STUDIES ON VITAL STAINING

I. Some Problems in Colorimetry. The Quantitative Analysis of Mixtures of Colored Substances in Solution

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INTRODUCTION

In the course of some experiments on vital staining a need arose for a method for quantitative analysis of various dyestuffs when in solution along with other colored substances which would interfere with measurement in any of the ordinary standard colorimeters. This problem was finally solved by use of the spectrophotometer. With this instrument one may measure the amount of light absorbed by the solution in any portion of the spectrum. A blue dye transmits blue light but absorbs strongly in the red, and by measuring the amount of this absorption in the red end of the spectrum one may calculate how much of the dye is present in the solution. Conversely, a red dye transmits red light but interferes with the passage of blue and green light, so that the dye concentration may be determined by ascertaining to what extent blue light is absorbed. In case both dyes are present simultaneously each may be determined separately by taking measurements in the two portions of the spectrum where each has a more or less specific effect. It is true that the absorption is not entirely limited to one particular region of the spectrum in the case of either dye, but in suitable cases the correction is small and can be applied in the way outlined below. Other workers (1) have considered this problem of color analysis, but from a somewhat different point of view. It is the purpose of the present paper to describe the method for the analysis of colored mixtures in general terms and to give simple equations which are applicable to the problem studied in the papers to follow. These same equations and the same method in general should be

applicable to a wide variety of problems. Spectrophotometric analysis of the sort here discussed has not received the attention which it deserves from biologists. The quantitative analysis of such mixed colored solutions should find many applications in biology. Dye solutions may be studied quantitatively, even though hemoglobin, bile pigments or urinary pigments may be present as contaminations—a condition often present in biological studies. Dyes excreted into the urine can be measured despite the presence of urinary pigments or even blood. Quantitative analyses by means of color reactions are often unsuccessful on account of extraneous pigments or side reactions. In such cases spectrophotometric analysis might be used to rule out the interfering effect of the foreign colored substance.

The method just outlined will be illustrated by the use of two dyes, brilliant vital red and Niagara sky blue, for they are the ones used in the work to be reported later, but the method of analysis of colored mixtures is applicable to a wide variety of substances, the chief condition being that the two substances mixed must be of different colors. Certain limitations of the method will be discussed more in detail later.

The Analysis of Simple Colored Solutions

There are several types of spectrophotometers which differ in various details. In all types a beam of white light is broken up by the instrument into a continuous spectrum of violet, blue, green, orange and red. If a glass cell of known thickness containing the unknown colored solution is placed in the beam of light, certain portions of the spectrum will be darkened more than others. By means of a slit in the eyepiece the view may be limited to any particular portion of the spectrum and the amount of dimming can be measured by means which vary with different types of spectrophotometer.

For ideal solutions the Lambert-Beer law states that

$$i_1 = i_0 \cdot 10^{-ECh} \tag{1}$$

or,

$$\frac{i_1}{i_0} = 10^{-ECh} = T \tag{2}$$

where i_1 is the intensity of light of any given wave length after the beam has traversed the colored solution, i_0 is the intensity before traversing the solution. C is the concentration of the latter (here expressed in terms of mg. per liter), h is the thickness of the layer of dye solution (expressed in cm.) and E is a constant which

varies with the nature of the colored substance and with the portion of the spectrum examined. The ratio of i_1 and i_0 represents the fraction of the light transmitted and is independent of variations in intensity of the light source. In some types of instrument this fractional transmission, T, can be read off directly on the scale of the instrument or can be computed by relatively simple calculation.

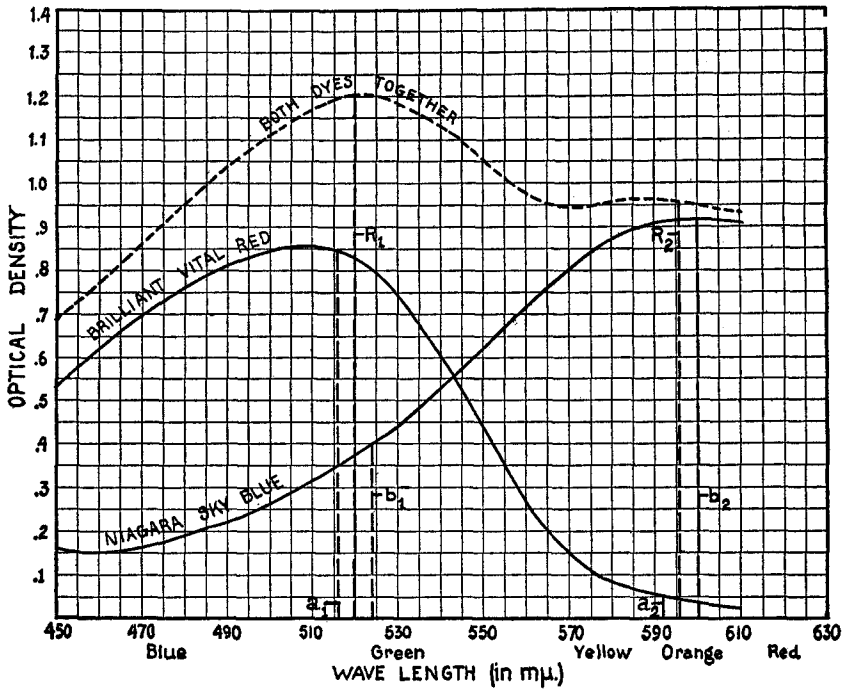

CHART 1A. Absorption curves calculated for 0.002 per cent solutions. Layer = 1 cm. thick.

Obviously our interest centers in the values of E, C and h. These factors may be grouped together by letting

$$D = ECh \tag{3}$$

Then,

$$\frac{i_1}{i_0} = T = 10^{-D} \tag{4}$$

D, the "optical density" of the solution may be looked upon as the obstructive power of the dye solution and is proportional to the concentration and to the

thickness of the layer. Its value varies in different parts of the spectrum depending upon the particular kind of light absorbed by that particular substance. In some types of instrument the calibration is such that D can be read directly on the scale of the photometer. In other instruments D must be calculated from the observed value of T. This calculation is very simple, since from equation 4 it is obvious that $D = -\log T$. In the Bausch and Lomb instrument used by us a control beam of light enters the instrument alongside the first beam and the two are matched by means of Nichol prisms. One glass cell containing the colored solution is placed in one of these beams of light. The other beam traverses a control cup filled with water. One should adopt the practice of reversing the cups and taking a second reading, thus avoiding errors arising from polarization and from slight maladjustment of the instrument. The readings of the Nichol prisms are shown in degrees. For purposes of precision the colored solution should be so diluted that the first reading lies between 10° and 25°. On reversing the cups a large angular reading is obtained (between 65° and 80°). From these two readings one may calculate the optical density from the formula,

$$D = \log \cot \text{small angle} + \log \tan \text{large angle}.$$

Having determined the value of D one may calculate the concentration of the colored substance by means of formula 3. This latter step necessitates knowledge of the value of E for that particular substance in that portion of the spectrum, but this value is readily ascertained by making a preliminary analysis of the colored substance in pure solution. In Chart 1A are shown the absorption curves of the two dyes, brilliant vital red and Niagara sky blue. For reasons to be discussed later the dyes were made up in a mixture of saline and normal dog plasma. The different portions of the spectrum are plotted according to their wave lengths expressed in $m\mu$ (1 $m\mu$ equals 10 Ångstrom units). The power of each dye to absorb light (D, the "optical density") is shown by the ordinates. It is seen that brilliant vital red absorbs almost none of the red light, but is quite effective in absorbing blue and green light. The maximum absorption is in the vicinity of 500–520 $m\mu$. In quantitative analysis of this dye it is obvious that the greatest precision is obtainable by basing the calculation on readings taken in the region of the maximum density. We note that at 520 the "optical density" of a layer 1 cm. thick of the 0.002 per cent solution is 0.84. If we express concentration in terms of milligrams of dye per liter of fluid we find from equation 3 that the value of E is 0.042. In case of the blue dye the maximum absorption is in the region of 600 $m\mu$ and the value of E at this particular point is 0.046. Once these values of E have been determined one may proceed to the analysis of unknown solutions of these substances, using equation 3 for purposes of computation. Using this method of color analysis one obviates the necessity of having to prepare frequent standard solutions of these substances as in ordinary colorimetric work. One avoids unnecessary labor and at the same time is assured of results which are independent of uncontrollable variations in the standard.

The Analysis of Two Admixed Colored Substances

When one desires separate quantitative analysis of two colored substances present simultaneously in solution one may use the method previously mentioned, i.e., one may measure the absorption of light in the two portions of the spectrum where each dye has its characteristic absorption band. The situation is illustrated graphically in Chart 1A by the absorption curves of Niagara sky blue and brilliant vital red. In case both dyes are present simultaneously in equal amounts the mixture becomes purple and the absorption curve is the sum of the two individual curves and is shown by the dotted line. It is with such mixtures of colors that one has to deal in experimental work and it is our problem to deduce from this summation curve just how much of each of the two dyes is present in the mixture. For purposes of making this analysis we may choose to make readings at wave length 520 and 600, for in these regions one dye shows approximately its maximum absorption while the other shows nearly the minimum. Readings taken nearer the ends of the spectrum would be somewhat more ideal theoretically, but it is difficult in these regions to match the colors accurately in the spectrophotometer.

Referring to Chart 1 A it is seen that R_1 and R_2 can be measured in the spectrophotometer. We wish to ascertain the values of a_1 and b_2 so that we may calculate from them the concentration of each dye in the mixture.

Inspection shows that

$$R_1 = a_1 + b_1 \tag{6}$$

$$R_2 = a_2 + b_2 \tag{7}$$

But the form of the absorption curve of each of the two dyes is very specific. Hence

$$\frac{a_1}{a_2} = K_a, \text{ or } a_2 = \frac{a_1}{K_a}$$

$$\frac{b_1}{b_2} = K_b, \text{ or } b_1 = K_b\, b_2$$

where K_a and K_b are constants which can be determined for each dye by study of the latter in pure solution uncontaminated by the other dye. Substituting these values in the equations above

$$R_1 = a_1 + K_b\, b_2 \tag{8}$$

$$R_2 = \frac{a_1}{K_a} + b_2 \tag{9}$$

from which it follows that

$$a_1 = \frac{K_a R_1 - K_a K_b R_2}{K_a - K_b} \tag{10}$$

$$b_2 = R_2 - \frac{a_1}{K_a} \tag{11}$$

These values of a_1 and b_2 are "optical densities" and by substituting their values for D in equation 3 the concentration of each dye in the mixture can be calculated.

Before undertaking the analysis of colored mixtures one should plot accurate absorption curves of pure solutions of each substance. This gives the various constants needed and at the same time allows one to proceed wisely in choosing the points in the spectrum where measurements should be made in order to give the greatest possible precision. The relative quantities of the two substances present influence the accuracy of analysis in a way which will be evident at once. In general the substance can be determined more accurately when it occurs in large amounts in the mixture. However it is possible to measure small amounts of a substance in the presence of large amounts of another colored substance provided the absorption bands of the two substances are fairly well separated. Inspection of Chart 1A shows that brilliant vital red absorbs very little light at 600. The large absorption by Niagara sky blue at this point permits very accurate analysis of the latter. On the other hand at 520 the light absorption is by no means all due to brilliant vital red; hence the amount of the latter cannot be measured so precisely as would be possible if the Niagara sky blue absorption band were more strictly localized to the red end of the spectrum.

In all spectrophotometric analysis it has been found desirable to take readings at several points in the region of the spectrum chosen for special study. On plotting these points one will discover and correct small errors due to inexact matching of the colors. It is also wise to take readings in neutral portions of the spectrum so chosen that suspected contaminating substances will be detected when the curve is plotted. In our own experiments to be reported later we

were concerned with the analysis of dyestuffs in plasma, and under these circumstances readings were taken in parts of the spectrum where hemoglobin and bilirubin would be detected if present.

It would seem hardly necessary to remind workers in this field that hydrogen ion concentration of the solvent may totally alter the form of the absorption curve of the dissolved coloring matter. In some

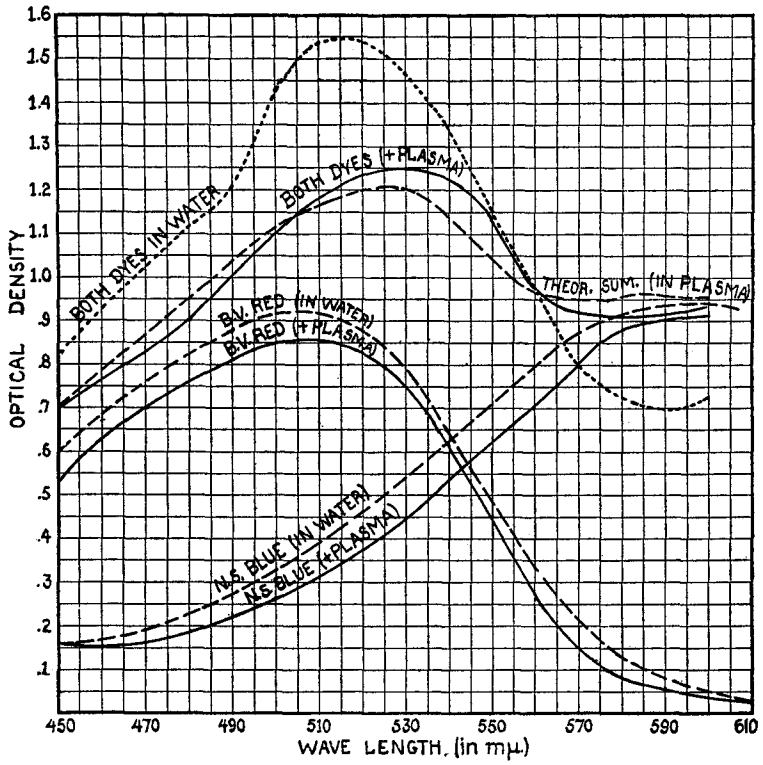

CHART 1B. Light absorption curves of brilliant vital red and Niagara sky blue (0.002 per cent solution) in water, and in plasma diluted with four parts of saline.

cases the salt concentration or protein concentration is also of great importance in its effect upon a dye. The preliminary control experiments should include a study of all such factors and their possible influence upon the color behavior of the dye. Okuneff (2), Seyderhelm and Lampe (3) and others have shown that whereas trypan blue dissolved in water is violet in color, the addition of plasma proteins

or egg-white gives the dye a more bluish color. In studying mixtures of colored substances the possibility of interaction between the two must also be kept in mind. In extreme cases precipitation may occur, but in other cases the reaction is much less evident and the only effect noted may be that the observed absorption curve of the mixture does not quite represent the summation of the absorption curves of the two components. A difficulty of this very sort arose in the case of mixtures of brilliant vital red and Niagara sky blue, and for a time it seemed as though it would be impossible to make quantitative studies of such mixtures. Curiously enough these difficulties existed only when the two dyes were brought together in aqueous solution. When a small amount of plasma protein was added to the mixture it was found that the combined absorption curve of the two dyes approached the theoretical summation curve in quite a gratifying manner. These facts are shown graphically in Chart 1B. The discrepancy from theory in pure aqueous solution is less marked in the blue than in other portions of the spectrum. In the green it amounts to about 30 per cent, and well over in the red near 590 the discrepancy is almost as great, though in the opposite direction. From the standpoint of quantitative analysis it is most fortunate that a little plasma protein will correct these abnormalities in light absorption and give values so closely approximating the theoretical summation curve shown by the interrupted line in the chart. It is rather remarkable that relatively small amounts of plasma need be added to bring about this result. Experiments have shown that one part of plasma to ten of the color mixture is adequate. The plasma of vitally stained dogs may contain large quantities of the two dyes and in such cases it may be necessary to dilute it preparatory to making spectrophotometric readings. In order to preserve an adequate concentration of the stabilizing proteins it is well to use saline mixed with normal dog plasma as a diluent. Normal plasma has a weak absorption curve of its own, but this may be ascertained in advance and proper correction made if necessary.

As yet we do not fully understand how the proteins bring about this stabilizing effect on the color absorption curve, though it is suggested that the proteins enter into some form of combination with the dyes and thus prevent or break up loose combinations which tend to form between the dyes themselves. It is noteworthy that either dye alone

in solution gives a color absorption curve which is largely independent of the presence or absence of plasma proteins. It would appear to be a combination of the dyes which is affected by the proteins.

It is clear that the effect on the dyes cannot be attributed to the buffer action of the plasma, for these particular dyes are relatively insensitive to changes in acidity unless very large amounts of acid are added. Furthermore, we can show that the two dyes made up together in selected inorganic buffer solutions show color absorption curves almost identical with those obtained in pure aqueous solution, and like the latter the deviation from the theoretical is quite marked unless plasma protein be added also.

SUMMARY

1. A spectrophotometric method is discussed which permits quantitative analysis of colored substances present in mixtures

2. Special attention is given to the analysis of mixtures of two dyes which are being used in a series of studies on vital staining.

3. It is shown that the method can be applied to quantitative analysis of mixtures of naturally occurring animal pigments or to mixtures of these with various other colored substances.

4. Certain limitations of the method and certain necessary precautions are discussed.

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