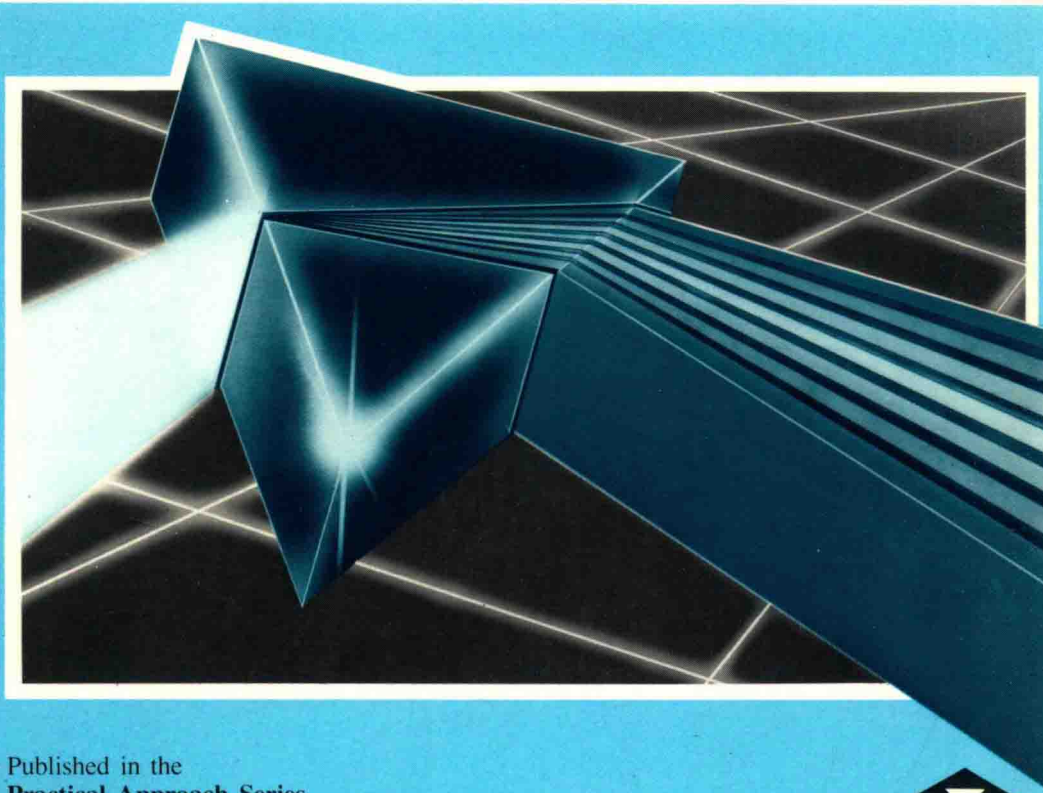
Spectrophotometry & spectrofluorimetry

a practical approach

Edited by
D A Harris & C L Bashford



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Preface

The spectrophotometer on the bench is a familiar feature of any biochemistry laboratory. It requires no great skill to use, is sensitive enough to handle materials at physiological concentrations and, best of all, it produces immediate data. As is so often the case, however, familiarity can lead to contempt – too many spectrophotometers are used without sufficient care, are rarely serviced or calibrated and, often, are underused (or even misused) in terms of the facilities they provide.

This book, therefore, is intended to help the reader get the most out of his spectrophotometer and its close relation, the spectrofluorimeter. Applications are described to the characterisation and quantitation of both small and large molecules (photometric assays), to the investigation of intermolecular interactions (ligand binding studies) and to the study of molecular conversions (monitoring chemical reactions) – these both in equilibrium (static) and changing (kinetic) systems.

At every stage, emphasis is placed on the capabilities and limitations of the instrument in use – how to select a machine for a given task, how to check if it is working satisfactorily, and what to do if it fails to produce the expected data. Applications of single, dual and multiple (spectral) wavelength modes of measurement are described, and their respective advantages explained. Chapter 5 also demonstrates the use of the microscope for measurements typically considered the domain of the conventional spectrophotometer or fluorimeter.

It is assumed throughout that commercially available instruments will be used by the reader – those who design a dedicated spectrophotometer for a specific task are unlikely to require this text to help them use it. Some possible workshop modifications of commercial spectrophotometers (e.g. low temperature and rapid mixing attachments) are however described. In some cases commercial instruments are named; the reader should note that in such cases, the specific instrument should be taken as representative of a class and that other manufacturers may well supply comparable instruments.

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D.A.Harris and C.L.Bashford

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Abbreviations

ABTS	2,2′-azino-di-(3-ethylbenzthiazoline)-6-sulphonate
ADH	alcohol dehydrogenase
ANS	1,8-anilino naphthalene sulphonate
diO-C_5-(3)	3,3′-dipentyloxacarbocyanine
FA	fatty acid
FCCP	carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
FP	flavoprotein
GOD	glucose oxidase
G6P	glucose-6-phosphate
GPDH	D-glyceraldehyde-3-phosphate dehydrogenase
G6PdH	glucose-6-phosphate dehydrogenase
HK	hexokinase
Hoechst 33258	[(2-[2-(4-hydroxyphenol)-6-benzimidazolyl-6(1-methyl-4-piperazyl) benzimidazole]
IF-3	initiation factor 3
αKG	α ketoglutarate
LDH	lactate dehydrogenase
MOPS	morpholinopropane sulphonic acid
OA	oxaloacetate
oxonol V	bis[3-phenyl-5-oxoisoxazol-4-yl]pentamethineoxonol
oxonol VI	bis[3-propyl-5-oxoisoxazol-4-yl]pentamethineoxonol
P_i	inorganic phosphate
PBS	phosphate buffered saline
PEP	phosphoenolpyruvate
6PG	6-phosphogluconate
2PGA	2-phosphoglycerate
PGK	phosphoglycerate kinase
PK	pyruvate kinase
PN	pyridine nucleotide
POD	peroxidase
TCA	trichloroacetic acid
thio ITP	6-mercaptopurine riboside-5′-triphosphate
TIM	triose phosphate isomerase

Contents

CHAPTER 1

An Introduction to Spectrophotometry and Fluorescence Spectrometry

C.LINDSAY BASHFORD

1. GENERAL CONSIDERATIONS

All biochemicals absorb energy from at least one region of the spectrum of electromagnetic radiation. The energies at which absorption occurs depend on the available electronic, vibrational and rotational energy levels of the molecule. When absorption is from the u.v./visible region of the spectrum (200−700 nm), transitions occur between electronic energy levels, and these electronic transitions form the physical basis for the techniques described in this volume. Spectrophotometry and fluorescence spectrometry (spectrofluorimetry) involve the measurement of these transitions in precise, analytical procedures which permit the characterisation and quantification of (biological) molecules.

A simple appreciation of the fundamental processes occurring when radiation interacts with matter is useful for understanding the operation of spectrophotometers, and is given below. However, a detailed theoretical understanding of these processes is not required for the laboratory application of photometric techniques. Readers interested in these aspects should consult physical chemistry texts.

1.1 **Absorption of Light**

Molecules absorb energy only when the incident photon has an energy precisely equal to the difference in energy between two allowed states, the photon promoting the *transition* of an electron from the lower to the higher energy state. Before another photon can be absorbed, the excited state must lose this energy and revert to the ground state. Commonly, this reversion is rapid ($< 10^{-12}$ sec) and occurs by loss of energy to vibrations and rotations within the same molecule and, by collision, to other molecules (especially the solvent). In short, energy is lost to the environment as heat. The rapidity of reversion is such that, at moderate light intensities, the number of photons absorbed is proportional to light intensity, and constant in time.

If the exciting beam is particularly intense, as it can be with laser light sources, the excitation rate may exceed the rate of decay of the excited state. The number of photons absorbed from a beam of given intensity will thus fall in time as the number of ground state molecules falls − a phenomenon known as *photobleaching*. Such intense sources are thus avoided in the measurements described here; the fraction of molecules in the ground state remains close to one (>99%) and absorption is constant with time.

Another possible cause of photobleaching is a chemical reaction of the excited state.

The chemistry of excited state molecules may differ from that of ground state molecules – they are in general more reactive – and during intense illumination unexpected 'photochemical' reactions may occur leading to incorrect measurements and, at worst, destruction of a valuable sample. However, light sources for absorbance measurements (see below) are rarely sufficiently intense to cause problems of this type. In favourable circumstances the wavelength-dependence of 'photochemical' reactions will provide useful 'action spectra' of complex systems (see Chapter 7).

1.2 **Emission of Light**

In some molecules, particularly rigid conjugated systems, loss of energy from the excited state by vibration or rotation may be slow. In this case, the excited state may lose energy, in addition, by radiative emission i.e. by emitting a photon. If emission is from a singlet excited state, this process is known as fluorescence; if from a triplet state, it is phosphorescence. For observable fluorescence, the lifetime of the excited state must be about 10^{-9} sec, and for phosphorescence it must be about 10^{-3} sec. Clearly, for radiative decay to compete significantly with energy loss as heat, vibrations and rotations within the excited state must be severely restricted to prolong its lifetime.

The competition between radiative and non-radiative decay means that fewer photons are emitted by a collection of molecules than are absorbed; the *quantum yield* (Q_f; Section 3.2) of fluorescence or phosphorescence is less than unity. In addition, during the lifetime of the excited state, some non-radiative loss of energy generally occurs to the environment before emission of the bulk of energy as a photon. This results in the *energy* of the emitted photon being lower than that of the absorbed photon; fluorescence or phosphorescence is at longer wavelength than the corresponding excitation. Factors affecting excitation and emission spectra are discussed more fully in Chapter 2.

While all molecules absorb photons, relatively few fluoresce or phosphoresce significantly at room temperature, so these latter properties are especially useful for resolving minor components in complex mixtures. Furthermore, the high sensitivity of photodetectors and the ability of monochromators or filters to resolve incident from emitted light makes fluorescence, particularly, an exquisitely sensitive analytical procedure. Nanogram amounts of fluorophores can usually be assayed fluorimetrically.

2. ABSORBANCE SPECTROPHOTOMETRY

2.1 **Types of Spectrophotometer**

All spectrophotometers comprise the following elements:

(i) A light source which provides illumination of the appropriate wavelengths. The most common lamps used are tungsten–halogen, for use between 350 and 900 nm, and deuterium, for the u.v. region (200–400 nm). Arc lamps, either of xenon or of mercury, usually contain lines of too great an intensity or fluctuate too much to be commonly employed in absorbance spectrophotometers.

(ii) A device, usually a monochromator or an optical filter, which selects the precise wavelength of interest. In most instruments wavelength selection occurs between the lamp and the sample; in a few instruments, such as the Hewlett-Packard 8450,

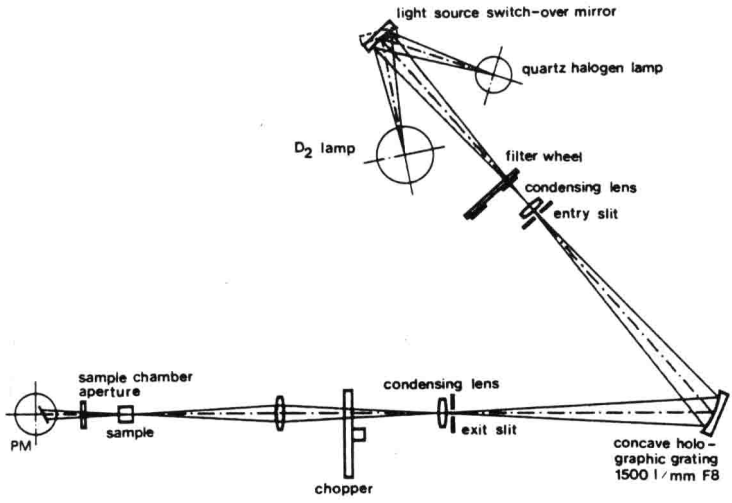

Figure 1. Optical diagram of a single beam spectrophotometer (UVIKON 610/710). PM represents the photomultiplier tube.

wavelength selection occurs between the sample and the detector (an arrangement described as reversed optics).

(iii) A compartment to house the sample to be studied.

(iv) A detector, usually a photomultiplier or a silicon diode, which measures the amount of light transmitted by the sample.

Commercially available photometers incorporate all these features in three main configurations.

2.1.1 *Single Beam Instruments*

These are the simplest type of spectrophotometer. A typical optical diagram of such an apparatus is shown in *Figure 1*. The chopper allows light to illuminate the sample (and the photodetector) intermittently (at a known frequency) and allows the incorporation of a.c. amplifiers into the electronic circuits. Such amplifiers have a better performance than the d.c. devices used if the chopper is omitted. Single beam machines have a single position for sample and reference material. The apparatus is zeroed and standardised with the reference material in the sample position and this is then removed before the sample is studied. Such apparatus is useful for routine assays, for example those described in Chapter 3, where measurements are required, at a single wavelength, of samples and standards. The most important requirement in single beam instruments is that the source output be stable, as changes in transmitted intensity due to variations in source intensity are not compensated.

2.1.2 *Double Beam Instruments*

Corrections for variations in source intensity can be made automatically if the excitation beam is divided between reference and sample materials. This is the strategy adopted by double beam instruments. An optical diagram of such an apparatus is shown in *Figure 2* and a three-dimensional view of the same apparatus is shown in *Figure 3*.

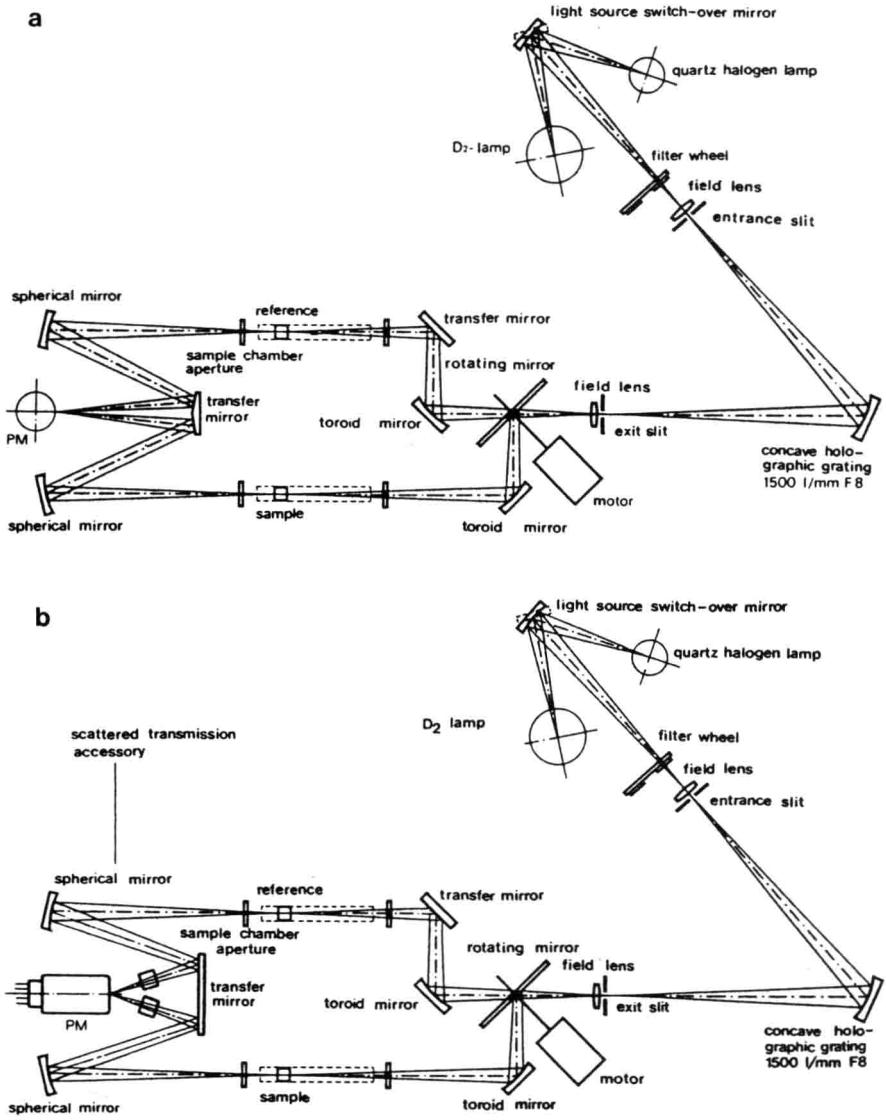

Figure 2. Optical diagrams of double beam spectrophotometers. (**a**) UVIKON 810/860 spectrophotometer with the sample and reference cuvettes in the conventional position. (**b**) UVIKON 810/860 spectrophotometer modified for use with turbid samples. Note that the cuvettes are placed much closer to the photomultiplier (PM) in this configuration.

The essential point is that light of the same wavelength illuminates both the sample and the reference material. In the system illustrated in *Figure 2* the beam is switched from sample to reference by the chopper; the optics for both the sample and the reference chambers are focussed onto the same area of the photodetector (to ensure that each is monitored with the same sensitivity) and a signal from the chopper instructs the electronics as to whether the sample or the reference position is being interrogated. It is