0.48, 0.44, 0.36, and 0.18.

2.3.3. Dielectric Dispersion

Masszi et al. (1976) studied the dielectric constant of frog muscle, leading to the conclusion that the permittivity of water in the muscle cells is lower than that in normal liquid water. Foster, et al. (1980) working with the much more fragile isolated barnacle muscle fiber with one end cut and at 10°C, came to the conclusion that 95% of the muscle water is free water. Subsequent studies of Dawkins et al. (1981) and Gabriel et al. (1983) at the Queen Elizabeth Hospital at London on various rabbit tissues fully confirmed Masszi's earlier conclusion. But perhaps the most important dielectric study was that of Clegg and his coworkers on brine shrimp cysts also at Queen Elizabeth Hospital. Extending the frequency to 70 GHz, they reached the unequivocal conclusion that there is no free water in even the most fully hydrated cells (1.4 g H₂O/1 g. dry matter) (Clegg, et al., 1984).

2.3.4. Quasi-elastic Neutron Scattering

Equally important work on the physical state of water in living brine shrimp cyst cells utilized neutron scattering. The studies of Trantham, Rorschach, Clegg, Hazlewood, Nicklow and Wakabayashi (1984) showed that the translational motion of the cell water was reduced from that of normal liquid water by a factor of 4. The rotational diffusion coefficient was reduced by a factor of 13. The authors recognized that their data are in agreement with the polarized multilayer theory of cell water. Just as interesting was the finding of Rorschach (1984) that a 35% solution shows very similar QENS properties as water in brine cyst cells. A study of the QENS properties of living frog muscle cells established that the correlation time is reduced by a factor of 1.8 when compared to a 0.15 M KCl solution (Heidorn, Rorschach, Hazlewood and Ling, 1986; Heidorn, 1985). This more modest rotational limitation agrees with results of model studies of highly hydrated, Na⁺ excluding, aqueous polymer solutions (Ling and Murphy, 1983).

2.4. ATP as the Cardinal Adsorbent

Earlier I have described a nail-iron filing model where interaction with a magnet raises the energy and decreases the entropy of the system. I have also mentioned that in the AI hypothesis the equivalent of the loosely tethered nails is the polypeptide chains of cell proteins, the equivalents of iron filings are primarily water and K^+ , and the equivalent of the magnet is the cardinal adsorbents which may be other proteins, Ca^{++} , but, above all, the ultimate product of cell metabolism, ATP.

Using the one-dimensional Ising method, Yang and Ling derived a cooperative adsorption isotherm for linear proteins chains carrying a succession of binding sites for K^+ , Na^+ and other solutes. The isotherm differs from the familiar Langmuir type of adsorption isotherm in that the binding sites exhibit nearest neighbor interaction, mediated by, for example, electrical polarization or inductive interaction between pairs of nearest neighboring sites. When this nearest neighbor interaction, represented by the symbol $-\gamma/2$, is positive, the adsorption isotherm is sigmoid, as is well known in the case of binding of oxygen on hemoglobin. Indeed it was shown that the empirical Hill coefficient, n , long suspected to involve some sort of cooperativity, in fact equals $|\exp(-\gamma/2RT)|$, where R is the gas constant and T the absolute temperature. Another key parameter of the isotherm is the intrinsic equilibrium constant $K_{j \rightarrow i}^{\infty}$ between the i th (e.g., K^+) and j th (e.g., Na^+) ions competing for the same binding sites.

The Y-L isotherm accurately describes the binding of oxygen on hemoglobin in vitro (Ling, 1984, p. 216) or in vivo (i.e., in intact erythrocytes) (Ling, 1966, p. 967). The Y-L isotherm also describes accurately the uptake of K^+ in frog muscle, canine carotid arteries, rabbit myometrium, guinea pig taenia coli, human lymphocytes, etc. (see Ling, 1984, Table 11.4 on p. 347). In these cases, values of $K_{Na \rightarrow K}^{\infty}$ ranging from 2.8 to 3.6 Kcal/mole and $-\gamma/2$ from 0.47 to 0.75 Kcal/mole were found. As mentioned above, these findings are also in full accord with the cooperative binding of Na^+ on hemoglobin in vitro, where a $-\gamma/2$ of 0.82 Kcal/moles was observed.

The Y-L isotherm in an extended form also describes accurately the changes in the binding of oxygen on hemoglobin in the presence of different concentration of 2,3-diphosphoglycerate and inositol

hexaphosphate (Ling, 1984, p. 223) where the effect on oxygen binding in consequence of the binding of these phosphates (as well as of ATP, see below) (Chanutin and Hermann, 1969; Klinger et al., 1971) was primarily a shift of $K_{O_2}^{OO}$. Of specific interest here is that the oxygen affinity of hemoglobin also reacts allosterically to ATP binding in a similar manner (Chanutin and Curnish, 1967), and it involves no ATP hydrolysis because hemoglobin has no ATPase activity.

We have already shown how K^+ binding on muscle cytoplasmic proteins (probably myosin) and oxygen binding on hemoglobin in vitro and in vivo are both quantitatively described by the Y-L isotherm. The findings just described above show that like the magnet and nail-filing model, ATP can indeed by binding onto suitable cardinal sites on a cytoplasmic protein, hemoglobin, allosterically control the affinity of a remote solute-binding site.

Given the proper environments (including other essential cardinal adsorbents adsorbed on their respective cardinal sites), we make the following postulates:

Postulate 1 - The binding of ATP exercises an allosteric effect on a "gang" of distant β - and γ -carboxyl groups, causing them to acquire an electron-density (measured by the parameter, c-value) where K^+ is greatly preferred over Na^+ .

Postulate 2 - The binding of ATP exercises an allosteric effect on the backbone NHCO groups, causing them to assume a fully extended conformation and polarize cell water in multilayers.

2.4.1. Results of Testing of Postulates

Since ATP has an enormous binding affinity on myosin (10^{10} - 10^{11} M^{-1} , Goody et al., 1977; Cardon and Boyer, 1978), one expects virtually all ATP in muscle cells to be adsorbed. Postulate 1 then directly leads to the prediction that at equilibrium, there should be a quantitative relation between the concentration of ATP in the cells and the concentration of K^+ in the cells.

This prediction has been verified in two ways. First, Gulati et al. (1971) showed that by exposing frog muscle to 11 different poisons of widely different pharmacological effects first at 25° C followed by a four-hour incubation at 0° C to permit equilibration, linear relations

were shown in all cases between ATP concentrations and K^+ concentrations confirming the prediction. The second confirmation used a different tactic and is described alongside with the testing of prediction based on Postulate 2, i.e., that at equilibrium there should be a direct quantitative relationship between the concentrations of ATP and the degree of polarization of the cell water as revealed by its q -value for such large and complex molecules as Na^+ and sucrose.

2.4.2. Results of Testing Postulate 2

The tactic we chose was to expose frog muscle at $0^\circ C$ to a very low concentration of a metabolic poison (0.2 mM Na^+ iodoacetate). Under these conditions, the ATP level slowly declined, reaching its final zero level in about 10 days. This very slow change permits equilibrium to be reached between K^+ , as well as Na^+ and labelled sucrose (10 mM) concentration in the external medium and in the cell at any specific ATP concentration reached in the cells.

The results once more confirm the relation between ATP concentration and K^+ levels. While K^+ concentration fell with ATP concentration decline; Na^+ concentration rose along a time course that looks like the mirror image of the K^+ time course. By itself the data could be interpreted in two ways: (1) with ATP depletion, the β - and γ -carboxyl groups decrease their relative preference for K^+ and increase their relative preference for Na^+ . As a result a stoichiometric displacement of adsorbed K^+ by adsorbed Na^+ occurs, hence the mirror image profiles; (2) with ATP depletion, the β - and γ -carboxyl groups decrease their preference for K^+ and increase their preference for fixed cationic ϵ -amino groups and guanidyl groups. As a result K^+ desorption goes para passu with loss of available K^+ or Na^+ adsorbing sites and the formation of salt linkages. Along with this change, the proteins involved also undergo conformation changes and a concomitant depolarization of the cell water. In this model, there should be an entirely parallel change of Na^+ concentration and sucrose concentration. Since these solutes do not compete for the same sites, their parallel changes can only be due to water solvency changes and their similar effects on the p -values of both. (Figure 5)

Since with ATP depletion, the change of Na^+ concentration was indeed found to parallel similar changes of sucrose concentration, one reaches the conclusion that Postulate 2 is confirmed: ATP depletion permits cell proteins to return from their high-energy, low-entropy state in which they

preferentially adsorb and polarize water to a low-energy, high-entropy state seen in native proteins, where the β - and γ -carboxyl groups are locked in salt linkages and the backbone NHCO groups in α -helical and other inter- or intramacromolecular H-bonds. K^+ then leaves the cell; Na^+ (and sucrose) enter. With these changes, the cell dies.

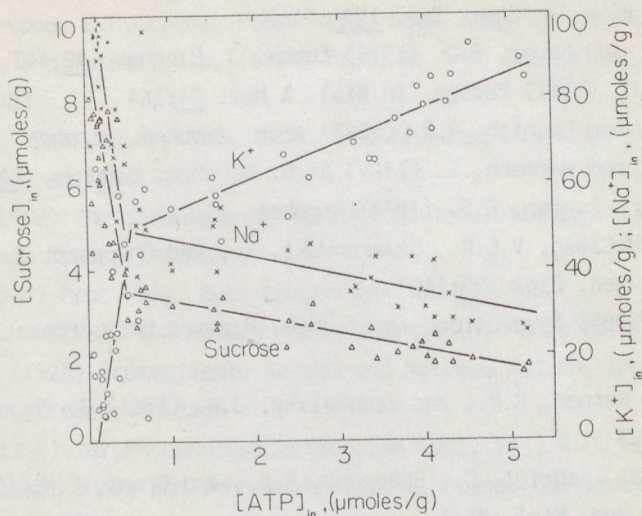


Fig. 5. Equilibrium distribution of K^+ , Na^+ , and labeled sucrose in frog muscles with different concentrations of ATP, having been exposed to 0.1 mM iodoacetate for up to 9 days ($0^\circ C$). (From Ling and Ochsenfeld, 1983)

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THE CRISIS IN THE MIDDLE EAST
 1947-1948

There is a general feeling of relief and satisfaction among the people of the Middle East, who have seen the end of a long and painful struggle. The crisis in the Middle East has been a major event in the history of the region, and its resolution has been a cause for celebration. The people of the Middle East have been suffering from the effects of the crisis for many years, and they are now looking forward to a peaceful future. The crisis has been a major challenge to the stability of the region, and its resolution has been a cause for celebration. The people of the Middle East have been suffering from the effects of the crisis for many years, and they are now looking forward to a peaceful future. The crisis has been a major challenge to the stability of the region, and its resolution has been a cause for celebration. The people of the Middle East have been suffering from the effects of the crisis for many years, and they are now looking forward to a peaceful future.

"DO WE NEED A NEW VIEW OF THE LIVING CELL ?"

MODERATOR: Gilbert N. Ling

Ling: We've now come to the last session of our meeting. Before we enter into the discussion of our topic, I would like to say how deeply I and the other participants of this symposium appreciate our Hungarian hosts' great efforts in making this memorable experience possible. It has been what I consider the best of such a small group meeting.

The topic of this section is, "Do we need a new view of the living cell?" I suspect that if everyone in the audience is asked this question, one may find widely different answers. I also suspect that if Professor Ernst were alive today, he would like many others here share with me the opinion that we do need a new view of the living cell. However as participants in a symposium and not respondents to an opinion poll, we need more than a yes or no answer. For myself, detailed accounts of my reasons for giving such a definitive answer are given in my book, In Search of the Physical Basis of Life published by Plenum Publishing Corporation of New York in 1984 and in a more up-to-date but much shorter book entitled A Revolution in the Physiology of the Living Cell, which I hope will be available either in late 1987 or early 1988. For the interested reader of the published version of this symposium who my books may not be accessible, I have prepared at the end of this volume an outline of the reasons for my rejection of the existing view of the living cell (Appendix I on p.325). With this outline available, I only need to point out that the existing view of the living cell, the membrane-pump theory, must be replaced because it has been long ago disproven. It is disproven because it violates one of the most inviolable laws of physics, the law of conservation of energy, because all the evidence once considered as unequivocally supporting the membrane-pump theory has been one by one shown to be either erroneous or equivocal and because a host of

other new experimental findings while contradicting the membrane-pump theory, are in full harmony with an alternative theory of the living cell, the association-induction hypothesis, which is in essence irreconcilably opposed to the membrane-pump theory.

With my answers given, I can now open the full panel discussion.

Pollack: I would like to know how you can explain an action potential.

Ling: The action potential was first explained by Bernstein as a transient increase of membrane permeability to ions leading to an annulment of the resting potential. Later it was discovered that the action potential does not merely represent an annulment of the inside negative resting potential but actually goes beyond the baseline to cause an "overshoot", transiently rendering the inside of the cell electrically positive. In 1949 Hodgkin and Katz discovered that the "overshoot" depended on the concentration of external Na^+ . They then introduced the Hodgkin-Katz-Goldman equation, based on the assumption that the cell membrane represents a homogeneous, isotropic medium and that the movement of one ion is independent of the presence of other ions (the "independence principle"). In this theory the resting cell behaves like a K^+ electrode because the cell membrane is overwhelmingly more permeable to K^+ than to Na^+ . During activity, the cell behaves like a Na^+ electrode because the cell membrane momentarily becomes more permeable to Na^+ than to K^+ .

In 1952 Hodgkin and Huxley described in detail an action potential comprising an initial surge of Na^+ into the cell followed by a rise of outward movement of K^+ . The driving forces for the inward Na^+ movement and outward K^+ movement were their respective concentration gradients. Thus a "Na potential" is defined as equal to $RT/F \ln (|\text{Na}^+|_{\text{ex}}/|\text{Na}^+|_{\text{in}})$. The sudden surges of ionic movements during an action potential were attributed to the opening of gates to specific "channels", which at first were more in the form of electronic circuit analogues, but became more physical and realistic as time went by even though there was no evidence in support of this view. Nor were molecular mechanisms for these events ever offered.

In 1952 I suggested a basic mechanism for selectivity between K^+ and Na^+ different from the Mond-Anson pore size idea. I proposed the idea that the cell membrane should contain as its major component fixed anion carrying proteins. In 1953 and years following I showed that the same ion selectivity mechanism offered for selective K^+ accumulation over Na^+ can explain related K^+ permeability. First, I demonstrated that ion permeation into resting cells did not follow "independence principle". Rather, the

movement of one ion (e.g., K^+) through the cell surface is inhibited by other ions present (e.g., Rb^+) (Ling, 1953). To explain, I suggested that the cell surface has attributes of a two-dimensional replica of the three-dimensional cytoplasm; both carry β - and γ -carboxyl groups. In both there is enhanced association of the counterion K^+ with the β - and γ -carboxyl groups of proteins in consequence of the "principle of enhanced counterion association with charge fixation". The result is that most K^+ entering and leaving the cell follows what I call the "adsorption-desorption route". That is, a would-be entrant K^+ must first combine with a β - or γ -carboxyl group at the cell surface before dissociating from it to enter the cell. In support of this view, I found that the rate of entry of K^+ into muscle cells follows qualitatively the theory derived on the basis of this assumption and demonstrated that the anionic groups mediating K^+ entry have a pK_a of 4.76 characteristic of β - and γ -carboxyl groups (Ling and Ochsenfeld, 1965).

In 1955, I announced what was to be called the "surface adsorption theory of cellular electrical potentials" as part of the AI hypothesis (Ling, 1955), in which the cellular potential arises from the presence of fixed anionic β - and γ -carboxyl groups at the outer cell surface. The different sensitivity of potential to K^+ and Na^+ arises also from the different adsorption concentration on the surface carboxyl groups. In 1957 I presented the theory of a key mechanism in the creation of an action potential: a change of the electron density at the singly charged oxygen atom of the cell surface β - and γ -carboxyl groups thereby altering the relative adsorption constant from preferring K^+ over Na^+ to preferring Na^+ over K^+ . This concept was supported by theoretical model computations. In 1965 I presented the polarized multilayer theory of cell water (Ling, 1965a) and immediately afterward extended the concept to the cell membranes. That is, like the cytoplasm, the cell membranes contain domains of intensely polarized water which are not "atomic sieves" but function as atomic sieves, because both the q -value and the diffusion coefficient (D) are size dependent and the permeability constant P , is equal to the product of q and D .

Ling, Proc. 19th Intern. Physiol. Cong, Montreal, p. 566, 1953;
Ling, Fed. Proc. 14:93, 1955; Ling, Fed. Proc. 16:81, 1957; Ling
and Ochsenfeld, Biophys. J. 5:777, 1965; Ling, Fed. Proc.
24:S103, 1965.

In 1968 Bertil Hille also suggested that protein carboxyl group with a high pK_a value acts as a "selective filter" for Na^+ in a specific Na channel and a low pK_a carboxyl group functioning as selectivity filter for K^+ in the K^+ channel. Hille did not then, nor afterwards refer or give credit to our earlier publication in which the same ideas were presented. In a 1975 review, Hille showed a titration curve of the " K^+ channel", which yielded a pK_a (4.5) very close to that of the pK_a (4.7) reported by Ling and Ochsenfeld ten ~~years~~ before, again without mentioning our earlier work. Hille and other like-minded workers introduced the two channel idea: sodium channel for Na^+ , potassium channel for K^+ . In his 1975 review, Hille said "I am beginning with the popular concept there are two channels, without proof" (Hille, 1975). That is just how it was and how it is: no proof. In fact, it is worse than that. As is, this dual channel model is not tenable for a variety of reasons. The most fundamental reasons are the natural consequence of the untenable condition of the membrane-pump theory of the living cell, on which the entire resting and action potential concept was built. However, for discussion, I can choose some more specific focal aspects. As an example, the action potential is interpreted as involving a sequential opening and closing of first, the Na channel and then the K^+ channel, both channels being completely closed in the resting cell. Yet we know perfectly well that the resting cell membrane is all the time permeable to both K^+ and Na^+ . There is no reason to expect that the "Na potential" to be maintained in the resting cell. The situation reminds us of one who goes through elaborate efforts to keep the front door securely locked at all times and opened only occasionally to admit certain specific guests on recognition, in the meanwhile the back doors to the same house are left open at all times.

The "big pore, small pore" model is also a classical, unworkable model. You don't make two holes, one big and one small, one to allow the cat to go through, one to allow the dog to go through. The cat will go through the dog hole too. Of course, Hille also incorporated my ion-selective carboxyl groups concepts. But I am afraid even with that addition, the two channel idea still would not work. Thus Hodgkin and Huxley showed that in squid axons bathed in sea water the application of a depolarizing voltage greater than 12-15 mV produces an action potential with an initial surge of inward Na current. This initial inward current

disappeared when sea water was replaced by choline chloride Ringer. Instead, a small outward hump takes its place and is attributed to the outward movement of Na^+ from the inside of the axon, now that the external Na^+ concentration has been reduced to nearly zero, and the Na potential has therefore reversed its polarity. Certainly, this observation has been a very convincing one in favor of their theory.

Chandler and Meves in 1965 studied squid axons externally bathed in the Na^+ free medium while internally perfused with only K^+ . As a result there is no Na potential. Yet Chandler and Meves found that the outward current Hodgkin and Huxley originally described persisted; concluding that K^+ as well as Na^+ can traverse the " Na^+ channel". In the two channel model a Na channel wide enough only to allow only the small (dehydrated) Na^+ (initial diameter 1.9 Å) to go through, but not the larger K^+ (diameter 2.66 Å) to go through. The fact that in the real world K^+ does indeed go through the Na^+ channel has thus made this big hole, small hole idea untenable; because it shows that the idea that the Na channel is rigid and narrow is incorrect.

In the AI hypothesis of the action potential, difficulty of this sort does not exist because first the polarized water domains in the cell membrane are much wider than the diameter of ions and secondly there is no standing "Na potential" in the resting state. Such a Na potential is created only during excitation, when two coordinated events occur *pari passu*:

(1) A sudden autocoperative shift of the c-value of the cell surface β^- and γ -carboxyl group toward a higher c-value with preferential adsorption for Na^+ over K^+ followed by a return to its normal low c-value thereby changing the cell surface from behaving like a K^+ electrode to one behaving like a Na^+ electrode.

(2) A concomitant all-or-none autocoperative depolarization of the cell surface water due to a conformation change of the water polarizing protein backbones to an internally "neutralized" (e.g., α -helical) conformation, followed by a reversal of the process of repolarization of the water which occurs in consequence. The depolarization of the membrane water at once creates the Na potential and the inward Na^+ current, creating the overshoot.

As Na^+ rushes in it displaces K^+ from its resting adsorbed state. The free K^+ then diffuses outward creating the delayed K^+ current. Reversible

water depolarization with simultaneous rise of the c-value of the surface β - and γ -carboxyl groups followed by reversal are the basic mechanisms of the action potential according to the AI hypothesis.

Hazlewood: How does it reverse?

Ling: Just the reverse of everything I said. Water becomes repolarized: c-value of β - and γ -carboxyl groups return to a lower value.

Pollack: What I'm missing is the link between the stimulus of the action potential, and physical events that you are suggesting, the change of water polarization, and the relative change in the adsorption of the two ions.

Ling: It is generally agreed that it is the advancing front of electrical depolarization that sets in motion the action potential, which in the AI hypothesis involves c-value increases of the β - and γ -carboxyl groups and water depolarization. These physical events are expressions of cooperative coordinated changes of the protein-water-ion assembly of the cell surface.

So your question boils down to three other questions. What are the key controlling agents that cause the cooperative c-value change of the β - and γ -carboxyl groups and water depolarization? How is this key controlling agent "turned on" by the electrical depolarization? Finally, how does the key controlling agent cause the cooperative changes?

Hazlewood: What is causing it, Gilbert, what is the cardinal adsorbent? Is it current, or is it Ca^{++} ?

Ling: Well, at this moment I don't feel any of the evidence in favor of one or the other one are truly compelling. Removal of Ca^{++} from the external medium simulates the effect of electrical depolarization (i.e., a reduction of the resting potential). A rise of Ca^{++} concentration simulates a rise of the resting potential. The electrical depolarization might cause a detachment of calcium and this calcium detachment causes the water depolarization and c-value change of the β - and γ -carboxyl groups. This is one hypothesis. In favor of this idea is the fact Ca^{++} removal does shift to K/Na equilibrium distribution in smooth muscle cytoplasm toward higher Na preference (Gulati, 1973). But there is another possibility. Recently a 25,000 D sodium channel protein was isolated and

its amino acid sequence determined. The channel protein has an excess of positive charge at one end. So electrical depolarization might cause this positive charge region to be detached from its negatively charged "cardinal sites" and rings about a cooperative c-value shift and water depolarization. It is very important at this moment to recognize that we do not have enough specific data to go much beyond speculations. Much more work in the right direction is needed.

Pollack: The experiment that Professor Tigy mentioned about passing current through a myofibril, now I know, that can give rise to change in the conformation of proteins, for example helix-coil transition. Suppose, it's just a remote possibility, when you apply a current to a cell, the current flow causes, in some region, near the membrane, on the membrane, a helix-coil transition. The fact that the protein has changed its conformation makes it selectively adsorb sodium instead of potassium. Is this a possible trigger?

Ling: Yes, I think it's possible. And precisely the kind of rich new terrains for future studies. I have recently finished a piece of work that has bearing on the subject. However, I must qualify it by telling you that I sent it to Nature and they said: "We have no interest." Then I wrote back: "Please, look more carefully into it." They sent another letter: "We still have no interest." I cite this to demonstrate another dimension of the difficulty in introducing new ideas. Publishers prefer to publish extensions of familiar subjects along well-worn paths then to deal with new concepts and new discoveries.

What satisfied me from this work was that I think that I began to understand how primary structure of a protein determines its secondary structure. I recent years good empirical rules have been established relating amino acid composition and secondary structure of proteins. For example, one finds that a glycine residue is a helix breaker. Alanine, on the other hand, is a helix maker. By why? The primary factor here has been called the "short-range interaction". The short-range interaction refers to how each amino acid residue determines the probability that the same residues own NH and CO group are engaged in an α -helical or β -pleated sheet conformation, etc. The short range interaction had no answer up to now beyond the AI hypothesis. I have discovered good correlation between the electron donating power of the side chains and the α -helical potentials of the 19 amino acid residues empirically determined. We were

also able to demonstrate that the inductive effect from one side chain can be transmitted through four amino acid residues each way giving support to the concept that electrical polarization or induction serves the key role in the behaviors of protein molecule to function as a coherent whole. These empirical rules between each amino acid's α -helical potentials and the electron donating strengths of the side chain of the amino acid residue also shed light on an apparent paradox: If amino acid sequence determines the secondary structure, then there should be only one secondary structure for each protein, because there is only one unique amino acid sequence for each protein. If so, how can one change the conformation of a protein as it is well known in response often to drugs, hormones, etc.? The answer is, "the cardinal adsorbent that changes the protein conformation does so by modifying the inductive effects of the side chains, which determine the protein conformation". In consequence of this, one may discharge electrically a positively charged region, the whole conformation may then change as a result. To support this view one recalls that in sickle cell anemia, a single amino acid residue difference in the beta chain of the hemoglobin molecules not only changes that hemoglobin, but changes properties of the entire red blood cell of which the protein, water, and ions are inseparable components. One cannot reasonably expect that such a change of the shape of the entire cell as a result of changing one single amino acid residue in one of its protein chains if the cell is truly a membrane enclosed solution of proteins. A goldfish in a glass bowl who suffers a deformation, no matter how great, cannot cause the whole glass bowl to change its shape.

Keszthelyi: I'm very simple minded. I just do not see the difference between a channel type of theory and a fixed charge model. When the electric potential comes, it moves some charges. This charge may be bound to protein side chain, so the protein side chains simply move a little bit, and open the channel. And you tell that when the potential comes, it moves some fixed charges and because of this, there is a conformational change in the protein. What is the difference between these two explanations of the same fact that the channel is opened?

Ling: It is true that there are some similarities between the two models. However most of the similarities arose from the appropriation of my prior ideas by Hille and others without giving me credit or even mentioning my name. Hence the confusion; in particular the concept that protein carboxyl

groups on the cell surface membranes providing a molecular mechanism for recognizing and distinguishing K^+ and Na^+ .

The rest is nothing but profound differences. The association-induction model is a self-consistent theory explaining all aspects of cell physiology of which the theory of the action potential is only a part and it is based on first principles from physics and corroborated by testing on inanimate model systems. The part of the channel model that was not appropriated from the AI hypothesis, was quite different. The rigid pore or atomic sieve idea has been disproven over and over again in history and there is no known inanimate model that corroborates the theory. However, it is beyond the time limitation to make a detailed indepth comparison of the two theories, but I have written quite a few reviews on the subject and they are cited below. However, I shall choose one specific subject for discussion. You don't have a driving force for the sodium to go in, in one case, and you have the driving force in the other. Why shouldn't that driving force or sodium potential have dissipated itself during the resting state since the resting cell membrane is permeable to both K^+ and Na^+ ? So one may say that this is due to the sodium pump, but electric potential is known to be independent of the sodium pump according to Hodgkin and other proponents of the membrane-pump model.

Keszthelyi: Yes, but then we have to accept that there is no sodium potential.

Ling: Not in the resting cell. But during the action potential, there is indeed a sodium potential. It is transiently created.

Keszthelyi: Now we are not speaking about a pump. We are speaking about a channel. And let's just take acetylcholine. Acetylcholine is a charged particles. It goes in, and it moves the charges. That is the explanation.

Ling: The correct answer to that question is "yes, there is no resting Na potential." Acetylcholine binds onto acetylcholine receptor sites, but the physiological effects are very specific to acetylcholine and not merely due to its possession of a cationic charge. Thus curare, the arrow poison, also carries a cationic charge. Its binding of curare on acetylcholine receptor sites does not create the increase of Na^+ permeability brought on by the binding of acetylcholine. Quite the opposite is the case. As the acetylcholine-curare comparison demonstrates, the mechanism is much more intricate. In the AI hypothesis the primary event is electronic polarization and depolarization and redistribution of electrons within the

protein-ion-water system produced by the binding of the cardinal adsorbent acetylcholine - an effect not duplicated by curare.

Keszthelyi: Then the channel opens in this case. It's just because charges move. The charges can be moved by voltage, or by simply adding a charged particle, which is acetylcholine in this case. And then there is a small change in the electric field distribution. Now I understood your point, that we create the driving force.

Ling: You are heading in the right direction. In fact, the AI hypothesis goes considerably beyond that. The theory must and does explain, though still in a tentative way, the different effects created by acetylcholine and curare. The primary event is a propagated electronic charge along protein chains that creates simultaneously an increase of the electron density, expressed as a c-value increase of the surface β - and γ -carboxyl groups and a coordinated depolarization of the intensely polarized water that occupies very wide membrane pores or domains. Acetylcholine, a strong electron-donating cardinal adsorbent or EDC is capable of creating the all-or-none change, while curare cannot. Here I believe is a major new direction for future progress which regrettably, at this moment, is being blocked by the continued dominance of the adherents of the obsolete membrane-pump theory.

Tigy: I have to confess to you, that I did not mention a very important result of Professor Ernst. He published about the sodium-potassium exchange in 1929. But one year after this, he repeated the same experiment, where he used an indirect stimulation, through the nerve. And he did not find sodium-potassium exchange. I, myself, repeated this experiment 20 years, later with radioactive potassium and sodium. And if you are stimulating your muscles in this physiological way, after even 10,000 stimuli, contractions, you cannot find larger sodium-potassium exchange, than 2%. It is not really significant. Finally Professor Ernst and I elaborated the "semiconductor theory" where no ionic movement is essential for the excitation process, but electronic changes in the structure. You can say conformational change from one state to the other

Ling, Bioelect. & Bioenerg. 5:411, 1978; Physiol. Chem. Phys. 14:47, 1982; in: Structure & Function in Excitable Cells, Plenum Publ. Corp., 1983; in: Modern Bioelectrochemistry, Plenum Publ. Corp., 1985.

one, but no net movement of ions. This was the message of Professor Ernst, I had to tell it honestly.

Clegg: Let me play the Devil's advocate, briefly, Gilbert. In the spirit of free exchange which you raised.

Ling: Absolutely!

Clegg: I'd like to ask two questions about your hypothesis that I think are central to it. You know the discussion is supposed to be: Do we need a new view of the cell. Well, I think the answer to this is: it depends on who we are listening to. But the two questions I have for you are these: the first one is based on your view that you need to polarize 8-10 layers at the most on these limited number of extended proteins. Now, I can guarantee you, that you'll never affect all the water that way. Not in a cell that has 70-80% water. My question is, how do you handle that? The second question is, do you really have any evidence for proteins in cells, that undergo helix extended, globular extended conformational changes that depend on ATP and cardinal adsorbents. These, I think, are two fundamental issues about the hypothesis.

Ling: Jim's questions are good questions. But they cannot be answered in a few sharply focussed sentences, much as one who is the one who is asked, "Have you stopped beating your wife?" cannot give a yes-or-no answer.

For this reason I have answered Jim's questions in proper detail in the form of a second appendix (Appendix II). Summing these detailed answers presented there, I only need to point out that Jim's guarantee notwithstanding, I do not agree with his view that "you'll never affect all the cell water that way" and my disagreements are based on logic and facts. In response to his second question, "Do I have evidence that ATP controls protein conformation in cells?", my answer is yes, although I am also the first to add that we need more people to get into this key problem. To the best of my knowledge my laboratory has been the only one pursuing the subject in the last thirty-four years.

Kellermayer: Jim, you were the one who presented Schliwa's data in detail. Concerning those observations I have presented, Dr. Cameron asked me whether I had definite proof for the globular-fibrillar transition inside the living cell. It may be that my answer was not convincing enough, but let us just accept that the microtrabecular lattice exists. If you extract proteins from that lattice, these proteins definitely turn to a globular phase because they stay in the supernatant even after a 100.000 g

centrifugation. If these protein were still in a more extended like in a fibrillar form, they would go down to the bottom of the centrifuge tubes. Until we have a direct proof for the globular-fibrillar transitions, the opposite: the fibrillar to globular transition may be considered as an indirect evidence for the existence of such processes. If Schliwa was correct and the microtrabecular lattice disappeared when the cells were treated with detergents, the proteins of the lattice must have gone through the fibrillar-globular transition. Whether ATP is needed when the proteins were extended in the microtrabecular lattice remains to be answered. But it could be that this is the key to the polarization of the water that Ling is talking about.

Osborn: I just want to comment about the Schliwa's experiment. I think there is another interpretation of Schliwa's paper.

One should read the paper by Kondo in Journal of Ultrastructure Research. Let me just mention two features here. He took different concentrations of BSA, and he could show that the spacing is very similar to that of microtrabeculae and it changes with the concentration of the solutions. So the argument to explain the Schliwa's result would be that there is too much protein for the fixation used. And when he extracted some of the proteins from the cell, it revealed the actin lattice, but before that, you're getting essentially a BSA effect, because you have high concentrations of protein in the cell. I personally don't accept that the experiment you just cited showed that you change the conformation of the protein upon extraction.

Kellermayer: Dr. Clegg was trying to tell us that the interior of the cell is not a crowded soup. It is an experimental fact, that when you treat the cells with detergent, 50% or more of the proteins get into the 100.000 g supernatant. Those proteins will stay in the supernatant.

Osborn: Right! because they depolymerized.

Kellermayer: The proteins coming out existed in another form inside the living cell.

Osborn: I would agree with that, but not in the way in which you are saying. I would go back to the picture shown of the edge of the cell by Small and Lindberg. It is of highly polymerized, highly concentrated actin, without the proteins bound to it.

And therefore I would believe in the essence what you are saying for the experiment, the result of extracting it. But I won't let you put the burden of the proof of that on the Schliwa's experiment, because I think, actually the edge of the cell is much more crowded than shown by Porter and Schliwa. And it is much more regular.

Kellermayer: All right! How do you see those proteins which come out? How do they exist inside the living cell?

Osborn: One has to consider that there is a whole biochemistry of some of these proteins. Some of these things one can duplicate in the test tube. Some of these things are regulated for instance by calcium, which hasn't received very much discussion. There are some elements of this process that you can build up and replicate in the test tube. There are some very-very simple systems, for example the microvillus system, which consists basically of actin and three or four other proteins. To think global theories of what happens in the cell are O.K. But I think we also have to consider what happens in specific situations with the proteins that we know, and try to integrate the biochemical properties of what we know, into the global theory.

Hazlewood: I think these questions are all addressing the very things that we will be addressing in the future.

And these are crucial things to find out in the light of the various hypotheses of the cell. Dr. Clegg, from what Dr. Ling has done, we know that extended structures, like the polyethylene oxide and other polymers, including denatured proteins, do exclude ions from the aqueous phase. Dr. Osborn, I think, you are completely correct about our not knowing what form proteins are in within a cell and it's like the old story of trying to confirm that a live animal exists in a box, yet if you open the box the animal immediately dies. The conformational state of cellular proteins is for the future; however, there has been one study of Dr. Ling that has fascinated me and it concerns the actomyosin gel. The gel can accumulate or exclude rubidium, depending upon the pH. It would be nice to know the conformational states and the ion selectivity of the proteins at various pH's.

Ling: The black box paradox may not be trivial at all. The Heisenberg principle has already proven that in some fundamental ways we can never know everything. When enzymology started to take the cells apart and study the enzymes isolated, the objection was raised, what has that got to do

with the living cell? The moral is: "how much had we known about the enzymes before?" But that's not the bottom line. The bottom line is: "can you put it back? And does it behave like that?" And that is a very-very difficult problem. I search hard to answer Dr. Clegg's question, and I ask myself, how would I be able to satisfy his question? In other words, what does Jim really want? Does he want me to go in there and see for myself, or what? It's like you want to know which letter is doing what in an alphabet soup. But if you take the cell apart, it loses mutual interaction. In other words, how can you find the characteristics of a "school of fish" after you have already taken the fishes apart?

Pollack: Are you saying that your ideas are untestable?

Ling: No, not untestable, just very difficult. It is not so straight forward. We are used to taking things apart, and to demonstrate the characteristics of enzymes persisting after isolation, but that does not prove that the enzyme is operating in that manner in the cell. I have demonstrated solute exclusion, freezing pattern behavior, which resembled the living cell, but he asked me what proof do I have, that is happening in the living cell. I would say the answer is asking back: what would you consider as a proof?

Cameron: I'd like to go back to the theme of our session: "do we need a new view of the living cell?". I would offer that polymerization and depolymerization, rather than, perhaps the opening of a globular protein to an extremely extended state might do the job. I think that's a very conceivable possibility. And I would also caution us about the thinking that globular proteins can do the job of layering several layers of water around them. I recently published study with Dr. Fullerton about lysozyme around which, water is perturbed up to 2-3 layers. And that is not an insignificant amount if you assume all of the cell consists of globular proteins on a weight basis. It seems to me that maybe the opening up of a protein is not really necessary. The polymerization of these globular proteins may be also significant, to order enough water to exclude sodium if its exclusion is needed at all. May be not.

Ling: Wait a minute, you are back to square one. If you say you don't need sodium exclusion, essentially you have to confront the energy problem. There is no way of shortening it. And I don't need that! Let me just add some more difficulty for the pump model. Professor Troshin has shown, that phenol red is excluded. The q-value of it is about 0.2. In the

conventional interpretation, you have to have a pump for the phenol red. High specificity. Phenol red didn't exist in nature until Kekule synthesized it, in 1872. So, do you say, that genetically the cell anticipated the production of this dye? That is obviously impossible. And yet, I'd bet, if you had complex molecules, they all would be excluded, but the model of the pump would demand, that the membrane has infinite space to accomodate pumps for a numberless set of new compounds not yet synthesized. I tell you another example. The pump has been postulated for E. coli for the handling of tetracycline. I would submit, there is no way for E.coli in his history to accumulate this information about tetracycline, because at the moment it meets tetracycline, E. coli is dead, there is no way for the bacteria to pass on that information. And so, the presence of E.coli-tetracycline-pump is out of the question. But you see, these are general problems, you cannot avoid, unless you face it.

Cameron: More than half of the living cells in the world obviously don't have a Na-K ATPase pump on their surface. They are plant cells. They have none, it has been shown. They have, perhaps other types of pumps, but these cells do fine without a Na-K-ATPase pump. I don't have any necessity to draw a pump into this, I'm talking about other mechanisms within the cells.

Hazlewood: Could I respond to you as to questioning why we should look at the extended proteins? The experiment provides an explicit test of the hypothesis. The hypothesis states that extended proteins organize the water such that sodium and other solutes are excluded. Now, if we are successful in opening up a protein, and exclusion does not occur, then we must go back, sit down, and rething things. I think we should open these proteins up, and see what their interaction with the water is like, in order to test our hypothesis.

Cameron: I was very impressed with your data on the use of NaOH to take a globular protein, hemoglobin, and open it up, and you could show that it would break salt linkages. Now, that is an extreme perturbation to cause it to do so. But it did it. I mean, I believe it was open. But the cell is not going to use NaOH. We don't want to have a mystical approach of what's going on in there. There's got to be an environment that will do this under what appears to be physiological. Perhaps, Dr. Ling you would make some suggestions, presence of ATP, or something like that.

Ling: I can continue on that. Because some way it is unfair to all of you,

and in some way it is very hard for me to communicate, because I can tell you only a very small bit of the information. And you see it like the top of the iceberg.

Tigy: In closing our meeting, I want to express that we are honored, that you all accepted our invitation. We have had a very pleasant and exciting two days in science, it was a true exchange of ideas. This was because all the invited colleagues are the best in their field, and we also had two giants among us, Professor Ernst in spirit, and Gilbert Ling. I'm very happy that you came, and I'm sure, if Professor Ernst were here you would debate three times as much as you did now. Really this is the atmosphere of science, to discuss and to debate, and we can improve our knowledge, and really want to find the real truth, but many times we believe that we have the truth, but is not always them.

Ling: It reminds me of Charles Darwin who said you just realize that the birth of new truth is a hard process. Contention and disagreement are only natural. Let us never change from that.

APPENDIX I

DO WE NEED A NEW VIEW OF THE LIVING CELL ?

G.N. Ling

1. What is the current view of the living cell?

The current view of the living cell, called the membrane-pump theory originated as the membrane theory of Wilhelm Pfeffer (1877), although when Schwann founded the cell theory in 1839 he already expressed the view that the containing membrane was prior in importance to its content and that the cell content nothing more than a homogeneous transparent liquid (see Hall, T.S., Ideas of Life and Matter, Vol. 2, p. 193-194, Univ. of Chicago Press, 1969). It is difficult if not downright impossible to describe the membrane-pump theory, because since Pfeffer's monograph Osmotische Untersuchungen, in which he presented the membrane theory, there has not been an authoritative document describing the membrane-pump theory as a self-consistent comprehensive theory of the living cell. Nevertheless, the following basic tenets of the membrane-pump theory can be counted:

- (1) All living cells are surrounded by a thin cell membrane which is primarily a lipid bilayer containing pockets of island-like proteins. The cell membrane is truly a membrane, separating two similar aqueous phases.
- (2) In the cell membrane are located a variety of pumps, whose continued activities at the expense of metabolic energy maintain the characteristic levels of ions and other solutes in the cell.
- (3) The bulk of cell water is normal liquid water.
- (4) The bulk of cell K^+ is free and dissolved in the cell water.

1. Evidence Once Widely Believed to Support the Membrane (Pump) Theory Now Shown To Be Erroneous Or Equivocal

1.1. A.V. Hill's Evidence of Free Water and Free K^+

A more complete description of the history is to be found in my monograph, In Search of the Physical Basis of Life (Plenum, 1984); a brief account will be given on the key events.

The dominance of the membrane theory from the 1930's on could be traced to the demonstration (or so it seemed) by A.V. Hill and his colleague Kupalov that all water and all K^+ in living cells are free. Hill first showed that urea distributed itself equally between frog muscle cell water and the bathing medium. This equality of urea distribution led Hill to conclude that in muscle cell there is no bound water, because at that time it was widely believed that bound water is "non-solvent" to all solutes. Hill and Kupalov then showed that frog muscle is in vapor equilibrium with a 0.118 M NaCl solution. Since Na^+ and Cl^- are free in this solution, K^+ which is the major ion in the cell must be free in order to match the osmotic activity of the 0.118 M of the free Na^+ and Cl^- . Thus the tenets of free water and free K^+ were widely believed to be proven.

Prof. Ernst in his monograph, Biophysics of the Striated Muscle (1963, p. 112) pointed out that it was these findings of A.V. Hill that abruptly changed the minds of the opinion-makers of the day, including Fenn, Höber, and Buchtal in favor of the membrane theory.

In my paper, "The Association-Induction Hypothesis and Life", I have pointed out that equal distribution of urea between cell water and external medium does not prove that the cell water is normal liquid water because water existing in the state of polarized multilayers does not discriminate against urea which is small and can fit into the polarized water lattice. Without water existing in the free state, Hill's free K^+ conclusion also falls apart.

1.2. Free K^+ from High K^+ Mobility Measured

Another set of experimental findings that had reinforced Hill's conclusion was due to Hodgkin and Keynes (1953). These authors demonstrated (or so it seemed) that K^+ diffusion in squid axon is virtually the same as that in sea water - a finding in turn confirmed by the demonstration later by Kushmerick and Podolsky (1969).

In 1966, Bernard Katz, Nobel laureate, wrote in his booklet, Nerve, Muscle and Synapse (McGraw Hill): "... but Ernst (1958), Troshin (1958), and Ling (1962) maintain that the greater part of the internal negative charges are provided by the polyelectrolyte proteinates. Moreover, these latter authors take the view that the potassium ions do not merely form counterions to the negatively charged colloidal structure but possess selective affinity and are chemically bound to the proteins... It seems, however, very difficult to support this view in face of the following pertinent observation by Hodgkin and Keynes (1953). These results are discussed here in detail because they are of crucial importance in the still persistent argument about the validity of the membrane concept..." (p. 42-43).

In 1973 Ling and Ochsenfeld introduced a new method for studying the K^+ diffusion in the intact part of muscle cytoplasm. Results of a total of 72 sets of experiments showed that only in injured and killed cells did K^+ diffusion approach those in free solution as found by Hodgkin and Keynes and by Kushmerick and Podolsky. In healthy cytoplasm, the K^+ diffusion coefficient is 1/8 of that in isotonic salt solution.

1.3. Free K^+ from High Activity Coefficient of K^+ Measured in Cells

Still a third type of experiments once widely hailed as providing unequivocal evidence in support of the membrane-pump theory relied on the use of intracellular K^+ or Na^+ specific electrodes. Early studies of squid axon and giant barnacle muscle fibers showed that the activity coefficients of K^+ (γ_{K^+}) measured was very close to that of an isotonic KCl solution. Subsequent extensive studies using the same technique but a variety of living cells, revealed widely different activity coefficients ranging from 0.27 (White, 1976) to 1.2 (Lee and Armstrong, 1972). (For a full compilation of data, see Ling, 1984, p. 255). If the membrane theory were correct, there should have been a uniform γ_{K^+} equal to that of an isotonic K^+ solution. This is certainly not the case. On the other hand, the data are readily interpreted in terms of the AI Hypothesis. The microelectrode can only "see" the K^+ activity in the thin layer of fluid immediately surrounding the electrode tip. Various γ_{K^+} were observed

Hinke, Nature 184:1257, 1959; Lev, Nature 201:132, 1964; White, Amer. J. Physiol. 231:1214, 1976; Lee and Armstrong, Science 175:1261, 1972; Hodgkin and Keynes, J. Physiol. 119:513, 1953; Kushmerick and Podolsky, Science 166:1297, 1969.

because the stability of the cytoplasm in response to the mechanical intrusion of the electrode varied. Higher stability in intestinal epithelial cells gave low γ_{K^+} ; low stability gives rise to γ_{K^+} close to that of an isotonic solution. Liberation of K^+ without a concomitant depolarization of the cell water (and hence maintained low q-value for K^+) in some part of the cytoplasm causes the liberated K^+ to diffuse to and accumulate in the depolarized water in the region of more completely destroyed cytoplasm in contact with the electrode surface (where q-value for K^+ is high); γ_{K^+} exceeding that of an isotonic solution like those reported by Lee and Armstrong would follow as a result.

1.4. Reputation of Alleged Demonstration of Active Transport in Cytoplasm-free Membrane Vesicles

A fourth type of evidence in favor of the membrane-pump theory was the claim made that membrane vesicles free of cytoplasm could accumulate K^+ and extrude Na^+ . After careful scrutiny, one finds that these claims have no valid basis. A full discussion was given by Ling and Negendank in 1980. Here I shall only briefly summarize the findings:

(1) Man-made vesicles: Active transport of Na^+ by phospholipids and vesicles containing the alleged Na pump (K , Na activated ATPase) was the result of an experimental artifact.

(2) Bacterial vesicles SR vesicles, etc.: These "vesicles" are not what the name suggests, i.e., empty membrane sacs. As a rule their solid contents equal or exceed that of intact living cells. Cytoplasm-free human red blood cell ghosts do not actively transport K^+ and Na^+ against concentration gradients (see below).

2. Disproof of The Pump Hypothesis Has Stood the Test of Time

Under rigorously defined conditions, the Na pump of frog muscle would require at a minimum from 15 to 30 times as much energy as the total energy available. Thus the pump concept violates the most basic, invariable law of physics: the law of conservation of energy. The fact that our experimental findings as well as our conclusion have remained unchallenged for nearly a quarter of a century after their publication but have been twice confirmed and, above all, that the Na pump at the plasma

membrane is only one of many more pumps required to keep the membrane-pump concept afloat, have already disproved the pump concept.

3. Other Evidence Against The Membrane Pump Theory

After the total unequivocal disproof, one should stop presenting still more evidence, if one were a legal counselor presenting a case in front of a jury; "diluting" the A^+ evidence with lesser evidence can only weaken the case. However, we are not legal counselors, nor our only purpose to convince our readers. As truth seekers, all evidence must be examined.

a. Persistent Selective K^+ Accumulation and Na^+ Extrusion in cells without a Functional Membrane Pump

A frog sartorius muscle consists of about one thousand single muscle cells each 60 to 100 μ wide and 3 cm long. They run parallel going all the way from one end to the other without interruption. By amputating one end of the muscle and exposing the cut end to a Ringer solution containing labeled K^+ and Na^+ while the intact part of the muscle is suspended partly in air and partly in vaseline, the postulated membrane pumps in the intact part of the cell membrane would be made nonfunctional, because air or vaseline offer no "sink" for the outward Na pump nor "Source" for the inward K^+ pump. Nevertheless in such an effectively membrane-pumpless open ended cell (EMOC) preparation, K^+ accumulation and Na^+ extrusion persist (Ling, 1978; 1984, p. 133).

Very recently Kellermayer, Hazlewood and their workers, whose work was presented at this meeting, showed that after treatment of the detergent Brij cell membrane intactness was destroyed. Yet K^+ was retained for a much longer period of time than if it exists in a free state in the cell (see Kellermayer et al, this volume).

b. Failure to Demonstrate Active Transport in Sacs of Axoplasm-Free Giant Squid Axon

In 1963, Dr. Richard Keynes of the Physiological Laboratory of Cambridge University announced at a seminar given at the Johnson Foundation at the University of Pennsylvania (which I attended) that efforts were made to demonstrate active transport of Na^+ against concentration gradient, in squid axon sacs freed of cytoplasm and with ends tied, supplied with energy sources. These experiments failed. To the best of my knowledge, this situation has not changed since then.

4. Decisive Experimental Evidence Supporting the Opposing Theory, the Association-Induction Hypothesis

4.1. A Summary of Some of the Most Basic Features of the AI Hypothesis Which Are Opposed to Those of the Membrane-Pump Theory

(1) The cell membrane is primarily protein and water existing in the state of intensely polarized multilayers. Lipids as a rule do not form a continuous barrier, often existing in the state of islands, while the outer surface of the cell membrane is bathed in an aqueous state, its inner surface is in contact with a cytoplasm having similar physical properties as the cell membrane. Therefore, in the AI Hypothesis, the cell membrane is more like a skin of an apple than a membrane like that of a balloon or bladder.

(2) The distribution of ions and other solutes are determined by the solubility in the cell water with or without adsorption on proteins. A metastable equilibrium phenomenon, solute distribution does not require to control energy expenditures.

(3) The bulk of cell water exists in the state of polarized multilayers.

(4) The bulk of cell K^+ is adsorbed on β - and γ -carboxyl groups of cell proteins.

4.2. Demonstration of the Close-Contact One Ion-One Site Adsorption of the Bulk of K^+ in Frog Muscle on the β - and γ -Carboxyl Groups of Protein Located at the A Bands and Z-Lines

a. Experimental confirmation of the principle of enhanced counterion association following charge fixation. This major postulate (Ling, 1952, 1962), on which the basic concept of K^+ adsorption is based, has been repeatedly confirmed (Kern, 1948; Ling and Zhang, 1983).

b. Experimental confirmation of the "salt linkage hypothesis". Most native

Kern, Makromol. Chem. 2:279, 1947; Ling, Physiol. Chem. Phys. 9:319, 1977; Ling and Zhang, Physiol. Chem. Phys. and Med. NMR 15:251, 1983; *ibid*, 16:221, 1984;

proteins do not adsorb large amount of K^+ , Na^+ , and other alkali metal ions. Ling and Zhang (1984) have now confirmed the salt linkage hypothesis first proposed by Ling in 1952; this failure to adsorb K^+ , and Na^+ arises from the competition for the β - and γ -carboxyl groups by fixed cations on the same or other proteins thereby forming, salt linkages. When the fixed cations are removed by titration with alkali hydroxides, stoichiometric adsorption of Na^+ and other alkali ions follow (see Ling, in this volume.)

c. Extensive evidence demonstrating the localized distribution of K^+ and its surrogates, Cs^+ , Tl^+ at the A bands (and Z-lines) where the bulk of β - and γ -carboxyl groups are located:

(1) Transmission electron microscopy of frozen dried, unstained sections of frog muscle, which were loaded with electron-dense Cs^+ or Tl^+ (Edelmann, 1977, see Edelmann in this volume).

(2) Radioautography of air dried muscle fibers previously loaded with ^{134}Cs or ^{204}Tl (Ling, 1977). Similar results were obtained by Edelmann in autoradiography of frozen hydrated frog muscles loaded with ^{86}Rb and ^{134}Cs (Edelmann, 1980a).

(3) Dispersive x-ray microanalysis (Edelmann, 1978, 1983, 1984; Trombitás and Tigyi-Sebes, 1979; Tigyi et al, 1980-1981).

(4) Laser microprobe mass spectrometric analysis (Edelmann, 1980b).

d. Persistence of preferential selective accumulation (e.g., $Tl^+ > Cs^+ > Na^+$) in muscle cells without a functional cell membrane and pumps (EMOC), indicating selectivity is not achieved at the step of membrane permeation or pumping. Since only differences among these ions are short-range attributes which cannot be "felt" without close contact, the preferential accumulation of these ions can only be due to close-contact adsorption.

e. Experimental confirmations of the general equation of selective solute distribution in living cells in a wide variety of living cells (Ling, 1984, Chapt. 11).

Edelmann, Microsc. Acta Supp. 2:166, 1978, Histochem. 67:233, 1980a, Physiol. Chem. Phys. 12:509, 1980b, Physiol. Chem. Phys. 15:337, 1983, Scanning Electron Microsc. II:875, 1984; Trombitás, Tigyi-Sebes, Acta Physiol. Acad. Sci. Hung. 14:271, 1979; Tigyi et al, Intern. Cell Biol., 1980-1981.

4.3. Experimental Confirmation of the Existence of the Cell Water in the State of Polarized Multilayers

According to the AI Hypothesis, all the protoplasmic part of the living cell (not including the contents of vacuoles) contain matrices of "fully extended" protein chains (or other macromolecular equivalents) with a linear array of suitably distanced and alternating positively charged (P) NH groups and negatively charged (N) CO groups directly exposed to, and polarize in multilayers the bulk of cell water. Water in the state of polarized multilayers has reduced solubility for solutes in a size-dependent manner. Methanol and urea are equally distributed between polarized water and normal liquid water with an equilibrium distribution coefficient (q) equal to 1, because methanol and urea are small and can fit into the polarized water lattice. Sugars and hydrated ions, being large complex do not fit into the polarized water lattice and are partially excluded from the polarized water ($q < 1$). Whereas a matrix of fully extended protein chains constitutes an NP-NP-NP system, matrices of linear chains containing only negative (or positively) charged sites but at distances approximately equal to two water molecules diameters are called respectively NO-NO-NO or PO-PO-PO systems. Both NP-NP-NP and NO-NO-NO systems have been shown to polarize water in multilayers. Thus far, no PO-PO-PO systems have been experimentally studied though, in theory, they should also polarize water but more weakly.

4.3.1. Model Studies

Isolated proteins in their native state with the NHCO groups locked in intra- or interchain H bonds have little or no effect on water solvency and on other properties of the water described below. When proteins for structural reasons (e.g., gelatin) or in response to secondary structure breaking denaturants (e.g., urea) exist in the "fully extended" conformation with their NHCO groups exposed to the bulk phase water as well as linear polymers which qualify as NO-NO-NO sites (e.g., polyethylene oxide, polyvinylpyrrolidone) exhibit the following

Ling, Ochsenfeld, Walton & Bersinger, *Physiol. Chem. Phys.* 12:3, 1980; Ling & Ochsenfeld, *Physiol. Chem. Phys. & Med. NMR* 15:127, 1983; Ling, *ibid*, 15:155, 1983; Ling & Murphy, *ibid*, 15:137, 1983.

properties:

(1) solubility in a size-dependent manner for large (hydrated) Na^+ salts, sugars, and free amino acids normally found in low concentration in living cells but normal solubility for urea in agreement with theory (Ling et al. 1980; Ling and Ochsenfeld, 1983).

(2) osmotic activity far in excess of molar concentration, a property of importance since adsorbed K^+ is osmotically important (Ling, 1983).

(3) greatly enhanced uptake of water vapor at very high relative humidity corresponding to that of a Ringer solution. Vapor sorption over entire range of vapor pressure follows Bradley's multilayer adsorption isotherm (Katz, 1919, Ling and Wu, to be published).

(4) concentration dependent freezing point depression (while solution of native proteins up to a concentration of 50% has no effect on freezing point of water) also in agreement with unusual freezing behavior of solutions of gelatin and PVP (Ling and Zhang, 1983).

For technical reasons, the following traits have been observed or studied thus far only in various NO-NO-NO systems of oxygen-containing polymers.

(5) reduced NMR relaxation times for water protons and a lowering of NMR correlation time of the water affected by a factor from 3 to 14 (Ling and Murphy, 1983).

(6) reduced characteristic frequency of dielectric dispersion of water corresponding to a reduction of the Debye correlation time of from 2 to 5 (Kaatze et al., 1978).

(7) reduced rotational and translational diffusion time of water molecules as revealed by investigations of quasielastic neutron scattering (Rorschach, 1985).

Katz, Kolloid Beihg, 1, 1919; Ling & Zhang, Physiol. Chem. Phys. & Med. NMR 15:391, 1983; Kaatze, Gottmann, Podbielski, Pottel & Terveer, J. Phys. Chem. 82:112, 1978; Rorschach in Water and Ions in Biological Systems, ed. Vasilescu, p. 729-736, Plenum Press, New York, 1985.

4.3.2. Living Cell Studies

Properties of cell water closely similar to those of model systems described above have also been observed:

(1) reduced solubility in a size-dependent manner. Low solubility for Na salts, sugars, and free amino acids but nearly normal solubility for urea.

(2) osmotic activity far in excess of molar concentration of free solutes inside the cell.

(3) greatly enhanced uptake of water vapor at very high humidity corresponding to that of a Ringer solution. Vapor sorption over entire range of vapor pressure follows Bradely's multilayer adsorption isotherm (Ling and Negendank, 1970).

(4) unusual freezing behavior (Chambers and Hale, 1932; Miller and Ling, 1970).

(5) reduced NMR relaxation times of water protons (see Ling and Murphy, 1983; Seitz et al., 1980).

(6) Reduced characteristic frequency of dielectric dispersion of cell water and longer Debye correlation time (Clegg et al., 1984; Gabriel et al., 1983; Masszi et al., 1976; for contrary conclusion, see Foster et al., 1980; and its criticisms, see Ling 1984, Clegg et al., 1982).

(7) Strongly reduced rotational and translational diffusion coefficients of water in *Artemia* cyst cells (Trantham et al., 1984) and reduced rotational diffusion coefficient in frog sartorius muscle (Heidorn et al, 1986).

Ling and Negendank, *Physiol. Chem. Phys.* 2:15, 1970; Chambers and Hale, *Proc. Roy. Soc. London B* 110:336, 1932; Miller and Ling, *Physiol. Chem. Phys.* 2:495, 1970; Seitz, Hazlewood, and Clegg in *Brine Shrimp Artemia*, *Ped. Persoone, Sorgeloos Rolls and Jasper, Universa, Wetteren, Belgium* 2:545, 1980; Clegg, Szwarnowski, Mc Clean, Sheppard, and Grant, *Biochim. Biophys. Acta* 721:309, 1976; Clegg, Mc Clean, Szwarnowski, and Sheppard, *Phys. Med. Biol.* 29:1409, 1984; Gabriel, Sheppard, and Grant, *Phys. Med. Biol.* 28:43, 1983; Masszi, Szijsjártó, and Gróf, *Acta Biochim. Biophys. Acad. Sci. Hung.* 11:129, 1976; Foster, Schepps, and Schwann, *Biophys. J.* 29:271, 1980; Trantham, Rorschach, Clegg, Hazlewood, Nicklow, and Wakabayashi, *Biophys. J.* 45: 927, 1984; Heidorn, Rorschach, Hazlewood, Ling, and Nicklow, *Biophys. J.* 49:92A, 1986.

APPENDIX II

HOW MUCH OF THE CELL WATER HAS SOLVENCY PROPERTIES DIFFERENT FROM NORMAL LIQUID WATER ? CAN WE HAVE A HYBRID MODEL IN WHICH THE MEMBRANE-PUMP THEORY AND AI HYPOTHESIS COEXIST ?

G.N. Ling

In the opening section of my talk, I pointed out that a central issue of cell physiology is the segregation of substances between the cell and its environment as highlighted by that of K^+ and Na^+ . I also pointed out that there are three ways such sustained differences in concentration can be achieved: an insurmountable energy barrier, pumps, and differences in the physico-chemical environment in the two contiguous phases. In the case of living cells, the first two choices have already been eliminated. The cell membrane is permeable to both K^+ and Na^+ disproving the original version of the membrane theory according to which either one or both of these ions are absolutely and permanently impermeant. The pump has also been eliminated from the studies of the energy requirement of the pumps. This too has been discussed in my talk. Therefore we are left no other alternative: somehow the cell must represent a different physico-chemical environment from the solutes. Our next question is, "In what way can the physico-chemical environment within the cell be different from the cell's external environment?"

Let us begin with a homogeneous saturated solution of, say, sodium sulfate. Lower the temperature, sodium sulfate crystals form. As a result there are now created out of a single homogeneous phase two separate phases in equilibrium. The crystal phase contains Na_2SO_4 at a higher concentration than that in the surrounding medium and it also contains water ($Na_2SO_4 \cdot H_2O$). Clearly the physico-chemical environment within the crystal phases are by and large different from the solution phase. Take Na^+ for example: it has access to three different environments: (i) as part of the Na_2SO_4 crystal lattice, (ii) dissolved in the water of hydration and (iii) dissolved in the external solution.

Let us consider another saturated solution of Na_2SO_4 . This time instead of changing the temperature, we introduced a piece of dried pig bladder. Soon the pig bladder swells up, taking up both water and Na_2SO_4 from the environment. However the pig's bladder did not take up water and Na_2SO_4 in the same proportion as in the original saturated Na_2SO_4 solution. Rather, more water is taken up than Na_2SO_4 . As a result, the external solution becomes supersaturated and Na_2SO_4 crystals began to form until once more the solution becomes a simple saturated Na_2SO_4 solution. Clearly the water taken up by the bladder must have less solubility for Na_2SO_4 than a normal liquid water. In other words, the equilibrium distribution coefficient or q-value of Na_2SO_4 in the water taken up by the pig's bladder is below unity. This experiment was first reported by Carl Ludwig in 1849.

Similarly dry gelatin will take up water from a saturated NaCl solution and cause NaCl to crystalize out and at equilibrium water taken up will contain less Na_2SO_4 than in the external medium.

Similarly dry (but living) seeds of the plant *Xanthium glutatum* will take up water from a saturated solution of LiCl , only here living cells, rather than dead cells and inanimate proteins and synthetic polymers, take up water preferentially over salts.

Thus dried pig's bladder, dry seeds, and dry gelatin can all change the nature of water in such a way that makes its solubility for Na^+ salts, and other solutes very much reduced - much as a living cells does to its water. What is the common factor shared by dried pig's bladder, dry seeds, dry gelatin, and living cells? The answer is protein. But not every protein, because pure native proteins do not possess this effect. Only when a protein exists in a fully extended conformation does it acquire this characteristic. In gelatin its existence partially in the fully extended state is due to the possession of proline, hydroxyproline, and glycine. But as also presented in my talk, any protein can be made to interact with water like gelatin if for one reason or another (e.g., exposure to urea), the proteins are prevented from forming intra- or intermacromolecular H-bonds, thus preventing the backbone NHCO group from interacting with the bulk phase water.

That native proteins with its NHCO groups locked in α -helical and β -pleated sheet conformation has minimal effect on water solvency and that synthetic polymers like polyethylene oxide (PEO) containing nothing more

Ludwig, Z. ration. Med. Henle 8:1, 1849

than repeating units of $(-\text{CH}_2-\text{CH}_2-\text{O}-)_n$ unable to form intra- or intermacromolecular H bonds are all able to achieve what gelatin, dry pig's bladder, dry seeds, and living cells do, suggest that it is an extended protein or polymer chain containing suitably spaced oxygen (and NH) groups that interact with the bulk phase and that it is the NH and particularly the CO groups that are the seats of this interaction with water.

The next question is "What is the minimal amount of water in living cells that must have it property so altered?" In the AI hypothesis, this amount is virtually all if not all the water in the cell, excluding water in vacuoles, etc. as in old plant cells. Let us examine the reasons for this and in the process also answer the question, why we cannot have a hybrid model of the living cells where both pumps and the AI hypothesis coexist.

Take frog muscle for an example. 80% of its weight is water. The q -value of Na^+ in the cell water is close to 0.1. Thus if all the water affected by the extended proteins has zero solubility for Na^+ , we would require 90% of the cell water to be affected to account for the q -value of Na^+ at 0.1. However, Na^+ is by no means the solute with minimum q -value. Nor is any solute likely to have a q -value of zero. Taken together, these failures more or less force us to a conclusion that virtually if not all of the cell water is affected by the extended proteins.

In many human affairs, compromise is often the clue to wisdom. One notable exception is science. In the search for the truth, there is absolutely no ground to barter.

Let us consider the frog muscle. If we arbitrarily assign 20% of its water to be free water, then a Na pump as well as all the other pumps must be installed to account for the q -value of Na^+ and other solutes with a volume smaller than 0.2. The same energy problems that has disproved the Na pump hypothesis will apply here as well. The law of parsimony is thus truly a law of great validity. There is no way that some creatures came by way of Darwinian evolution and some others by the theory of divine origin. Having eliminated the absolute membrane impermeability, as a means for excluding Na^+ from the cell and having demonstrated the key to creating the kind of water with reduced solvency for Na^+ salts, sugars, and free amino acids is the fully extended polypeptide chains and that these

extended protein chains must be able to alter not just a fraction of the cell water but all of the cell water, one is then face to face with the first question raised by Dr. J. Clegg. "Can there be enough protein that can polarize all the cell water especially in the most extensively hydrated cells like frog muscle?"

Let us first make clear that multilayer polarization of gaseous molecules has been a well recognized physical phenomenon. The attempt to explain the condensation of argon molecules in terms of the theory of polarized multilayer by DeBoer and Zwicker (1929) elicited the severest criticism of Brunnauer, Emmett and Teller in 1951. However, these critiques were very explicit in conferring their criticism to molecules like argon which has no permanent dipole moment. For other molecules with large permanent dipole moment like water (dipole moment= 1.834×10^{-18} esu) Brunnauer et al. stated clearly and unequivocally, "many layers may be successfully polarized..." However, multilayer polarization is not only theoretically feasible but easily demonstrated experimentally. Thus Ling and Ochsenfeld have shown that as many as 10 to 20 water molecules are made nonsolvent toward Na citrate around each oxygen atom of the oxygen-carrying polymers like PEG, polyvinylmethyl ether (PVME), etc., even though in these cases, the polymer chains are randomly oriented and lacking in the kind of orderly orientation found commonly in biological systems.

The consequence of multilayer polarization is in loss of motional freedom of the water molecules, in particular the loss of rotational motional freedom. In the important quasi-elastic neutron scattering study of water in *Artemia* cyst cells by Tranthem, Rorschach, Clegg, Hazlewood, Nicklow and Wakabayashi, the rotational diffusion coefficient of water in these cells has been shown to be reduced by a factor of about 13 and that of the translational diffusion coefficient by a factor of 3, in full harmony with the polarized multilayer theory of cell water. There is no indication that any of the water in the *Artemia* cyst does not suffer these severe motional restrictions. To the best of my knowledge, the cell has no other component in all living cells than proteins that can be offered as an alternative source of this action on the Artemia cyst water. With no

Brunnauer, Emmett, and Teller, J. Am. Chem. Soc. 60:309, 1938;

DeBoer and Zwicker, Z. Phys. Chem. Abt. B. 3:407, 1929

such alternative in sight, we can only conclude that it is the cell protein that produces this profound motional restriction. With no other known mechanism how the qualitatively smaller component (protein) can affect all the units of the large component (water), we can only draw the conclusion that all the water in the Artemia cysts are polarized in multilayers by cell proteins.

The final word on the all inclusiveness of the Artemia cysts cells could not have, for the benefits of my presenting a defense against Dr. J. Cleggs' criticisms come from a better source, than Dr. Clegg himself.

Thus in his, what I believe important paper of 1984, Clegg, McClean, Szwarnowski, and Sheppard concluded from their studies of high frequency dielectric dispersion up to 70 GHz in these words, "This result implies the almost complete absence of cyst water that has bulk dielectric properties, even in cysts at the maximum water content of about 1.4 g/g..." (p. 1417)

A 1.4 g/g water content corresponds to a water content of 58%. The water content of some cells like frog muscle has a water content of 80%. However, I shall demonstrate that this difference in percentage water content is not nearly as large when one considers the number of layers of water between the fully extended polypeptide chains.

Figure 1, reproduced from a 1983 paper shows the theoretically calculated distances in Ångstrom units (right ordinate) or number of layers of water molecules between adjacent fully extended chains (left ordinate), under the assumption that all protein considered are fully extended. They as well as other not fully extended protein molecules occupy no space.

Note that the number of layers of water molecules corresponding to the Artemia cysts at 1.4 g H₂O/g, (i.e., 42%) is about 4 water molecule layers between the nearest neighboring chains. At 20% protein, it is 5 layers. Even at a protein content of 5%, it is still no more than 12 layers. Since in reality, proteins take up space, the number of layers of water polarized by each chain must be no more than 5 layers even if only a small part of the total cell proteins, say 5%, exists in the fully extended conformation. This then is the answers to Dr. Clegg's first question. It

Clegg, McClean, Szwarnowski, and Sheppard, Phys. Med. Biol.
29:1109, 1984; Ling, Physiol. Chem. Phys. and Med. NMR 15:155,
1983

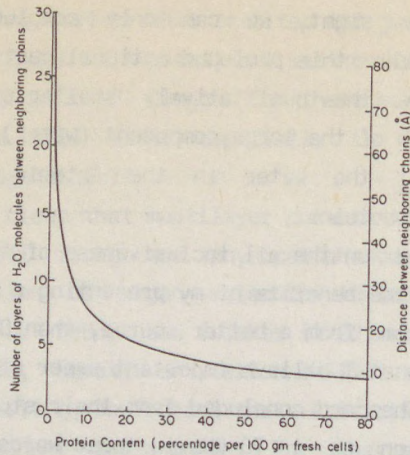


Fig. 1. The theoretical distances both in Ångstrom units (right ordinate) and in number of water molecules between nearest neighboring (fully extended) protein chains in hypothetical case when the entire protein contents (abscissa, in % (w/w) are in the fully extended state and occupy no space.

may be mentioned that recently Heidorn, Rorschach, Hazlewood, Ling and Nicklow had made quasi-elastic neutron scattering studies of isolated frog muscles. It was found that the measured rotational diffusion coefficient of water in the muscle is more restricted than in an isotonic KCl solution. Again there is no evidence of a fraction of cell water that has rotational diffusion coefficient equal to that of free water.

Heidorn, Rorschach, Hazlewood, Ling, and Nicklow, *Biophys. J.* 49:92A, 1986

Evidence That ATP Maintains the Key Intracellular Proteins in the Conformation Where β - and γ -carboxyl Groups Selectively Adsorb K^+ Over Na^+ and Extended Backbone NHCO Polarize the Bulk of Cell Water

Polarized water provides one physico-chemical environment in the cell different from the external aqueous medium that maintains low levels of Na^+ , sugars, and free amino acids. There is a second type of intracellular "environment" that is provided by the β - and γ -carboxyl groups and other adsorption sites. Selective adsorption on the β - and γ -carboxyl groups elevates the level of cell K^+ markedly from that in the plasma. There is now such extensive evidence for the adsorbed state of cell K^+ ably reviewed by Edelman in this volume, that I need not reiterate them again. I do need to point out that both the ability of the β - and γ -carboxyl groups selectively to adsorb K^+ and the ability of the NHCO groups of the fully extended polypeptide chains to polarize water in multilayers are lost upon cell death. Thus the proteins that selectively adsorb K^+ and polarize water have reverted to a state which most native proteins assume. Nevertheless, as I pointed out in my article, exposure of isolated native proteins can be made, not only selectively to adsorb alkali metal ions stoichiometrically on its β - and γ -carboxyl group but to polarize water on its NHCO groups when the proteins are exposed to NaOH or other alkali halides. But clearly it is not high pH that endows the living cell proteins with a similar ability. What then is this agent that endows cells with life?

It is common knowledge that for life to continue, there must be water. There must also be foods. From foods life acquires its building materials, proteins, DNA, etc., as well as the ATP.

The work of Podolsky, Morales, George, Rutman and others have long ago demonstrated that ATP does not contain an extra package of energy in an unusual phosphate bond, even though textbook writers are reluctant to let go this once highly cherished now totally obsolete idea.

Among the major new concepts that were introduced in the AI hypothesis is the new interpretation of how ATP helps to maintain the living state. Again as explained in my presentation, like the magnet polarizing the chain of soft nails and iron filings, ATP acts as a bona fide "cardinal adsorbent". By adsorption onto key cardinal sites, ATP controls and thus enables the β - and γ -carboxyl groups selectively

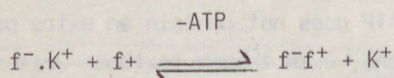
to adsorb K^+ and the backbone to assume the fully extended state to polarize the bulk of cell water. Even though much remains to be made clear in the future, I am convinced of the general validity of the concept. The following are some evidence already on hand:

(1) ATP strongly reacts in vitro with two cytoplasmic proteins - myosin in muscle, hemoglobin in red blood cells. There are evidences that these proteins participate in the K^+ binding and water polarizing in the respective cells.

(2) In keeping with its role as a cardinal adsorbent, ATP binds onto myosin strongly and specifically in vitro. Thus the association constant is 10^{10} - $10^{11} M^{-1}$ while that of ADP is only $10^5 M^{-1}$.

(3) The in vitro binding of ATP on hemoglobin allosterically controls the affinity of heme groups for oxygen. Hemoglobin is not an ATPase, its effect on hemoglobin does not involve ATP hydrolysis but is the direct result of its binding onto the protein.

(4) In the presence of the metabolic poison, Na iodoacetate (IAA), the ATP content of frog muscle falls rapidly at $25^{\circ}C$. To near zero level in about 6 hours ATP content declines much more slowly at $0^{\circ}C$ retaining 75% of its initial concentration even after 10 hours (Ling, 1962, p. 446-448). Exposure to $25^{\circ}C$ of the IAA-poisoned muscle also causes rapid contraction and with it rapid loss of cell K^+ ; cooling to $0^{\circ}C$ arrests contraction as well as K^+ loss; warming once more causes accelerated contraction and K^+ loss, etc. Since contraction definitely involves conformation changes of the major protein (myosin) which also provides major share of adsorption sites for K^+ , the correlated change of K^+ loss and contraction suggests that ATP prevents the β - and γ -carboxyl group (f^-) normally adsorbing K^+ from forming salt linkages with fixed cations (f^+) thereby releasing the K^+ :



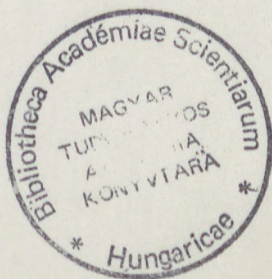
In other words, ATP binding onto the K^+ adsorbing protein myosin produces an effect similar to that produced by the removal of the electric charge of the fixed cation by alkali hydroxides in making the β - and γ -carboxyl groups available for K^+ adsorption.

This concept was corroborated by the demonstration that the K^+ contents of frog muscles exposed to widely different poisons are

indifferent to the nature of the poison used and the targets of the poisonous activities but depends only on the resultant level of ATP concentration in a stoichiometric consistent manner.

(5) In native proteins exposed to alkali halides (e.g. NaOH) not only are the β - and γ -carboxyl groups unmasked due to the neutralization and elimination of the fixed cation, there is also a profound change in the solvency of the bulk phase water that is similar to that brought about by the exposure of the native proteins to 8 M urea. The effect of 8 M urea on native proteins is well known, it causes the dissociating of α -helical and other inter- and intramacromolecular H bonds, and the unmasking of the backbone NHCO groups to react with the bulk phase water reducing its solvency for Na^+ , sucrose, etc. By analogy, the alkali halide also causes the unmasking of the backbone NHCO groups (as well as the β - and γ -carboxyl groups).

Thus if ATP binding onto cardinal sites of myosin and other cell proteins produces the same overall effect as alkali hydroxides one might expect an ATP concentration dependent water polarization and lowering the q -values for Na^+ , sugars, etc. In fact, this expectation was also confirmed. In the IAA-poisoned muscles there is also an ATP-concentration dependent change of the concentration of sucrose (and of Na^+). In parallel with this ATP concentration decline, there is a qualitative rise of sucrose concentration from a low level corresponding to a ρ -value of 0.25 in muscle containing normal concentration of ATP to a ρ -value of 1.0 when all ATP is gone.





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The Symposium "The Physical Aspect of the Living Cell" was devoted to the memory of late Eugen (Jenő) Ernst who was characterized by Albert Szent-Györgyi as a real apostle of scientific knowledge.

Ernst was a unique intellectual giant of this century and he had a significant influence on world biophysics and on the whole of Hungarian biology. His admirable scientific achievements in biophysics ranged this meeting among the outstanding researchers of the world who accepted the invitation and whose findings were consistent with Ernst's concept concerning the complexity of the protein-water-inorganic element interaction in the living cell.

This book contains the comprehensive lectures and the free discussions of numerous distinguished scientists who examined and argued about the mechanisms of life which involves the exact three dimensional organization at the submolecular, the molecular, and the supramolecular levels.

The Proceedings of Eugen (Jenő) Ernst Memorial Symposium reflects Ernst's idea that science is a search for truth.



Akadémiai Kiadó, Budapest

THE HISTORY OF THE
PROGRESS OF THE
ARTS AND MANUFACTURES
IN GREAT BRITAIN
FROM THE EARLIEST
TIMES TO THE PRESENT
PERIOD