The QUANTITATION OF MIXTURES of HEMOGLOBIN DERIVATIVES BY PHOTOELECTRIC SPECTROPHOTOMETRY

FRANCIS T. HUNTER, M.D.
Associate in Medicine
Harvard Medical School
Associate Physician and Clinical Pathologist
Massachusetts General Hospital
Boston, Massachusetts

The methods for the quantitation of pigment mixtures using narrow wave bands in the visible spectrum described in this book should effect a considerable saving in time and labor for the laboratory worker. These methods require a minimal number of chemical procedures, and they are of the simplest sort.

To lessen the probability of error, volumetric pipetting has been reduced to a minimum. More important, the need for cumbersome apparatus, requiring prolonged chemical treatment or resulting in fleeting color reactions or time delays, is eliminated. 248 pages, 55 illustrations.

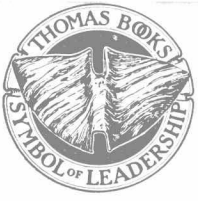
CHARLES C THOMAS • PUBLISHER • SPRINGFIELD • ILLINOIS

THE QUANTITATION OF MIXTURES OF HEMOGLOBIN DERIVATIVES BY PHOTOELECTRIC SPECTROPHOTOMETRY

By

FRANCIS T. HUNTER, A.M., M.D.

Associate in Medicine, Harvard Medical School
Associate Physician and Clinical Pathologist
Massachusetts General Hospital
Boston, Massachusetts



CHARLES C THOMAS · PUBLISHER
Springfield . Illinois . U . S . A .

CHARLES C THOMAS · PUBLISHER

BANNERSTONE HOUSE
301–327 EAST LAWRENCE AVENUE · SPRINGFIELD · ILLINOIS · U.S.A.

Published simultaneously in The British Commonwealth of Nations by
BLACKWELL SCIENTIFIC PUBLICATIONS, LTD., OXFORD, ENGLAND

Published simultaneously in Canada by
THE RYERSON PRESS, TORONTO

First Edition

Printed in the United States of America

PREFACE

BECAUSE OF THE increasing employment of photoelectric methods in biological chemistry, it was thought that a small handbook setting forth the principles underlying the quantitation of pigment mixtures in general, and of mixtures of certain hemoglobin derivatives in particular, might be useful. In the present work, no attempt has been made to cover exhaustively the whole subject of spectrophotometry, as such an ambitious project would not only duplicate existing published material, but would frankly be beyond the author's capabilities. It is hoped, however, that the mathematical and geometric fundamentals of spectrophotometric quantitation of pigment mixtures have been detailed clearly enough to enable the laboratory worker to develop procedures and to construct conversion charts and nomograms suited to his own needs.

It seems strange that nomography has been so little used in the field of medicine, but perhaps that is because most individuals instinctively recoil from even the simplest mathematical processes. Be that as it may, by utilizing this graphic method, once the necessary calibrations have been made and nomograms constructed, the simultaneous quantitation of two or more pigments reduces to the simple task of reading a scale and drawing a straight line—a task demanding certainly no more than average ingenuity.

With the exception of the war period, the data here presented have been accumulated over the past several years—chiefly at odd moments and as biologic material became available. The quantitation of oxygen saturation, however, had to await the development of a method for the anaerobic dilution of blood samples. As might be surmised, this was preceded by a discouragingly long series of failures. It became obvious, finally, that dilutions could only be made in a closed system, and the apparatus described is the result. Despite its imperfections, from a physical point of view it is believed to be basically sound.

The opportunity cannot be allowed to pass without thanking

those who have so generously contributed material, suggestions, and criticisms. To the Macalaster-Bicknell Company of Cambridge for one of the line sketches (Fig. 3) and construction of the diluting apparatus, and to the Central Scientific Company for frequent technical assistance, a debt is owed. Thanks are due to Dr. William J. Brickley, Medical Examiner for Suffolk County, Northern Division, and to Dr. Joseph T. Walker, State Police Chemical Laboratory, Massachusetts Department of Public Safety, for the opportunity of quantitating a number of blood specimens for carbon monoxide. Dr. Walker also was kind enough to read the first section of the manuscript, and has made valuable criticisms and suggestions.

Dr. M. N. States of the Central Scientific Company has been more helpful than he realizes. In addition to reading the manuscript and checking the mathematics, by his calling attention to Heilmeyer's monograph, embarrassing errors in the quantitation of bilirubin have been avoided. His suggestions and material assistance in regard to the spectrophotometric instrument employed, must also be acknowledged. And lastly, to the publisher—Mr. Charles C Thomas—for his painstaking workmanship, a "Well Done"!

F.T.H.

INTRODUCTION

WITHIN LIMITED ranges of concentration, clear colored solutions of unit thickness transmit monochromatic light of a given wave length in accordance with the Bouguer-Beer Law.* This is expressed by the equation

$$C = -1/K \log I/I_0$$

in which C is the concentration of the substance, K (the extinction or absorption coefficient) is a constant characteristic for each substance, and I_0 and I are the intensities of the incident and transmitted light, respectively. Since this law applies equally to each of the components of a homogeneous mixture, the spectrophotometric quantitation of two (or more) pigments in the same solution is both possible and practicable.

Historically, spectrophotometric determination of mixtures of biologic pigments began 70 years ago with the researches of Vierordt.† In the course of his investigations, he developed a method for quantitating two pigments in a common solvent by measuring the absorption ratios of the two components at two different wave lengths. Later (in 1900), Hüfner† worked out modified procedures for determining the proportions of reduced and oxyhemoglobin, and of carboxy- and oxyhemoglobin in mixed solutions of these pigments. Employing the same principle, by 1932 Heilmeyer[1] had measured the concentration of a number of mixtures, including reduced hemoglobin-oxyhemoglobin, carboxyhemoglobin-oxyhemoglobin, and methemoglobin-oxyhemoglobin.

Up to this point the photoelectric cell had not yet reached a satisfactory stage of development, and all transmission measurements were necessarily made with visual spectrophotometers. Although these had become instruments of high precision, in the last analysis accurate measurement with them required consider-

* This presupposes that the solutions are non-fluorescent at the wave length in question, are homogeneous, that molecular aggregation and configuration remain undisturbed by dilution, and that the absorption cells present plane parallel surfaces to the light path.

† Quoted by Heilmeyer.

able skill and experience on the part of the observer. Moreover, the visual observations were not only time consuming, but optically fatiguing. It is therefore not surprising that 60 years elapsed without practical application of spectrophotometry to clinical problems.

Perfection of the photoelectric cell, however, brought about a complete change and awakened general interest in the use of this means of measurement. Yet, to isolate portions of the spectrum, the first instruments produced had to depend upon colored glass filters. As a result, the wave bands were quite wide, construction of simple conversion charts required transmission measurements on different concentrations of each pigment, and unusual difficulties arose whenever the determination of pigment mixtures was attempted.

Notwithstanding this, in 1938 Evelyn and Malloy[2] using a color-filter photoelectric photometer, proposed a method for determining oxyhemoglobin, methemoglobin, and sulphemoglobin, on a single sample of blood. The procedure outlined by these investigators, however, exhibits two serious defects, in that turbidity is present during one transmission determination, and an unknown amount of the deeply colored ferricyanide reagent is used up in reaction. Furthermore, this method has the disadvantage common to all filter photometers: conversion charts on semi-log paper are usually curves and not straight lines.

With the development of instruments capable of isolating narrow spectral bands, the quantitation of pigment mixtures became greatly simplified. Nevertheless, until recently few significant papers on this subject have appeared in the literature. Austin and Drabkin[3,4] and Drabkin[5] have described a method (essentially the same as Vierordt's) by which the simultaneous use of extinction coefficients at different wave lengths gave differences proportional to the amount of each component present. In 1943–44 Horecker[6] and Horecker and Brackett[7] made simultaneous determinations of methemoglobin, carboxyhemoglobin, and oxyhemoglobin mixtures by measuring the transmission at isobestic wave lengths and at wave lengths in the infra-red region of the spectrum. But since infra-red and ultra-violet spectrophotometry require specially designed instruments, and because there are

several types of instruments available that are limited to the visible spectrum, the quantitative determination of mixtures within the wave length range 400 to 700 mμ has a wider field of usefulness.

More recently de Duve[8], Beznák[9], and Biörck[10] have utilized measurements of density at isobestic wave lengths and density differences at other wave lengths in the visible spectrum for quantitating mixtures of carboxyhemoglobin and carboxymyoglobin. They were handicapped, however, by the failure of their instrument to reproduce the wanted wave lengths. Although this is a subject of fundamental importance, mechanical defects of this type cannot be discussed here. It must therefore be taken for granted that the instrument used is reliable in this respect.

From the foregoing considerations, it is evident that routine spectrophotometric quantitation of pigment mixtures is *practicable* only when certain requirements are fulfilled. The method should:

1. Use narrow spectral bands in the visible spectrum.
2. Require a minimal number of chemical procedures.
3. Employ colorless reagents.
4. Result in clear solutions.
5. Be sufficiently accurate for clinical purposes.

By utilizing narrow wave bands (obtainable with the Beckman apparatus, the Cenco-Sheard "Spectrophotelometer," or for that matter with any properly constructed and calibrated prism or diffraction-grating instrument), per cent transmission values plotted on *semi-log* paper against pigment concentration result in straight lines passing through the point of origin (100 per cent transmission, zero concentration). Similarly, density values plotted on *rectangular* coordinate paper against concentration give straight lines through the point of origin (zero density, zero concentration). This effects a considerable saving in time and effort; because in order to construct a conversion line chart it is necessary to measure these values for only *one* concentration of the pigment. As has already been mentioned, employment of wave lengths outside the visible spectrum requires special light sources—

quartz absorption cells for ultra-violet determinations, etc. Hence, it should be apparent that measurement of pigment concentration at wave lengths within the *visible spectrum* possesses several advantages.

Reduction in the number of chemical procedures not only saves time, but lessens the probability of error. Even with extreme care, quantitative pipetting is subject to a certain degree of variation, particularly when used in conjunction with micro-techniques. Moreover, the amount of biologic material available must be taken into consideration (blood or stains for instance), since quantitative tests cannot be used for clinical or medico-legal purposes until the amount of material required decreases to a reasonable order of magnitude. Lastly, procedures necessitating cumbersome apparatus, requiring prolonged chemical treatment or resulting in fleeting color reactions or time delays, are usually too complex for routine use, and become practicable only after considerable simplification.

Employment of *colorless reagents* in procedures involving light transmission measurements has more importance than has been given it by most investigators. It should be obvious that if a color development depends upon the reaction of a substance with a colored reagent, the reagent remaining will often materially lessen the light transmission. Hence, unless the transmission of the *remaining* reagent can be measured directly, an error results that no "corrections" can correct for.

Finally, the subject of *clear solutions* is a most important one. So far as has been recorded, the laws describing the scatter of light by colloidal and larger particles are as yet unwritten. Although Stratton and Houghton[11,12] have described the behavior of light of different wave lengths directed through a controlled fog, they were able to write theoretical equations for scatter by uniform-sized spherical particles *only*. It appears, therefore, that *direct* "corrections" for turbidity have no mathematical basis, and that all such "corrections" are scientifically absurd.

In summary, it is apparent that methods for the quantitation of pigment mixtures using narrow wave bands in the visible spectrum, requiring a minimal number of chemical procedures, employing colorless reagents, giving clear solutions, and proving

sufficiently accurate for clinical and medico-legal purposes, should effect a considerable saving in time and labor.

Of the procedures to be described, it is believed that each one meets the above requirements:

1. The chemical procedures are of the simplest sort.
2. Volumetric pipetting has been reduced to a minimum.
3. None of the reagents are colored.
4. Clear solutions result if the outlined technique is followed.
5. Quantitations made by these methods compare favorably in accuracy with the results obtained by chemical determinations.

NOTES ON SYMBOLS AND TERMINOLOGY

THE SYMBOLS AND terms used in this study are defined as follows:

I_0 = the intensity of the *initial* light.

I = the intensity of the *transmitted* light.

$I/I_0 = T$ = the *transmission*, *transmission factor*, or *transmittancy;* and is always expressed as a decimal fraction (see below).

$I/I_0 \times 100$, or $T \times 100$ = the *per cent transmission*, or *per cent transmittancy*.

Transmission value refers alike to transmission factor, transmittancy, and to per cent transmission.

(After completion of the manuscript and charts, attention was called to a technical misuse of the term *transmission*. According to the definition of the Progress Committee of the O.S.A., this term should be restricted to "the transmission of a given cell containing the solution (T_{sol})," or to the "transmission of the same (or a duplicate) cell containing pure solvent (T_{sov})." The ratio of of the transmission of these two cells should properly be called *transmittancy*.) (*J. Optic. Soc. Am. and Rev. Sc. Instrum., 10;* 177, Feb. 1925.)

D = the (optical) density; and by definition,

$D = \log I_0/I = -\log I/I_0 = -\log T$.

K = the extinction coefficient (or absorption coefficient).

(This is a constant *only* when the wave band width employed is strictly monochromatic, or when a *linear* relationship exists between pigment concentrations and the logarithm of corresponding transmission values. For the sake of brevity, expressions such as "the value of K" or "K values" have been frequently used in place of the proper, but longer expression, "the value for the extinction coefficient K.")

Line Conversion Charts are charts upon which density or log transmission values are plotted linearly against pigment concentrations.

An Isobestic Wave Length is one at which solutions of different pigments at equal concentration, have identical transmission values.

A Total Isobestic Wave Length is one at which *all* components of a mixture have identical transmission values, when at equal concentration.

A Partial Isobestic Wave Length is one at which two or more, *but not all*, of the components have identical transmission values, when at equal concentrations.

"Isotranslucent" Wave Lengths are wave lengths at which a given pigment solution shows the same transmission values.

"Reversed" Transmission occurs when pigment *a* shows a higher transmission than pigment *b* at one wave length, and less, at another wave length.

Nomograms are defined as "diagrammatic representations of mathematical laws which are expressed analytically by means of equations with any number of variables."

Points of Origin are defined as follows:

(a) On *semi-log* line conversion charts and nomograms, the point of origin is 100 per cent transmission, zero concentration. This point is located at the *left upper* corner of the chart. On "double" nomograms, a second point is located at the *right upper* corner.

(b) On rectilinear (cartesian) nomograms the point of origin is 100 per cent transmission, zero concentration. This point is located at the *left lower* corner of the chart.

(c) On d'Ocagne nomograms, the *constructional* points of origin are located at the left and right lower corners of the chart. The horizontal line connecting these two points ($x = 0$, $y = 0$) is the d'Ocagne *base line* (equivalent to the cartesian point of origin, $x = 0$, $y = 0$).

The use of millimicra ($m\mu$) to express the wave length has been employed throughout. On some of the charts, however, the symbol *lambda* (λ) has been used instead; this is because standard lettering sets do not carry the greek alphabet.

Oxygen (O_2) capacity in volumes per cent is the usual unit of quantitation for all hemoglobin derivatives. By this, one should not assume that the particular derivative could bind or carry that many volumes of oxygen per 100 cc., but that the pigment was derived from a quantity of hemoglobin capable of combining with that amount of oxygen.

CONTENTS

Part II—Spectrophotometric Procedures

Appendix I—Continuous Transmission Curves and Quantitative Line Conversion Charts

Appendix II—Quantitation of Pigment Mixtures

(Charts and Nomograms)