PHOTOELECTRIC METHODS IN CLINICAL BIOCHEMISTRY

by

G. E. DELORY M.Sc., Ph.D.

Assistant Professor of Biochemistry
University of Manitoba, Winnipeg, Canada
Formerly Biochemist, Royal Infirmary, Preston, Lancashire, England

WITH A FOREWORD BY

PROFESSOR E. J. KING M.A., Ph.D., D.Sc.

洪盛書店

上海虹橋路三七五弄五號

1949

PHOTOELECTRIC METHODS IN CLINICAI BIOCHEMISTRY 1949

原著者 DELORY

影印發行者 洪盛書店 上海虹橋路 375 弄 5 號

印刷者 祥泰印刷廠

裝訂者 文江製本所

照相者 克勤印務局

定價 (白道林紙)25,000

一九五四年四月印 (1-200)

ERRATA

"Photoelectric Methods in Clinical Biochemistry"
by G. E. Delory, M. Sc., Ph. D.

Page 58. Reagents Required.

(3) Aminonaphtholsulphonic acid reducing agent *should read :—*

" Dissolve 0.1 g. of 1-2-4 aminonaphtholsulphonic acid with 6 g. of sodium metabisulphite and 1.2 g of crystalline sodium sulphite in 50 ml. of water, filtering, if necessary. Do not use after two weeks."

also

Page 44, line 7. For " creetinine " *read* " creatinine."

Page 62. Procedure—*line 1. For* " blood " *read* " serum."

Page 64. Principle—*line 2. For* " washing " *read* " ashing."

Page 70, 4th line from bottom—" Add 2 ml. of concentrated sulphuric acid . . . . "

should read :—

" Add 21 ml. of concentrated sulphuric acid . . . . "

Page 84. Notes (b)—*line 3, delete* " 0.5 ml. of."

FOREWORD

by Professor E. J. KING M.A., Ph.D., D.Sc.

THE art of the analyst has been practised for many centuries ; and has followed many directions. From the first simple weighings of crude substances to the refined modern measurements of micro quantities of accurately characterized derivatives, of chemical reactions, and of various properties of matter, analysis has been dependent on its tools. The analytical balance, liquid and gas burettes, potentiometers, polarimeters and polarographs, colorimeters and spectrophotometers for the ultraviolet, visible and infra-red, all have contributed to the development of analysis from an art into a scientific discipline. For beauty of performance as well as precision, and for ingenuity of conception and design of his instruments, the analyst is as well served as any modern scientist or technologist.

Chemical developments in analysis have been not less important than instrumental. New and complex reactions have been devised which are capable of accurate assessment in a variety of ways, and the quantities of material necessary for measurement have become less and less. The present-day emphasis on micro-analysis has been made possible not only by the manufacture of instruments which can measure smaller quantities more accurately, but by careful exploration of the conditions under which chemical reactions can be counted on to go to an invariable conclusion and to yield well-defined products, by the discovery of reactions which yield multiple quantities of analyzable product, and by the invention of means to measure properties of the substances being analyzed or of their products in chemical reactions. There is a limit to the amount the present micro-balance will measure, but it is possible to titrate smaller quantities than we can weigh, and by using such reactions as that of the iodate method for chloride it is possible to multiply the size of the titration several times. Colorimetric analysis often pushes the limit still further, in that the amount of a substance necessary to yield a measurable colour may be much less than that which can be weighed or titrated ; and with those substances which fluoresce it is possible to estimate their concentration at dilutions so high that their colour would not be apparent.

The instruments described in this volume have been developed by Messrs. Hilger and Watts Ltd. to enable these two last branches of analysis, colorimetric and fluorimetric, to be applied with greater ease

and accuracy than has been possible in the past with visual instruments, and to increase their scope of application in new fields. Colorimetric methods are not only often more sensitive than other forms of analysis, they are quicker and simpler to execute; and with instruments operating on the photometric or absorptiometric principle they may be quite as accurate as many of the classical gravimetric and volumetric procedures. Indeed, most of the criticism which used to be levelled at colorimetric analyses is no longer valid, because it is now possible to measure colour as accurately, or nearly as accurately, as we can measure any other property of matter.

Over the past several years the number of photoelectric colorimeters in use in hospital biochemical laboratories has steadily increased and their advantages over instruments of the Duboscq type have become increasingly realized. This book has, therefore, been written in the hope that it will be of use to the workers in such laboratories.

Its aim is threefold: (1) to give in as simple fashion as possible the theoretical background needed for the intelligent use of photoelectric colorimeters, (2) to give descriptions and instructions for the use of the two instruments manufactured by Messrs. Hilger and Watts Ltd. namely the Spekker Absorptiometer and the Biochem Absorptiometer, and (3) to give practical details for the colorimetric procedures most commonly undertaken in clinical biochemical laboratories.

The methods are based on those which have been used with marked success in the routine biochemical laboratories of the Postgraduate Medical School of London and the Preston Royal Infirmary. The author, Dr. G. E. Delory, has contributed much valuable data, and many useful procedures, modifications and inventions to British analysis in the biochemical field. It is a pleasure to record my appreciation of his sustained and valuable efforts, and of this book of biochemical analysis which should have a wide usefulness as a manual of colorimetric methods for use with photoelectric instruments.

E. J. KING

PREFACE

THE part played by colorimetric analysis in the progress of biochemistry needs no emphasis. The methods are, on the whole, simpler than gravimetric or volumetric procedures and require smaller quantities of the material to be analyzed; an obvious advantage where we are dealing with body fluids.

By means of the modern photoelectric colorimeter, which is rapidly superseding the older visual instruments, colorimetric measurements can now be conducted with a greater degree of precision and even more simply.

The first three chapters of this book deal with the theory of colorimetry, chapters four and five describe and give directions for the use of the two instruments manufactured by Hilger and Watts Ltd., namely the Spekker Absorptiometer and the Biochem Absorptiometer, while the final chapter gives details of recommended analytical procedures.

In the theoretical and descriptive sections, the aim has been to provide as simply as possible the background needed to enable the analyst to use his photoelectric colorimeter to the best advantage.

The analytical procedures given are mainly adaptations of the microchemical methods developed by Professor King and his colleagues which have proved their value in the course of routine hospital biochemical analysis. Typical calibration curves have been given for most of the methods described, since, although the relation between the reading and the concentration of the substance being analyzed may vary with the particular instrument being used, it is felt that the inclusion of such curves will be of value to the analyst as an indication of the kind of readings he may expect to find.

A word should be said on the question of terminology. Since we are concerned here with the measurement of light absorption rather than colour the writer would support those who seek to replace the terms colorimetry and colorimeter with photometry and absorptiometer respectively, but he feels that, since the former terms are so firmly established in the routine hospital laboratory, they should be retained in a manual such as this at least until the underlying principles are more widely appreciated.

A book of this type owes much to the knowledge garnered by many workers in various countries and published over a period of many years.

It is clearly impossible to refer to all these publications, but every effort has been made to give an adequate number of references from which earlier work, not specifically mentioned, may be traced.

It is a pleasant duty to thank those who have helped at one stage or other in the preparation of this manuscript. First and foremost, thanks are due to Professor E. J. King for editing the manuscript, writing the foreword, and for all the help and encouragement he has given me during the thirteen years we have been associated together.

Among the other people I wish to thank are the following :

Mr. F. Twyman of Hilger and Watts Ltd., London, at whose suggestion the book was written.

Mr. H. G. Bevan of the City Hospital, Nottingham, who has read through and commented in detail on the whole manuscript. Mr. Bevan has also compared the calibration curves given here with those for the Spekker Absorptiometer in his possession.

Mr. F. Keyser of the Postgraduate Medical School, London, who has compared the calibration curves given here with those obtained with the Spekker Absorptiometer and Biochem Absorptiometer in Professor King's department.

Dr. F. L. Warren of St. Mary's Hospital, London, and Dr. C. A. Mawson of the Royal Berkshire Hospital, Reading, for advice on the ketosteroid method. Dr. Warren has also read through the section on multiple absorption.

Mr. D. Livingstone Ward for technical assistance.

Dr. F. B. Smith, formerly of the Royal Infirmary, Preston, whose kindness and encouragement have done much to make the preparation of this book easier.

And finally, Miss B. Whalley for her willing, patient and competent secretarial assistance.

Grateful thanks are also due to :

Messrs. Evans Electro Selenium Ltd. for permission to quote from their advertising matter and for the loan of the blocks illustrating the principles and properties of the EEL photocell, and to

Messrs. Chance Ltd., and Messrs. Ilford Ltd., for permission to reproduce particulars of the transmission properties of their respective light filters.

G. E. DELORY

CONTENTS

LIST OF ILLUSTRATIONS

Photoelectric Methods in Clinical Biochemistry

CHAPTER I

THE PRINCIPLES OF COLORIMETRY

Introduction.—Colorimetric procedures have long enjoyed a well merited popularity in the analysis of body fluids, since they are time-saving and easy to carry out, require small amounts of material, and possess an accuracy well within the limits required for most purposes. In principle, such methods depend on the measurement of the amount of colour produced during a chemical reaction in which the test substance takes part.

For the most accurate work, this measurement is carried out by studying the amount of light absorbed by the coloured substance under standard conditions. Strictly speaking, therefore, the estimations to be described here should be called "photometric" or "absorptiometric" rather than *colorimetric* methods, but the term *colorimetry* has established itself so firmly in the routine biochemical laboratory that it will be used in this sense throughout this book. Similarly, it has seemed desirable to retain the term *photoelectric colorimeter* except when referring specifically to the two Hilger instruments which are manufactured under the names of the *Spekker Absorptiometer* and the *Biochem Absorptiometer*.

With these reservations, the terminology and symbols recommended by the publications committee of the Society of Public Analysts have been adopted.

Laws of Light Absorption.—The two fundamental laws are

(i) *Lambert's law*, which states that the proportion of light absorbed by a substance is independent of the intensity of the incident light; and

(ii) *Beer's law*, which states that the proportion of the light absorbed depends only on the total number of absorbing molecules through which it passes, independently of their concentration.

The following simple but important fact follows from Lambert's law: Suppose that a beam of light is passing into a solution of an absorbing medium. We can think of the total path as being made up of a whole series of paths of equal lengths. As the light passes through each layer,

a certain amount of light is absorbed and consequently the intensity of the incident light reaching each successive layer will be progressively less. But since, from Lambert's law, the proportion of light absorbed is independent of the intensity of the incident light, and since by definition, each layer is identical, it follows that each successive layer of the medium absorbs an equal proportion of the light passing through it.

If the light is passing through a given depth of solution of a coloured substance in a non-absorbing medium, then, from Beer's law, the proportion of the light absorbed is dependent upon the concentration of the coloured substance.

The next section gives a mathematical treatment of the laws of light absorption. Readers not familiar with the integral calculus should omit this and accept equation (6) as the fundamental equation of colorimetry.

Mathematical Treatment of Lambert's and Beer's Laws.—Suppose that a beam of monochromatic light of intensity I passes into an infinitely small thickness dl of a solution of an absorbing substance of molecular concentration c expressed in gram-molecules per litre. Then, if the amount of light absorbed be dI,

$$\frac{dI}{I} = -kcdl, \tag{1}$$

where k is a constant for a given wavelength*.

In order to determine the amount of light absorbed by a layer of thickness l, the intensity of the incident light being I_0 and that of the emergent light I, it is necessary to integrate this expression between the given limits. We then have

$$\log_e \frac{I}{I_0} = -kcl. \tag{2}$$

This may be transformed to give

$$I = I_0 e^{-kcl}; \tag{3}$$

or logarithms to the base 10 may be substituted, giving

$$\log_{10} \frac{I}{I_0} = -\epsilon cl, \tag{4}$$

where ϵ is a constant differing in value from k because of the change to common logarithms. Eliminating the negative sign,

$$\log_{10} \frac{I_0}{I} = \epsilon cl. \tag{5}$$

* The amount of light absorbed will clearly be dependent upon the wavelength of the light, and consequently the so-called constants will have different values with different wavelengths of the light.

Expressing the concentration of the absorbing substance in milligrams per 100 millilitres instead of in gram-molecules per litre and calling this concentration C, we have

$$\log_{10}\frac{I_0}{I} = E_s Cl, \tag{6}$$

where E_s is another constant such that $\epsilon c = E_s C$.

This equation (6), which is of fundamental importance, may be stated as follows:

When light passes into a solution of an absorbing substance in a non-absorbing solvent, the logarithm of the ratio of the intensity of the incident to that of the transmitted light is directly proportional to the depth of the solution and the concentration of the absorbing substance.

Technical terms in common use:

The *optical density* d, also called the *extinction* E, is the logarithmic ratio of the intensity of the incident light to that of the emergent light; or, in mathematical symbols,

$$d = \log_{10}\frac{I_0}{I} = E_s Cl = \epsilon cl$$

The *extinction coefficient* K * is the optical density when the layer of solution is 1 cm. thick; consequently

$$K = E_s C = \epsilon c.$$

The *molecular extinction coefficient* is the optical density when the layer of solution is 1 cm. thick and the concentration of the absorbing substance is one gram-molecule per litre. This term is by definition ϵ in equation (5).

The *specific extinction coefficient* † E_s may be defined as the extinction coefficient for unit concentration. In medical biochemistry it is convenient to take 1 mg. per 100 ml. as the unit of concentration, although in other branches 1 g. per 100 ml. may be used as the unit.

* Some workers in the U.S.A., e.g. Michel and Harris (1940), call this term the *transmissive index*.

† In characterizing a particular coloured substance it is better to record the extinction and to state the concentration of the coloured substance and the cell depth.

Thus $E_{1\text{cm.}}^{1\text{mg./100ml.}}$ would be the extinction of the given coloured substance in a concentration of 1 mg. per 100 ml. and in a layer of 1 cm.

The term *transmission* is often used. This is defined as the ratio of the intensity of the transmitted to that of the incident light: thus

$$T = \frac{I}{I_0}.$$

Transmittance, a term widely used in the U.S.A., may be defined as the ratio of the transmission of a cell containing the coloured solution to that of an identical cell containing water or a " blank " solution.

Multiple Absorption.—If we have two coloured substances present in the same solution, then, providing that there is no chemical interaction, the observed extinction coefficient will be equal to the sum of the extinction coefficients of the two substances taken separately. This fact is of value for calculating the concentration of one substance in the presence of another.

The estimation of blood creatinine will be taken as an example. In this method, advantage is taken of the fact that creatinine gives an orange colour with alkaline sodium picrate. It is, however, necessary to use an excess of the reagent, which is itself coloured. Allowance can be made for this by preparing a " blank " solution, that is to say a solution containing the same quantity of reagent but without creatinine, and measuring the colour of this solution at the same time as that of the test solution.

Now, if K be the extinction coefficient of the creatinine–picrate coloured complex, and K_T and K_B the observed extinction coefficients of the " test " and " blank " respectively, then

$$K_T = K + K_B \qquad (7)$$

Thus a more accurate measure of the creatinine concentration may be made by subtracting the extinction coefficient * of the blank from that of the test. This can be done automatically in the photoelectric colorimeter by placing the blank solution in the glass cell or test-tube instead of the water normally used to set the instrument.

This principle may be extended to determine the concentration of one substance in the presence of another where it is not possible to prepare and read a blank.† Let $K_{X,a}$, $K_{X,b}$, $K_{Y,a}$, $K_{Y,b}$, be the extinction coefficients of substances X and Y at wavelengths a and b respectively, and

* Since the extinction coefficient is the optical density for unit depth of fluid, the colorimetric readings may be substituted here, when the photoelectric colorimeter is used.

† For references to some of the original papers and for some applications to the study of blood pigments see Michel and Harris (1940)

$K_{M,a}$ and $K_{M,b}$ the extinction coefficients of the mixture at these wavelengths. Then

$$K_{M,a} = K_{X,a} + K_{Y,a} \tag{8}$$

and

$$K_{M,b} = K_{X,b} + K_{Y,b}. \tag{9}$$

If, $\dfrac{K_{X,a}}{K_{X,b}} = R$ and $\dfrac{K_{Y,a}}{K_{Y,b}} = S$

substituting, $K_{M,a} = K_{X,a} + SK_{Y,b}$ and $K_{M,b} = \dfrac{K_{X,a}}{R} + K_{Yb}$.

Eliminating $K_{Y,b}$, $\dfrac{K_{M,a} - K_{X,a}}{S} = \dfrac{RK_{M,b} - K_{X,a}}{R}$;

whence,

$$K_{X,a} = \frac{R(SK_{M,b} - K_{M,a})}{S - R} \tag{10}$$

This means that if the ratios of the extinction coefficients for two wavelengths be known for two substances taken separately, the observed extinctions of the mixture can be correlated and the concentrations of the constituents calculated.

This method has been employed for the estimation of 17-ketosteroids in urine (see p. 50). The ratio of the extinction coefficients in the green and violet has been found to be 2·2 (Talbot *et al.*, 1942) for pure androsterone and 0·6 for interfering substances. Substituting these figures in equation (10) we have

$$K_{X,a} = \frac{2{\cdot}2(0{\cdot}6K_{M,b} - K_{M,a})}{0{\cdot}6 - 2{\cdot}2} = \frac{K_{M,a} - 0{\cdot}6K_{M,b}}{0{\cdot}73} \tag{11}$$

Thus by reading the mixture with the spectrum violet and the spectrum green filters the corrected green reading may be calculated by subtracting six-tenths of the reading in the violet from the reading in the green and dividing this result by 0·73. This corrected green reading may then be used to calculate the concentration of urinary 17-ketosteroid.

Validity of the Laws of Light Absorption.—Until it became the practice to carry out colorimetric measurements with monochromatic light, the view was widely held that the laws of light absorption were true in an ideal sense only over a restricted range. For example when using the Duboscq colorimeter, analysts were careful to apply Beer's law only when the test and standard solutions were not markedly different in concentration. When there was likely to be a marked variation in the concentration of the tests it was customary to prepare

a series of standard solutions and to read each test against that standard nearest in concentration to it. With the development of more accurate procedures for colour measurement, it has become evident that the amount of light absorbed by a coloured solution is proportional to the concentration of the coloured substance over a remarkably wide range, provided that the wavelength of the light in which the analysis is conducted is carefully chosen.

There still remain certain cases in which the absorption does not closely follow the course predicted by the Beer-Lambert law, but these anomalies are probably due to changes in the absorbing molecules themselves and not to invalidity of the law. For further discussion on this point and for references, see Twyman and Allsopp (1934).

The laws of light absorption are, of course, applicable only to monochromatic light, and may apply only approximately to those instruments which use a whole band of wavelengths.

CHAPTER II

COLORIMETRIC MEASUREMENTS

Introduction.—The previous chapter has dealt with the relationship between the amount of light absorbed by a coloured solution and the concentration of the coloured substance. We now come to the application of these principles to quantitative analysis.

The history of the colorimetric analysis of substances of biochemical interest dates back over a hundred years, to the estimation of ammonia by Nessler using his well-known mercuric-iodide reagent. It was, however, the system of colorimetric analysis developed by Folin and Wu in the second decade of this century that popularized these methods for the analysis of blood and other biological fluids. Colorimetric methods of analysis are especially applicable to such material, since not only is the amount of substance to be estimated often very small, but it is clearly desirable to be able to carry out estimations on blood with minimal quantities. Side by side with the development of analytical techniques, there has been progressive improvement in the design of instruments for colour measurement, upon which the value of these methods depends.

Instruments for Colour Measurement.—The *Duboscq type of colorimeter*, which has been the instrument most commonly used for colorimetric analysis in the past, enables a comparison to be made of the colour intensities of two solutions. A beam of light divides into two equal parallel paths, one of which is allowed to pass through a standard and the other through a test solution. The two beams emerge into an eye-piece, where they are seen as equal halves of a circular field. The standard solution is now set at a known depth, and the depth of the test solution is varied until the two colours appear of equal intensity to the eye.

The *Pulfrich photometer* has been used with great success by a number of biochemists, although it has never achieved popularity in Britain. As with the Duboscq colorimeter, the light divides into two paths, one of which passes through a known depth of the coloured solution and the other through the same depth of water. Between the light source and the eye a filter is placed to render the light monochromatic. By adjustment of a diaphragm the amount of light reaching the eye from the water side is cut down, until the two halves of the field appear of equal brightness. The diaphragm is calibrated to give the optical density of the coloured solution, from which the concentration of the chromogenic substance may be calculated (see p. 3).