

EXPERIMENTAL METHODS IN BIOPHYSICAL CHEMISTRY

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Experimental Methods in Biophysical Chemistry

Edited by

Claude Nicolau

*Department of Molecular Biology,
Craiova University, Romania*

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Preface

The ever increasing number of books appearing year after year somewhat discourages projects of editing or writing new ones. With this book, it obviously was not the case, and that for several reasons.

Biophysical Chemistry, though a subject read in a few universities, may still be considered a young area of Science 'lying between Physics, Chemistry and Biology'. It has developed into a successful realm of the Life-sciences and very interesting work was and is currently reported along this line. It deals mainly with sizes, shapes, structure and interactions of biomolecules, and these results have been of great significance to a large spectrum of disciplines.

One might wonder how the methods described in this book have been selected, why only these and not others. Most of the methods presented here have yielded already very valuable information, are theoretically well-founded and their use will probably be a lasting one. A few others are of significant potential interest and although they have produced a number of interesting data, their full development is, at least partly, to come. A very few of the methods dealt with in this book are slightly less used now than they were sometime ago. But, in those particular cases, we feel that there is a definite chance for them to be used again, in connection with others and that new developments are to be expected.

All the authors in this book are well-known contributors to the areas which they describe and their personalities emerge out of their work. The book is intended as a source of informations and references for those engaged in biophysicochemical research but as well for graduate students and for people working in hospital- and biological laboratories at large.

The speed of publication of a contributed volume is, unfortunately, dictated by the delivery date of the last manuscript. When this was received all contributors were given the opportunity to up-date their manuscripts. It is a great pleasure to acknowledge the lasting patience displayed by the publisher with the editor during the processing of the material as well as the excellent cooperation, which, notwithstanding the distances, characterized our relations.

Foreword

Rather than to attempt to define biophysics here, I should like to evoke its general meaning. There can be no better approach, I feel, than to consider its origin and its historical development. It is no paradox to claim that biophysics was born several centuries before this era, under the impulsion of Leucippus, Democritus and Epicurus, who progressively reduced the phenomena of the Cosmos to atoms. It was Lucretius the Roman, a contemporary of Julius Caesar, who brought the character of a universal vision to this hypothesis in his remarkable poem entitled 'De Natura Rerum' in which he describes how atoms which come together in every possible manner during their movement finally adopt stable configurations which correspond to the observable inanimate world and to the living world. What an admirable way of expressing a unitary theory, which to-day, after many vicissitudes, imposes itself more and more.

It is not however possible to follow step by step the evolution of this great idea, which brought biology into the realm of physics; I should like, however, in transferring the scene to the fertile 19th century, to recall a few examples of particularly productive interactions between the two sciences. It was to Julius Robert von Mayer, doctor of medicine and philosopher, and then to his contemporary H. von Helmholtz, who was originally a physiologist and later became an innovator in physics, that we owe the establishment of the principle of the conservation of energy in its universal meaning. Nor can I leave unmentioned the case of Ludwig Boltzmann, who was the founder of statistical physics, and who in establishing this was inspired by the law of natural selection which had been discovered a little earlier by the great biologist Charles Darwin, to account for the evolution of living things.

The latter, had himself drawn from the theories of the British economist Malthus, who worked out his population theory in the first quarter of the nineteenth century. For his part, Ernst Mach, who was a sceptical empiricist, precursor of Einstein, wrote: 'Physics is not the whole world: biology has also its place there, and is essential in any depicting of the world'. These remarks throw a special light on the openness of mind of a great physicist towards biology. Louis Pasteur, Agrégé ès sciences physiques, had already made royal presents to this science, as we know. More recently, Schrödinger with his book entitled 'What is life?' made a considerable contribution to the

bringing together of the two sciences. And finally, to-day, the Institut de la Vie of France, amongst other functions, ensures a truly efficacious liaison between the physicists and biologists.

But if, in an individual form, biophysics has always existed, it is only recently that it has been transformed into a truly independent science, comparable to physical chemistry, geophysics, or astrophysics. How has this come about? To understand this, we must once more return to the period towards the end of the nineteenth century, when on the one hand Kékulé and on the other hand van't Hoff succeeded in giving a geometrical form to chemistry by bestowing a spatial structure on such molecules as benzene and methane. Around this period also, first Newlands and then Lothar Meyer and Mendeleeff in working out the periodical system of chemical elements, which they arranged in function of increasing atomic weight produced the 'arithmetical' aspect of chemistry, with the well-known law of octaves. Later, with the discovery of isotopes by Fajans and Soddy, it was seen that the effects of degeneracy completed the whole picture. To understand and interpret these fine discoveries, which in fact establish the whole strategy of the inorganic world, knowledge had to be extended to a universe as yet unexplored. When first G. N. Lewis and then Rutherford penetrated into the electronic and even the nuclear structure of matter, scientists succeeded in accounting for the fundamental characteristics of the periodical system. It then became clear that there existed an ineluctable law which demanded the exploration of the universe firstly to dimensions of 10^{-8} cm and then to 10^{-13} cm, to make possible the interpretation of macroscopic regularities.

And what about biology? The first attempts to spatialize biomolecules by means of X-ray diffraction occurred from the 1940's onwards. The fine work of J. D. Bernal and Astbury, disciples of Bragg, on the structure of DNA, and that of Linus Pauling on proteins, must especially be cited. It was however in 1953 that the structure of the double helix of DNA was established thanks to the imagination and the combined efforts of Wilkins on the one hand, and of Watson and Crick on the other. This was the point of departure for a relationship between tridimensional structure and biology which was of very fundamental importance, and which in principle can be compared with that established in an earlier period between the spatial structure of methane or benzene and chemical activity. Further, the genetic code, with its concept of codon or triade, which gives an arithmetical form to the strategy of life, recalls the octaves of the periodical system. From this, and by analogy, it is not difficult to predict an aspect of to-morrow's evolution. It is on the electronic level that the profound meaning of the genetic code will have to be interpreted, in particular its stability, its specificity, the curious effect of degeneracy, the separation of the two helices, order-disorder, etc. Work in this direction is already well under way, in particular that done by B. and A. Pullman, who grasped this ineluctable phenomenon very early and who are

every day succeeding in bringing the language of wave mechanics more and more into the realm of biology. But beyond the electronic scale, exactly as in the case of the periodical system, the nuclear scale takes form. The variations in the isotopic ratios were established about ten years ago for the atoms of carbon 12 and carbon 13, according to the species or class, for plants and invertebrates. Quite recently the author and his associates wished to explore the incidence of nuclear properties in the higher animals, in the case of a specific mechanism. With this aim in view, the CO_2 exhaled during breathing was chosen, taking the case of batracians, dog, man and birds. They were surprised to find that the ratio $\text{C}^{12}/\text{C}^{13}$ is substantially altered through the sequence, so that between the two extremes the difference in the ratios reaches nearly 1 per cent, with a progressive growth in the concentration of C^{13} . The profound meaning of such a result, which throws into relief the effect of the animal class, is still not quite clear. Within the framework of this research, it has recently been discovered by us—so far only in the case of rats—that under the impact of specific pathological conditions the isotopic ratios may also be significantly altered. This new fact awaits further development in the field of medicine. Yet, despite these successive changes in scale, which open up unsuspected horizons, we retain the impression, as the fine mental picture of Newton has it, that we are still like children who are playing on the beach while before them there lies the expanse of the ocean of truth.

Alongside the progress which has taken place in the field of the structure of biomolecules, electron microscopy has brought a new light to bear on the organization of cells, as, for example, the work on mitochondria, which are energy generators, and on ribosomes, sites of protein synthesis. The aim must be to account for this remarkable cellular heterogeneity through the knowledge of the interactions between constituent molecules. This is doubtless one of to-morrow's great problems, and shows up the gulf which separates isolated molecules from large associations, for basically the perspective must be the understanding of life in its most fundamental characteristics. Indeed, the study of biogenesis, of cryobiology and of the phenomena of reviviscence by emphasizing the importance of organization represent very encouraging tendencies. Following up the fine research carried out in France by Paul Becquerel, scientists have been successful in maintaining seeds, lichens and tardigrades in a latent state of life, submitting these to profound dehydration and maintaining them in a vacuum at temperatures near to absolute zero. Thus treated, the complete arrest of metabolism and the occurrence of apparently appreciable modifications of the cellular structures do not prevent reviviscence. This means that the fundamental factor for life is organization. But at once the problem can be posed as to what is the limit beyond which life fades away. Experiments undertaken in the laboratory of the author on dried lichens, dating from about two hundred years up to quite

recent periods, brought to light a decrease of the intensity of paramagnetic signals as age increased. The study of this phenomenon, which appears to be tied up with a progressive disorganization of the structure of the whole entity, is still proceeding.

As for biochemistry which is already well established its aims were, and remain, on the one hand the identification of biomolecules and their morphological analysis, and on the other hand an examination of the processes in living organisms in relation to functions. Its mission is carried further by biophysics within the perspective which has just been stated, to the point where these two tendencies, which express in a profound way all that the most refined resources of scientific thought can produce, constitute closely related and complementary branches of what is known as molecular biology. This, then, is a convergence which is entirely analogous to that undergone by physics and chemistry when these condensed around the year 1880, in the form of physical chemistry. This is perhaps the moment to pose the question as to whether the most fascinating properties of life, such as that of consciousness may be interpreted one day thanks to the carrying further of the line of thought which we have just evoked. This means in fact the posing anew of the problem which was stated so authoritatively by Lucretius and in which was implicit the idea that the final goal of physics was to explain all cosmic phenomena, including life itself. Our own replay must contain more nuance than this. It does not seem to me to be evident that there is no limit to our explanatory possibilities, in particular when we endeavour to reach the most subtle expressions of life. Can it be, then, the consciousness, which is the product of a prodigious cosmic emergence, could be reduced to simple concepts when, at the same time, these arise from consciousness itself? To say the least, these questions are irritating ones, and molecular biology does not seem to me to be ready to reply to them so far.

And yet the development of this science enlarges our vision in every direction. I cannot discuss all the aspects of this here, such as those connected with what I would call the defects in nucleic acids, in a sense which is entirely comparable to that concerned with deficiencies in crystalline structures. I still recall the time when it would have been considered a heresy not to believe that a crystal was a product of the implacable repetition of an elementary unit. And yet it is the deviations from these fine regularities, that is to say the defects in the lattice, which have become the agents responsible for exciting crystalline properties. Biological mutations should be set within this framework, and it is then possible to grasp how far we are from having understood the whole wealth of the possibilities connected with DNA. There is no doubt that we now have methods at our disposal which seem to me to be first class, and which are far from having been entirely explored, by which perturbances in DNA can be produced. I should particularly like to draw attention here to phenomena of photodynamic action in biology, the

study of which, using electron spin resonance, has been undertaken by the author and his associates and which allows one to envisage the possibility of directed mutations. This would of course be an exciting success!

There is also the whole field of molecular medicine which is still to be developed, as Linus Pauling has pointed out, in spite of some remarkable first successes such as the discovery of 'Sickle cell anaemia', which is connected with molecular perturbances in haemoglobin. And certain pathological states manifest themselves in a very subtle way at the level of the tissues. Thus, cancerous tissues seem to give rise to paramagnetic signals well before the effects of the tumours can be detected by the usual methods. Recent *in vitro* syntheses, firstly of RNA and secondly of DNA, both of which are biologically active, permit one to be extremely hopeful, perhaps even allowing one to envisage the possibility of correcting certain insufficiencies in the genetic code.

But completely undeciphered fields remain, such as that of olfaction which is perhaps linked to very subtle molecular interactions, as are, for example, charge transfers. The whole realm of pharmacodynamics remains largely to be understood, where analogous forces probably play a significant part, according to recent research by the author and his associates.

But where life is concerned the dominant note, which must never be lost sight of, seems to depend on the fact that the phenomena which occur cannot be isolated from their context, and from the notion that each emergent structure reacts on others, and vice versa. Life, then, would be global phenomenon, the reduction of which into separated forms would represent only an approximation which is valid up to a certain point, beyond which the phenomena of consciousness are probably situated.

The building up of theoretical biology, the extension of the fundamental concepts of statistical mechanics to the interpretation of biogenesis in the direction shown by I. Prigogine and his school, permits us to hope that life may be looked at in its own perspective.

Moreover, the experimental methods of modern physics, not only those based on X-ray diffraction, but also on electron spin resonance, nuclear magnetic resonance, mass spectroscopy, photochemistry techniques, and lasers, are all not only promising, but they have already achieved important progress.

In this book, the Editor has brought together a comprehensive set of articles by eminent scientists which admirably illustrate many essential aspects of biophysics. One realizes however that if the road is still a very long one, we are nonetheless embarked in a vessel, the direction of which will not cease for a long time to stimulate the ardour and the imagination of this new category of research workers.

Contributing Authors

- H. ALTMANN Institute of Biology, Reactor Centre, Seibersdorf, Austria
- G. P. ARSENAULT Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts, U.S.A.
- L. G. AUGENSTEIN Late of Biophysics Department, Michigan State University, East Lansing, Michigan, U.S.A.
- J. S. COHEN Physical Sciences Laboratory, Division of Computer Research and Technology, National Institutes of Health, Department of Health, Education, and Welfare, Bethesda, Maryland, U.S.A.
- G. ECKHARDT Institute of Organic Chemistry, University of Bonn, Federal Republic of Germany
- G. L. GRIGORYAN D. I. Ivanovsky Institute of Virology, The U.S.S.R. Academy, Moscow, U.S.S.R.
- S. GUINAND Laboratoire de Biologie Physico-Chimique, Université Paris-Sud, Orsay, France
- W. HABERDITZL Berlin Humboldt University, G.D.R.
- U. HAGEN Institut für Strahlenbiologie, Kernforschungszentrum, Karlsruhe, Federal Republic of Germany
- A. E. KALMANSON D. I. Ivanovsky Institute of Virology, The U.S.S.R. Academy, Moscow, U.S.S.R.
- R. L. MUNIER Institut Pasteur, Paris, France
- E. POSTOW Department of the Navy, Office of Naval Research, Arlington, Virginia, U.S.A.
- B. ROSENBERG Biophysics Department, Michigan State University, East Lansing, Michigan, U.S.A.
- H. W. SHIELDS Wake Forest University, Winston-Salem, North Carolina, U.S.A.
- G. SNATZKE Institute of Organic Chemistry, University of Bonn, Federal Republic of Germany

- J. TONNELAT Laboratoire de Biologie Physico-Chimique, Université Paris-Sud, Orsay, France
- J. O. WILLIAMS Edward Davies Chemical Laboratories, University College of Wales, Aberystwyth, Wales
- R. WURMSER Institut de Biologie Physico-Chimique, Paris, France
- P. ZUMAN Department of Chemistry, Clarkson College of Technology, Potsdam, New York, U.S.A.

Contents

I. STRUCTURES OF BIOMOLECULES

1. **Mass Spectrometry of Biomolecules** 3
G. P. Arsenault
2. **Use of Activation Analysis in Molecular Biology** 49
H. Altmann
3. **Optical Rotatory Dispersion and Circular Dichroism of Biomolecules** 67
G. Snatzke and G. Eckhardt

II. SHAPE, SIZE AND WEIGHT OF BIOPOLYMERS

4. **Light-Scattering** 135
J. Tonnelat and S. Guinand
5. **Ultracentrifugation** 163
U. Hagen

III. SEPARATION OF BIOMOLECULES

6. **Chromatography of Macromolecules of Biological Origin** ... 209
R. L. Munier

IV. ELECTRONIC PROPERTIES OF BIOMOLECULES

7. **Semiconductivity in Proteins and Nucleic Acids** 315
B. Rosenberg and E. Postow
8. **Magnetochemistry: Methods of Measuring Static Magnetic Susceptibility and their Applications in Biochemistry** 351
W. Haberditzl

- 9. Polarography of Biomolecules** **393**
P. Zuman

V. ACTION OF PHYSICAL AGENTS ON BIOMOLECULES

- 10. Electron Spin Resonance of Irradiated Biomolecules** **417**
H. W. Shields
- 11. Thermoluminescence in Biological Materials** **483**
L. G. Augenstein and J. O. Williams

VI. REACTIONS AND INTERACTIONS OF BIOMOLECULES

- 12. Nuclear Magnetic Resonance Investigations of the Interactions of Biomolecules** **521**
J. S. Cohen
- 13. Spin Labels in the E.p.r. Investigation of Biological Systems** ... **589**
A. E. Kalmanson and G. L. Grigoryan
- 14. Thermodynamic Study of Antigen-Antibody Reactions** **613**
R. Wurmser
- Author Index** **649**
- Subject Index** **673**

I

Structures of Biomolecules

CHAPTER 1

Mass spectrometry of biomolecules

G. P. Arsenault

Department of Chemistry,
Massachusetts Institute of Technology,
Cambridge, Massachusetts, U.S.A.

I. INTRODUCTION	3
II. SOME FUNDAMENTALS OF MASS SPECTROMETRY	5
III. INSTRUMENTATION AND TECHNIQUE	10
A. Ion Source	10
1. Electron Impact Ionization	11
2. Field Ionization	12
3. Chemical Ionization	14
B. Analyser	16
C. Detector and Recorder	17
D. Sample Introduction System	18
1. Heated Inlet System	19
2. Direct Introduction System	20
3. Gas Chromatograph	21
E. High Resolution Mass Spectrometry	23
IV. SELECTED APPLICATIONS	26
A. Determination of the Amino Acid Sequence in Oligopeptides	27
B. Determination of the Nucleotide Sequence in Oligonucleotides	33
C. The Use of High Resolution Mass Spectrometry in Determining the Structure of Antheridiol, a Sex Hormone in <i>Achlya bisexualis</i>	36
ACKNOWLEDGMENT	44
REFERENCES	45

I. INTRODUCTION

Mass spectrometry is one of the oldest and possibly the most versatile physical method of analysis. The first mass spectrometer bearing a strong resemblance to instruments in use today was built by Dempster.¹ The investigation of naturally occurring substances by mass spectrometry was begun in the nineteen fifties by K. Biemann, R. I. Reed, R. Ryhage and E. Stenhagen. Great impetus was given to this field of investigation in the nineteen sixties

by the successful development and commercial production of direct introduction systems (section IIID2), gas chromatograph-mass spectrometer combinations (section IIID3) and high resolution mass spectrometers (section IIIE), and by the use of digital computers and the development of computer techniques to record, reduce and interpret the wealth of data produced by mass spectrometers (Biemann and Fennessey,² Hites and Biemann^{3,4}). Today mass spectrometry is available to most organic chemists as one of several instrumental methods of analysis and should become a routine tool in biochemical and biomedical laboratories in the nineteen seventies. The long time span between the birth of mass spectrometry and its widespread use by everyone is a reflection of the cost, complexity and commercial unavailability of the instruments.

Mass spectrometry is the most sensitive instrumental method of analysis. It gives more information per microgram of sample than any other method, and satisfactory spectra may be obtained on as little as 1×10^{-12} g of material.

The sensitivity of the mass spectrometer is so great that sample isolation and handling techniques often lag behind. A common occurrence in taking the mass spectrum of a sample isolated in amounts of a few milligrams or less is to find that it contains as much impurity as desired compound. Fortunately, the sample does not necessarily have to be pure, and herein lies an advantage of mass spectrometry over other instrumental methods of analysis, since mass spectrometry provides several alternatives for dealing with interfering impurities. Firstly, purification without the necessity of further handling of the sample prior to analysis may be accomplished by the tandem use of the gas chromatograph and the mass spectrometer (section IIID3). Secondly, fractionation of microgram samples frequently occurs when they are vaporized slowly and directly into the ion source of the mass spectrometer (section IIID2). This is equivalent to a high vacuum fractional distillation or sublimation in which the mass spectrometer is used to analyse each fraction; the number of fractions taken is determined by the scanning speed of the instrument, which is not a limiting factor since complete spectra may be obtained in less than a few seconds if necessary. Finally, the high resolution mass spectrum (section IIIE) of a sample, which consists of the exact masses and, hence, the elemental compositions of all ionic species in the spectrum, may permit differentiation between ionic species originating from the impurities and the compound present in the sample.

The principal limitation of mass spectrometry lies in the vapour pressure requirement placed on the sample to be analysed. The minimum vapour pressure must be about 1×10^{-6} torr at the maximum temperature at which the sample may be heated without undergoing decomposition (section IIID). The vapour pressure of the sample thus drastically limits the molecular weight of compounds amenable to mass spectrometric analysis. The vast