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THE APPLICATION OF TRANSMITTANCY MEASUREMENTS TO BIOCHEMISTRY[1]

INTRODUCTION

MANY years ago at Troas, a visioned Macedonian came to Paul seeking assistance. From that hour the Macedonian left no doubt in Paul's mind as to the way he might best serve. So he took a straight course for Samothracia, and then on to Philippi.

To-day, we who are engaged in biochemical work have long sought help in the solution of our problems. As the Macedonian came to Paul, so we come to you for assistance in our labor. Meanwhile we have been marking time, pyramiding our evidence and quibbling about many of the non-essentials, while the real problem all the time unattacked has been lying before us. Our mutual problems are as full of spiritual significance and humanitarian interest as were those of the Macedonians, and should be considered no less seriously. The correct interpretation of every experiment has tremendous spiritual significance and every experimenter should most courageously guard the interpretation of his experimentation. Every interpretation should be pictured as a grain of truth, which, when put with other grains of truth to form a unified whole, will make the world in which we live one of reality and not one of superficiality. The biochemist again and again has looked to one branch of science and then to another for suitable methods of investigating his problems. He is beginning to realize that something not found in a test-tube or in any mechanistic physical-chemical philosophy of life must come to aid him in his efforts. New procedures, new interpretations and independent thinking seem imperative. The relation of light to matter appears on the horizon offering assistance in many of the modern problems which confront those investigating the chemistry of life. This paper is an attempt to show how properties of light will assist us in the solution of problems which as yet remain unsolved after many years of struggle. Light and chlorophyll, which are both so commonly known and so little understood, are worthy of our most critical examination.

EARLY INVESTIGATIONS

Approximately one hundred years ago David Brewster discovered fluorescence in fluorspar. His

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[1] Paper given before the Optical Society of America at the Bureau of Standards, November 3, 1928.

discovery was reported to the Royal Society of Edinburgh in 1833. In 1846 he reported the discovery of fluorescence in an alcoholic solution of leaf-green. One year before this, John Herschel discovered that quinine sulphate when placed in sunlight "gave off a blue color."

OBSERVATIONS OF STOKES[2]

G. G. Stokes repeated the experiments of these two workers, and the results obtained caused him to take a most lively interest in the phenomenon. He made many interesting observations, which were reported to the Royal Society in 1852. Using the light of a candle and a single isolated prism, he observed five absorption bands in an alcoholic solution of nettle leaf-green. He made the suggestion that the intense band No. 1 be used as a chemical test for the presence of chlorophyll. Later he observed that absorption commenced at about the fixed line B, and continued onward throughout the visible spectrum and beyond. The absorption was noticed to be subject to fluctuations, which were intimately connected with the absorption bands exhibited by this medium. Again he noticed that when a tube of quinine sulphate solution was plunged into the invisible rays of the spectrum the phenomenon had something of an unearthly appearance; it was literally darkness visible. It was observed that absorption of light, or change of refrangibility as he called it, was extremely common, especially in organic substances.

In 1864 he writes further: "I find the chlorophyll of land plants to be a mixture of four substances, two green and two yellow, all possessing highly distinctive optical properties. The green substances yield solutions exhibiting a strong red fluorescence; the yellow substances do not."

At the age of forty-five he addressed the fellows of the Chemical Society with the following words: "The optical properties of bodies, properly speaking, include every phenomenon in which ponderable matter is related to light by virtue of its molecular constitution, and not merely of its external form." He states further that spectral analysis is most valuable for the detection of elementary bodies and can not of course be applied to organic analysis. Absorption, fluorescence and reflection, however, are optical properties of bodies which can be used in organic analysis, for these properties vary markedly from one wavelength to another. Again he says:

> But in most cases, especially with substances of intense colorific power, the mode of absorption is eminently characteristic. Two or more dark bands are seen in the spectrum, indicating maxima of absorption; and the position of these bands, their relative intensity, and their other features, form altogether a series of characters the distinctive nature of which is such as those who have neglected the use of the prism have little conception of. They render it perfectly easy in many cases to follow a particular substance among a host of impurities. For each colored substance produces its own absorption, independently of the others, so that, unless the part of the spectrum in which the distinctive bands, or most of them, occur is wholly absorbed by the impurities, the presence of the substance can still be recognized. Not only is an examination of the absorption spectrum of a substance useful for enabling us to follow the substance through mixed solutions, but it sometimes reveals relationships in cases in which they might not be suspected, if the origin of the substances were unknown.
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> The phenomenon of fluorescence gives in some cases material assistance, but in general it is only by combining spectral analysis with processes of chemical separation, especially fractional separation, that a satisfactory conclusion can be arrived at. When a mixture is thus tested in various ways, a conviction is gradually arrived at that those bands of absorption which are always found accompanying one another belong to one and the same substance.
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> For convenience and rapidity of manipulation, especially in the examination of very minute quantities, there is no method of separation equal to that of partition between solvents which separate after agitation. . . .
>
> Bisulphide of carbon in conjunction with alcohol enabled the lecturer to disentangle the colored substances which are mixed together in the green coloring matter of leaves.

From this last paragraph it is revealed that Stokes was the first to make use of the fractionation method to separate the green chloroplast pigments from the yellow ones, and to roughly separate chlorophyll *a* from chlorophyll *b*.

INVESTIGATIONS OF WILLSTÄTTER[3]

The writings of Willstätter reveal that valuable observations of students of natural science, to whom the application of chemical methods was not familiar, exerted no influence upon the development of our knowledge of chlorophyll. Chemical literature ignored statements and suggestions of the great physicist and those of botanists, or treated them disrespectfully. As a consequence, important hints on the existence of two components of chlorophyll, which occur in the optical treatise by Stokes, fell on unfruitful soil and the fascinating microscopical observations of J. Borodin went unheeded by analysts.

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[2] G. G. Stokes: (1) "On the Supposed Identity of Biliverdin with Chlorophyll with Remarks on the Constitution of Chlorophyll." Proc. Roy. Soc. London, Feb. 25, 1864. (2) "Mathematical and Physical Papers," Vol. 3, 1901. 413 pages. (3) "On the Application of the Optical Properties of Bodies to the Detection and Discrimination of Organic Substances," *Jour. Chem. Soc.* 17: 304–311, 1864.

[3] Richard Willstätter and Arthur Stoll, "Investigations on Chlorophyll," 1928.

Willstätter and his coworkers, by making use of suggestions of the physicist Stokes and those of the botanist Borodin, successfully accomplished what many chemists before them had failed to do.

It was from the writings of Borodin that Willstätter learned how to prepare ethyl chlorophyllide, which was used as the basis for determining the purity of the chlorophyll preparations. From the writings of Stokes he learned of the possibility of separating chlorophyll *a* from *b* by means of non-miscible solvents. In 1913, using a new fractionation method, petroleum ether and methyl alcohol, with ethyl chlorophyllide as a basis for his colorimetric determinations, he obtained the two chlorophyll components. Preliminary spectroscopic investigation revealed eight bands belonging to chlorophyll *a* and ten belonging to chlorophyll *b* in the visible spectrum. No record is made of any observations beyond the visible. Colorimetric and spectroscopic methods were available to Willstätter for determining the amount of chlorophyll present in a solution. The former was used by him because component *a* optically masks *b*, the pigments were highly unstable and the absorption properties may be considerably altered by the influence of even a small amount of plant acid in the extract. In the preparation of chlorophyll, colorimetric tests alone were not sufficient to establish its purity. Chemical tests, such as the determination of carbon, of hydrogen or nitrogen, of magnesium, or of the melting-point, were of little value. Other chemical tests were devised to tell if transformations had taken place in the molecule. It was Willstätter's belief that when better methods exist for the isolation of the pigments, quantitative transmittancy methods could be used for improving the determinations.

The investigator of chlorophyll must realize also that Willstätter has found that many important chemical changes of chlorophyll and its derivatives exert little or no influence upon the absorption spectrum, while, on the other hand, certain insignificant changes of constitution produce rather large changes in the spectrum.

Willstätter tells us that future investigations on the constitution of chlorophyll will still find important problems. The relations between *a* and *b* chlorophyll and the transformations which led from chlorophyll to the chlorophyllins and from the chlorophyllins to the dibasic phyllins are yet to be explained, and important details of the structure of etioporphyrin remain to be investigated. The strange names used here are only names for derivatives of chlorophyll.

On still another page, he concludes from chemical analysis that the difference between the *a* and the *b* series probably consists in a molecule of oxygen, two hydrogen atoms of chlorophyll *a* being replaced by one oxygen atom in chlorophyll *b*. This assumption he admits has by no means been proved. The assumption is only a provisional one to render the relations between the two components understandable, not forgetting that further investigations are needed to fix and strengthen this explanation.

THE SITUATION TO-DAY

Many of us to-day are under the impression that Willstätter has written the last word on chlorophyll, but from his own writings it is learned that our knowledge regarding the most important organic nitrogen compound, which is produced by nature annually in the United States alone at the rate of many million tons, is very elemental. It is well known that light is related in some way to chlorophyll, and we are beginning to become conscious of the fact that both in some important way are related to your life and mine.

Our knowledge regarding this substance has reached the stage where chemistry alone will not solve the problem—chlorophyll, what is it? As a substance or quality is weighed or measured our knowledge concerning the thing investigated is mastered in proportion to the accuracy of our measurements. So if more about a quality or a substance would be known, it must be measured very precisely. So long as a substance exists as a mixture or so long as we mentally mix two or more qualities, little can be learned about either of them. Our first problem then is to highly refine the thing investigated or to get it in as pure a state as possible. Before a thing can be obtained pure a standard of its purity must exist in our minds. Purification is one of the most difficult problems confronting any investigator. Purity is of first consideration when absorption measurements are made of any substance. To make absorption measurements and know that they belong to a substance, that substance must first be isolated from all other substances which would in any way affect its absorptive properties.

FUTURE WORK ON CHLOROPHYLL

Specifically, to fractionate chlorophyll *a* from *b*, more than a colorimeter and more than chemical tests are necessary. Transmittancy methods alone appear sufficiently critical for the undertaking. Future work on chlorophyll will progress only as fast as the work of the chemist is correlated with that of the physicist. Purer chlorophyll can be prepared and chlorophyll *a* can be separated from chlorophyll *b* only as step-by-step investigation is made of the purity of the substances. This may best be done in cooperation with the physicist by testing the per cent. of light that is absorbed at different wave-lengths from the extreme infra-red to the extreme ultra-violet. We will then know positively that we have pure chlorophyll *a* and *b*, as they exist in the plant when every band is accurately known to have the same magnitude, shape and position that it has in living green leaves or know why there has been any alteration in the absorption curve. The data obtained will be invaluable in accurately estimating the two pigments in problems concerning plant physiology.

Future investigations on preparations that to-day we know are impure will be of little value compared to investigations made on preparations of the highest purity. Commercial centrifugals already developed will perhaps be of great value in speeding up every stage of the preparation of chlorophyll. To get a pure product, speed is of primary importance, for chlorophyll evidently undergoes decomposition at every stage of its preparation.

To-day, in addition to a study of the two chlorophyll components, there is need of a study of the absorption properties of all the important chlorophyll derivatives. Every possible chemical compound produced by breaking down the most important nitrogen compound in the world is worthy of examination by the most critical methods available. Who is so wise as to know in what form some natural product formed from chlorophyll may appear in every living cell? Once the characteristics of these compounds in a pure state are known, their presence may be easily established.

Already transmittancy measurements have been used to control the preparation of two important biochemical compounds. Transmittancy data of carotin and of xanthophyll, the purity of which was tested spectrophotometrically, will be presented to-day by McNicholas, as evidence of what has been done already in the use of such data as a control in the preparation of pure chemical substances. What has been done with carotin and xanthophyll can be done with chlorophyll *a* and chlorophyll *b* and any other organic substance.

One man highly trained and with a little assistance, by making use of the spectrophotometric data already obtained on optical instruments at the Bureau of Standards, could find out more in one year about the carotinoid pigments present in plants and animals than a host of workers have discovered in a hundred years. With Hardy's recording color analyzer I now find that this work could be done in a single day. Instead of using 6,000 eggs or 10,000 ovaries, the presence of xanthophyll or carotin could be conclusively proved by using a single egg or a single ovary. To-day, the final proof of the identity of two chemical compounds is furnished by transmittancy data of substances which need not necessarily be highly purified.

APPLICATION OF TRANSMITTANCY MEASUREMENTS TO OTHER FIELDS

The physicist of to-day has rendered a great service to all biochemists, for by the use of his instruments traces of organic compounds may be quite easily detected. Every chemical compound absorbs light in some portion of the spectrum, and this fact should be taken advantage of by all who have biochemical problems worthy of solution. Chemical tests are often not sufficiently critical to aid us in the solution of many of our problems in which transmittancy measurements are very helpful.

There is every reason to believe that spectrophotometric methods can be used to solve problems in protein synthesis, nutrition of plants and of animals, problems in photochemical processes, and many others which are most fundamental. Every phase of nitrogen chemistry is worthy of our deepest study, for our very lives seem to be tied up in an intimate way with nitrogen. In an American laboratory plans are already well under way for an intensive spectrophotometric study of certain proteins.

In the field of systematic botany for hundreds of years attempts have been made to find a natural system of classification for plants. Evidence is accumulating that each group of plants has a chemistry peculiarly its own. The chemist too for a long time has sought to know order in nature, in the inorganic and organic worlds. The present system of classification of biochemical compounds is most probably as artificial as have been those proposed in an attempt to find a natural system in plant classification. Botanists by cooperating with chemists should discover much that will aid them to understand nature's chemistry. Fractional separation methods using organic solvents and the new physical instruments offer hope of a solution of this important problem.

A study of the absorption bands of ergosterol has already revealed much regarding its chemical nature. The instruments developed are valuable too for use in routine chemical analysis of inorganic substances as well as substances in the organic world. Ferrari recently has used transmittancy measurements in studying very accurately the effect of various bleaching agents on the carotinoids in flour. Shapiro[4] has used transmittancy measurements to solve problems in organic chemistry involving hydroxyl substitution and the presence of the meta-quinoid structure in organic molecules. Otto Warburg[5] has made use of these measurements to show that the respiration

[4] *Jour. Am. Chem. Soc.*, 50: 1772.

[5] SCIENCE, No. 1767, 68: 437–443. 1928.

ferment is in many respects quite similar to all known hemin compounds. The absorption maximum of respiration ferment is slightly displaced toward the red in respect to the other hemin compounds. Spectroscopically, chlorophyll in solution has about the same relation to chlorophyll in the cell as the hemin compounds have to respiration ferment. Warburg has also made use of a new principle whereby the absorption spectrum of a substance can be indirectly determined from the photochemical activity of the given substance. Frank Wokes[6] has made a study of the transmittancies of the colorations which have been attributed to vitamin A. Vitamin A has been shown by Morton and Heilbron[7] to possess an absorption band with a maximum at 328 μμ while one of the decomposition products of this vitamin has an absorption band near 275–285 μμ. A study of the vegetable oils by Priest[8] has revealed the presence of chlorophyll and its decomposition products in all the oils examined. Olive oils were high in chlorophyll-like substances while cottonseed oil contained varying amounts. The velocity with which oxygen combines with reduced hemoglobin has been measured by Hartridge and Roughton[9] with a spectrophotometer. Rates of other chemical reactions can be measured. Measurements of the color of iodine solutions in typical solvents and of the effect of ultraviolet light on iodine solutions have been made by Getman.[10] The value of these instruments once fully comprehended will make them indispensable to every chemical laboratory.

If we are ever to learn much more about the structure of molecules the information is likely to come from a study of the absorption bands of compounds. The spectra of compounds can only be understood by finding order in their arrangement and relations of the absorption bands to the structure of the molecules.

In the biochemical field to-day there is need for a Rowland to map accurately the great variety of absorption spectra of pure organic compounds. The physicist already in one generation has brought amazing order out of an utter chaos of wave-lengths and he too can assist us in bringing order out of a mass of organic compounds which to-day is nothing but confusion.

If transmittancy measurements were made of every important biochemical compound highly purified, at once there would exist a standard basis of comparison for all biochemical work. The question—how much?—could be easily answered for every problem. A few minutes' examination of any given solution could settle immediately the presence or absence of a certain organic compound. Such measurements will assist us in discovering new organic substances as well as teach us to give only one name to substances now supposedly different.

A spectrophotometer is already being used by Shull to demonstrate conclusively that there is reason to question classical studies which have been made in the field of plant physiology. They are being used by others to show the physical condition of the human body and the progress of disease by simply analyzing the body fluids. Specific changes going on in ripening processes can be followed as well as many other chemical transformations involved in plant growth.

Biochemists need no longer be content to report results in millimeters on a colorimeter nor should they be longer content to report the results of tissue analysis in terms of ether and alcohol extract when it is possible to follow the course of any compound in a plant or animal. It is far better to know something definite about the complete life relations of a single organic compound than to talk glibly about many, as we now do.

The time has come when we must no longer use terms to cloak our ignorance or labeled pigeonholes into which we can stuff anything about which we know little or nothing. Error and ignorance will and must be discovered. Annually we are spending hundreds of thousands of dollars and taking much time to make practical applications in science without making certain of the soundness of the fundamentals upon which these practical applications are based. We are beginning to learn that transmittancy measurements are as valuable in the field of biochemistry as emission spectra are in the field of inorganic chemistry. As the spectroscope opened a vast unexplored territory for the analytical chemist as well as the astronomer, so the spectrophotometer opens a vast unexplored territory for the biochemist. It gives us a new instrument which should greatly assist in solving the relation of light to matter. We as biochemists would like to know more about the radiation hypothesis and more about Einstein's equivalence law, and about the chain reactions that are undoubtedly going on everywhere about us. Attempts should be made to correlate data for inorganic substances with data for organic substances, thus bridging or wiping out the gap between living processes and those assumed to be not living.

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[6] "Spectroscopic Study of the Colorations Attributed to Vitamin A," *Biochem. Jour.*, 22: 987–996. 1928.

[7] R. A. Morton and I. M. Heilbron, "The Absorption Spectrum of Vitamin A," *Biochem. Jour.*, 22. 1928.

[8] Irwin G. Priest, "The Color of Soy-Bean Oil as Compared with That of Cottonseed Oil." The Cotton Oil Press, III, 37–40, 1920.

[9] H. Hartridge and J. W. Roughton, *Proc. Roy. Soc.*, 107: 654–683, 1925.

[10] Frederick H. Getman, *Jour. Am. Chem. Soc.*, 50: 2883–90, 1928.

To do this, the Englishman must talk Chinese to the Chinese and the man from China must talk English to us. We are not effectively doing this now, but your society is doing more than any other agency to standardize procedures and make intelligible to those in other fields the work of the optical physicist and your president is doing his share of the work. We can only make use of your instruments as you make it possible for us to understand and use them. But we too are to blame, for our words and phrases so often must be only meaningless words to you, for so often we discover that they mean very little to us also.

Conclusion

In a very brief way I have tried to show how absorption spectra have been intimately related to the development of our knowledge of chlorophyll. One hundred years ago Stokes discovered several laws that are very applicable to-day. For apparatus he used a boarded-up window, a slit in a plank, a lens and a prism. Instead of thinking how much we have accomplished with highly refined instruments we biochemists should rather humble ourselves by thinking how little use we have made of the instruments which you have so nobly perfected.

For fifteen years the work of Willstätter has lain almost unnoticed. Perhaps much more time must elapse before we become conscious of how to apply the principles and procedures which he has developed for fractionally separating an organic substance from many others. He has successfully isolated four chemical substances from masses of organic substances most difficult to manipulate. What has been done with chlorophyll *a* and *b*, carotin and xanthophyll can be done with other organic substances. By spectrophotometric methods any substance can be traced through any number of fractional separations. Transmittancy measurements combined with centrifugal methods and fractional separations in solvents should make it possible to separate organic substances of which our knowledge as yet is very incomplete. Delay in using fundamental principles discovered by others is a question of psychology and not one of biochemistry or physics. Our delay is measured by the amount of time that it takes us to become wholly conscious of that which most unconsciously lies immediately before us—much that we have learned must be temporarily forgotten before we can become conscious of the real situation. It is further measured by our stubborn unwillingness to give up ideas which we have held from our youth. Once we get the viewpoint our sails will be set in a straight course to study the things that are fundamental and so full of spiritual significance for us all. Our concern is with life and how to live it more normally and completely by living in complete harmony with nature's laws and not forever opposing them. That is my motive for a critical study of biochemical compounds, using light, a fundamental something which can be accurately measured, as a final test.

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CHARLES DEPÉRET

The sad news comes from the University of Lyons of the sudden death of Professor Charles Depéret, dean of the science faculty of the University of Lyons and one of the most distinguished leaders in the long line of paleontologists of France who have succeeded Cuvier, the founder of this great branch of biology. According to a note received from M. Frédéric Roman, professor of geology in the University of Lyons, the death of Professor Depéret occurred, following a geological excursion in the south of France.

Charles Depéret was eminently a collector and field observer both in vertebrate and invertebrate paleontology. He naturally succeeded Albert Gaudry in the series of monographs on the fossil mammalia, especially of Southern France, so that we owe to him the scheme of geologic and paleontologic correlations between the Upper Tertiaries of France, England and Italy, which have been elaborated through the work of the present writer into very close comparisons of the Tertiary faunal horizons. Both in descriptive and in philosophic paleontology, Charles Depéret was a master. His talent in invertebrate paleontology and geology was not only shown in his great correlation scheme of the Tertiaries but was extended in recent years into the correlation of the Pleistocene through the rising and falling sea-levels and shore-lines surrounding the Mediterranean and extending up along the eastern borders of France and Great Britain. The establishment of four great *Étages* dividing the Pleistocene by marine stages similar to those by which the Pliocene and preceding Tertiaries are divided is his last and perhaps one of his greatest contributions to geology and paleontology.

He was a man of most genial and delightful personality, generous in the exchange of ideas and materials and inspiring to all the younger men who came in contact with him. Thus he leaves a school of younger geologists and paleontologists, many of whom have collaborated with him and participated in his field excursions and observations, and will sustain the high traditions of French paleontology and geology. To all these colleagues in the University of Lyons and to members of his family we extend our most