Biochemical Spectroscopy

by

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Volume 1

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Richard Alan Morton, Ph.D., D.Sc., Sc.D., F.R.I.C., F.R.S.
Emeritus Professor of Biochemistry
University of Liverpool

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Preface

The first edition of my book on *The application of absorption spectra to the study of vitamins and hormones* (1939) included in its Introduction the following paragraphs:

> Studies in absorption spectra interest chemists from many different points of view. Applied to diatomic molecules and the simpler polyatomic molecules, they yield valuable information on the intimate structure of the molecules, so that moments of inertia, internuclear distances, valency vibrations, valence angles and characteristic frequencies may be deduced. For the large polyatomic molecules with which this booklet is concerned information of such precision is at present out of the question.
>
> Spectroscopic research in this field is thus much more empirical, and its value lies in its use *as an adjunct* to biochemical and organic methods of study. In spite, however, of this serious limitation, the recent developments in knowledge concerning physiologically active substances owe much to spectral absorption curves in the two directions of elucidating photochemical changes and in providing characteristic 'labels' for substances the existence or importance of which rests on biological methods of experimentation. Vitamin research must always accept the animal test as the first and last court of appeal; the main service of absorption spectra lies in the possibility (which may not, of course, always eventuate) of supplementing the physiological description of an X-substance by means of a physical criterion capable of aiding in identification and analysis.

The Preface to the second edition (1942) acknowledged my debt to E. C. C. Baly and I. M. Heilbron, and to the Medical Research Council which, through Edward Mellanby, supported my investigations on vitamins A, D and E and, incidentally, introduced me to inter-laboratory cooperation and the committee-round, from which escape is so difficult. In this Preface I wrote:

> In the last twenty years the subject of Absorption Spectra has ceased to be a minor and auxiliary speciality within chemistry and has become a very versatile tool, almost indispensable in many fields of research. The subject thus affords a good vantage point. More and more workers, having other main interests, find it necessary to make use of absorption spectra, and they need a certain minimum acquaintance with the methods of experimentation and interpretation. Similarly, the spectroscopists (of whom there are several varieties) need to see their subject against a wider background. This book is a contribution to the work of liaison. It reviews many brilliant papers, distinguished by patient work, great skill and insight, but it is much more a record of small advances, the cumulative effect of which is prodigious in its implications. The great days of line spectra are over, with the developments of quantum theory in the first third of the century, sub-atomics has become neat

and tidy. Similarly, quantum mechanics is providing an adequate theory of the absorption spectra of simple molecules. A great clarification of the theory of large molecules has begun to take shape and in the next few years the study of absorption spectra will play its part in a process destined to affect the outlook of all chemists.

Much of the quotation needs no apology today, although the naive reference to sub-atomics must raise a smile. (My first war-time work concerned gas-warfare, which, to the leading chemists of the day, was a more immediate threat than atomic energy.)

At the head of the first chapter of the second edition was the following quotation:

> 'Les données numériques qui caracterisent l'absorption inégale des diverses lumières conduiront peut-être prochainement à une méthode d'analyse chimique universelle.'
>
> (BERTHELOT, *Science et philosophie*.)

In 1941 nobody could have foreseen how today ultraviolet and infrared spectroscopy, nuclear magnetic resonance and mass spectroscopy together with optical rotatory dispersion, circular dichroism and other techniques have lightened the labours of chemists and biochemists. Berthelot's hopes have come true.

I have often been asked by the Publishers and by many scientific friends to write a third edition. My failure to do so was due first to pre-occupation with the work of a long succession of research students to whom I owe much. In the second place it was due to the burden of service on committees academic, official and semi-official, and in particular to the task of reading and understanding the relevant literature. Such work is doubtless necessary and I have felt it to be part of the price the academic scientist has to pay for the privileges he enjoys.

A good deal of water has flowed under the bridges since 1942 and it seems that an introduction to many aspects of spectroscopy has become necessary in undergraduate courses for chemists, biochemists and, indeed, many biologists. Thanks to advances in electronics and in the design of instruments generally, users are now more concerned with what an instrument can do rather than how it does it. The making of measurements has become easier, quicker and more accurate than was formerly the case. Moreover, there is no lack of introductory textbooks dealing with the theory and practice of the main spectroscopic methods.

I have therefore in the present volumes used a new title and have aimed at displaying various spectroscopic approaches in action, sometimes concerted and at other times working selectively, in the solution of biochemical problems. The modern biochemist needs a very wide background knowledge in both biology and chemistry, but he cannot be a master of every trade. The designers of scientific instruments have relieved him of many tasks and specialists in physical methods have assembled a body of classified information which he can put to good use.

Those of us whose interest in biochemistry began some fifty years ago have seen immense advances and have enjoyed participating in them and in learning about them. I have tried in this book to *interest* the reader in both the biochemistry and the spectroscopy, but I have also borne in mind the worker in a particular field who needs rather full references to the literature.

In the past twenty-five years new Schools of Biochemistry have multiplied all over the world and the number of students has increased greatly. The prevailing pattern of biochemical research is a phase of surging advance leading to rapid and large-scale consolidation, followed by diminishing returns and redirection of effort. In preparing this book I found plenty of 'applied' problems requiring attention and holding out promise. A mature science has responsibility to undertake research that is necessary, timely and practicable. It is encouraging to know that very many biochemists are doing that kind of work and gaining satisfaction from it.

I am indebted to the Leverhulme Trust for the award of an Emeritus Fellowship which permitted me to continue writing after retirement. Mrs M. Hilditch acted as part-time secretary under this arrangement and her assistance has been invaluable. The late Professor R. J. Pumphrey kindly offered me a room in the Zoology Department and Professor A. J. Cain extended the hospitality. I have throughout had the use of the University Library and have often visited the Library of the Chemical Society at Burlington House. I wish also to thank the various societies and authors whose diagrams I have used and to apologize for any inadvertent failure to contact authors for permission. The sources of most diagrams are given in the figure captions, with a complete reference at the end of the chapter, but the following should also be included: Fig. 2.4, Golterman & Clymo (1967); Figs. 4.1–4.7, Morton *et al.* (1934); Fig. 4.10, Morton & Stubbs (1939); Figs. 4.11–4.23, Morton & Stubbs

(1940); Figs. 6.6–6.8, 6.11–6.12, Mayneord & Roe (1935); Fig. 8.9, Wetlaufer *et al.* (1959); Fig. 8.23, Bendit (1967); Figs. 8.30–8.31, Shifrin (1965); Figs. 9.6–9.11, Mason (1959); Fig. 9.14, Hearn *et al.* (1951); Figs. 13.24–13.25, Siegel *et al.* (1959); Fig. 15.18, Crawford & Jensen (1971); Fig. 16.10, Dowling (1960); Fig. 17.1, White *et al.* (1963); Figs. 17.3–17.4, Kishi *et al.* (1966).

Finally I must thank my wife for her tolerance and patience as well as her encouragement over the years.

The University of Liverpool

October 1974

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1 Introduction

1.1 Introductory

In recent years the task of establishing the structures of most new natural products has been made far easier than once seemed possible. High resolution mass spectroscopy leads to accurate molecular weights and sets definite limits to possible empirical formulae. Fragmentation patterns, nuclear magnetic resonance, infrared absorption, Raman spectra, ultraviolet absorption, optical rotatory dispersion and circular dichroism all provide evidence such that chemists today enjoy a flying start. X-ray crystallography too makes its own superb contribution.

The designers and makers of elaborate scientific instruments have greatly facilitated the gathering of reliable information. Most of the techniques are now explained, at least in principle, in undergraduate courses. Each physical method has, however, specialists thrusting into new territory and revealing more and more sophisticated aspects of structure and function. An important aspect of the new situation is that a good part of the effort once needed for recognizing and characterizing the molecules participating in complex biological processes is now deflected to elucidating modes of action. Biochemists should be able to interpret the information derived from a wide range of spectroscopic investigations. They will then be more free, and more competent, to clarify the roles of individual compounds in living organisms.

In the period immediately after the 1914–1918 War experimental work on infrared absorption spectra of organic compounds was a task for specialists. Coblentz had laid down firm foundations but serious technical difficulties persisted and even as late as 1938 it could be said that 'the technique for accurate work is neither easily nor cheaply acquired'. The advances in instrumentation which came later gradually reduced to simple routine much of the gathering of data.

The pioneers of absorption spectroscopy in the ultraviolet and visible regions (Hartley, Dobbie, Hantzsch, Baly and others) managed to obtain only semi-quantitative curves. They had to use photographic methods for recording spectral transmission by solutions of organic substances, and although the blackening of the developed photographic plate could be measured accurately, both theoretical and practical difficulties remained. In particular when the product It was constant (I = light intensity, t = time of exposure) the blackening was not constant. In fact a relation It^n = constant held, but n (the Schwarzschild exponent) showed considerable variation between different kinds of photographic plates. The advent of suitable photometers permitted reasonable accuracy to be achieved in the measurement of absorption spectra in the visible and ultraviolet regions. Twyman's sector photometer (invented just before the 1914–1918 War) came into wide use among specialists. The light source was a high tension condensed electric spark between metallic electrodes. A long-focus sector photometer was made specially for Baly's group at Liverpool so as to allow an intense arc between iron and nickel rods to be used instead of a spark. Various improved photometers

were devised (Judd-Lewis, Spekker) but the Twyman instrument proved easier to use. It was not theoretically impeccable (because of the Schwarzschild factor) but there was a compensatory intermittency effect on the blackening of the plate. The measurements remained tedious and some skill and experience was needed for $\pm 2\%$ accuracy in measuring extinction coefficients. The light sources moreover emitted line spectra, a fact which militated against the measurement of absorption displaying fine structure, particularly in vapour spectra. Light sources with continuous spectra were available for work in the visible and near-ultraviolet regions but there was still need for a stable light source having a spectrum continuous down to 200 nm. An early attempt in that direction was a condensed spark between aluminium electrodes under water. The spectrum was continuous but the spark was erratic and very noisy. An underwater spark between tungsten electrodes (Fulweiler & Barnes, 1922) was perfected at the Bureau of Standards by Brode. It involved a rotatory auxiliary spark gap in air and a spark under water actuated by a Tesla discharge at a very high voltage. This light source, in conjunction with a short-focus Twyman sector photometer, proved highly satisfactory apart from its noise and the fact that it was a source of electrical interference.

A hydrogen discharge tube associated with the names of Bay and Steiner was a further development. Different versions of hydrogen 'lamps' were all at first quite sizable and had to be connected to a source of (purified) hydrogen; they were however noiseless and provided a continuous spectrum down to 210 nm.

Considerable advances in electronics led to the use of small and highly reliable hydrogen 'lamps' which today form part of standard photoelectric spectrophotometers. They are now taken very much for granted but they were however the result of a prolonged effort to find the best light source for a new generation of spectrophotometers.

Visual spectrophotometery over the range 440–760 nm continues to have some advantages and the combination of the Hilger constant deviation spectrometer and the Nutting (or König–Martens) photometer has a fine record of work done. Nevertheless this technique is obsolescent and for many purposes recording spectrophotometers meet the needs better.

Photoelectric spectrophotometery began with very 'individual' assemblies using a mercury vapour lamp as light source and the work of von Halban (von Halban & Siedentopf, 1922) overcame numerous difficulties. Historians of the subject will find many other significant developments (cf. Suhrmann & Kollath, 1928; Warburg *et al.*, 1929). The work of Michaelson & Liebhafsky (1936) assisted Hardy in his important studies which led to recording ultraviolet spectrophotometers.

The appearance of the Cary & Beckman spectrophotometers, followed by many rather similar instruments, led to an enormous widening of the field of users of ultraviolet absorption. The manually operated photoelectric spectrophotometers were capable of an accuracy of $\pm 0{\cdot}2\%$ and this allowed a fresh approach to analytical work particularly in respect of correction for irrelevant absorptions. On the technical side Edisbury's book (1966) is of great value to new entrants to this field.

With respect to infrared spectroscopy applied to organic substances, here too remarkable advances in instrumentation have been made with parallel developments in the interpretation of spectra. Rock salt prisms remain widely used although prisms of KBr, LiF, CaF_2 and CsBr are also used. Prism-grating spectrophotometers and precision-grating spectrometers are now available. Selection of an instrument depends on the nature of the problem to be tackled. Some relatively cheap recording spectrophotometers are easy to use and maintain and give excellent service for semi-quantitative work, while the better performance of more expensive instruments meets special needs. The literature distributed by manufacturers is very helpful and most centres of organic or biochemical research now have workers experienced in the field. Infrared instrumentation is discussed briefly by Rao and more fully by Conn & Avery (1960), Goddu (1960) and West (1960), and many others.

Nuclear magnetic resonance was first observed in 1946 but its advantages in the study of organic structures only began to emerge in 1953 (Meyer *et al.*). In twenty years a vast amount of work has been done and the technique has undergone progressive improvements. The main reasons for this effort are that the information provided is different from, but complementary to, that obtained by ultraviolet and infrared investigations. Nuclear magnetic resonance (n.m.r.) has added a new dimension to structural studies. In many problems it can be used easily but the subject has complications demanding subtlety and expertise.

At the time that Aston carried out his early work on mass spectra, the notion that a molecular weight could for example be observed as 346·177 and calculated to