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(With Two Plates)

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THE DETERMINATION OF SMALL AMOUNTS OF CHLOROPHYLL—APPARATUS AND METHOD[1]

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INTRODUCTION

The present paper describes a photometric method for the determination of small amounts of total chlorophyll present in plant tissue, and the apparatus employed. The method is based on the fact that chlorophyll has an absorption band in a certain region of the spectrum that does not overlap the absorption bands of other soluble pigments such as carotenoids. Although it is fully realized that other methods have been employed and described, it is nevertheless felt worth while to point out some advantages of this method which is adapted very nicely to certain problems under investigation in this laboratory.

APPARATUS

The apparatus is illustrated in plate 1 and figure 1. Essentially it consists of a light source properly shielded in a housing and a horizontal optical path in which the chlorophyll solution may be interposed.

A single-filament street-series lamp (1,000 lumens and 6.6 amperes) serves admirably as the light source. This is connected to 10 storage batteries in such a manner as to give 12 volts. A battery source of current is more desirable than the commercial city supply because of its steadiness. Inside the lamp housing is a metal cylinder which may be raised or lowered so as to transmit or intercept the light in its passage through the optical system. From a condensing lens 3 inches in diameter (shown in fig. 1) the light is passed through a Corning heat-resistant, heat-absorbing light shade glass filter 2.66 mm. thick (F_1), a Corning heat-resistant pyrometer red, number 241, 48 percent filter, 4.85 mm. thick (F_2), and a glass cell 2 cm. thick containing

[1] Presented before the Division of Biological Chemistry of the American Chemical Society, Baltimore, Md., Apr. 3-7, 1939.

[2] The authors wish to acknowledge the assistance of L. A. Fillmen in the construction of the apparatus, and of E. R. Brydon in carrying out the determinations.

distilled water. This water cell (F_3) is connected to a small reservoir kept at room temperature and by thermosiphon action the accumulation of heat is minimized. By means of these filters the radiation utilized in the transmission measurements is restricted to the range from 6240 A. to just beyond the visible in the near infrared.

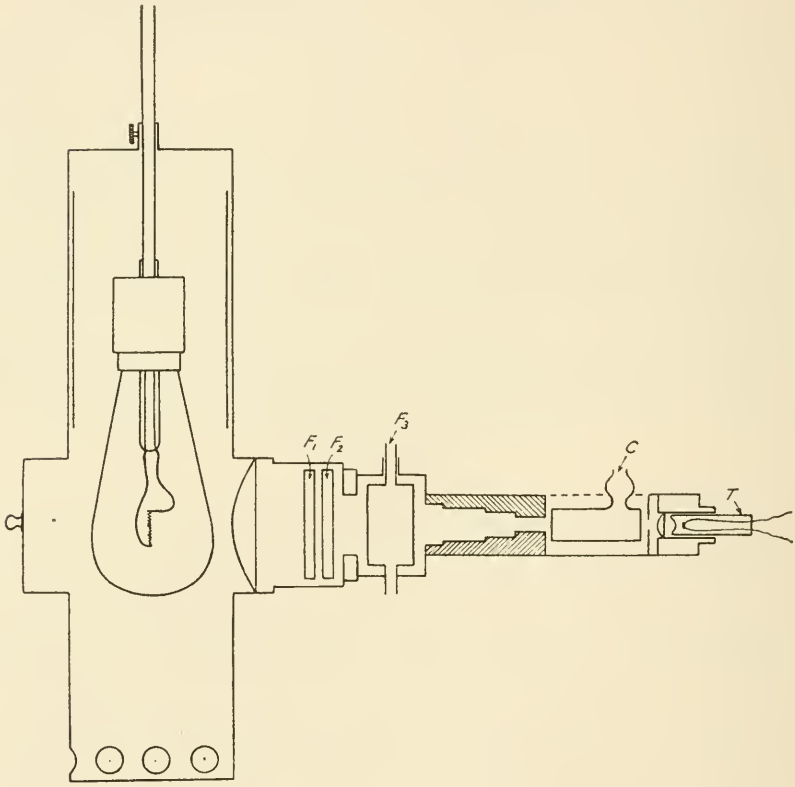


Fig. 1.—Diagram of apparatus used for the determination of small amounts of chlorophyll. F_1, Corning heat-resistant, heat-absorbing, light shade glass filter, 2.66 mm. thick; F_2, heat-resistant, pyrometer red, No. 241, 48 percent filter, 4.85 mm. thick; F_3, glass water cell, 2 cm. thick; C, glass absorption cell 5 cm. long used for holding chlorophyll solutions; T, vacuum thermocouple.

A thermocouple (T) is employed as the energy receiver. The brass thermocouple housing is thermally insulated from the lamp housing by a cylinder of Bakelite whose interior diameter is decreased in steps to reduce internal reflections.

The thermocouple has been developed and built in this laboratory by L. B. Clark. The receiver is a circular disk 1 mm. in diameter and 0.00127 mm. thick. The couple is permanently evacuated to a pressure of less than 10^{-4} mm. mercury which increases its response 20- to

30-fold. It is a rugged type of high sensitivity and has a uniform response over the wide range from 2500 A. to 6.5 μ. This transmission range has been obtained by the use of a so-called "bubble window" which is a thin (.025-.050 mm. thick) disk of glass that is fused to the cell body and then sucked in. The couple, whose zero stability is excellent, has a resistance of about 15 ohms and gives 3.3 microvolts per microwatt per square millimeter. Its time response is less than that of commercial galvanometers.

The couple is connected directly to a Moll galvanometer without intermediate means of amplification. This galvanometer has a period of 1.3 seconds with an internal resistance of 50 ohms and the external resistance for critical damping may be varied from 120 to 0 ohms. At a scale distance of 1 m. a deflection of 1 mm. corresponds to a current of 6×10^{-9} amperes.

The absorption cell (C) is 5 cm. long and has a volume of 10 ml. It is constructed of Pyrex glass with fused-on ends.

PREPARATION OF EXTRACT

The plant material is thoroughly ground by hand in a mortar with sand and acetone, and the solution decanted and filtered under reduced pressure. The residue, which is retained in the mortar, is ground and the solution filtered twice more, all the filtrates being combined. This requires from 10 to 15 minutes. Carotenoids are not separated from the chlorophyll. It is essential that all suspended material be removed from the solution since the slightest turbidity reduces the transmitted energy and introduces an error in the chlorophyll determinations. After trying several kinds of filters the one illustrated in plate 2 has been found very satisfactory. Several layers of close-grained filter paper are placed in position between the ground faces of the upper and lower portions of the filtering tube. A brass collar fits against a shoulder on each part of the filter tube and by means of screws the ground faces are held tightly against the filter paper.

DETERMINATIONS

Before making determinations of unknown chlorophyll solutions it was necessary to construct a calibration curve (fig. 2) from known concentrations of purified chlorophyll solutions.[3] It has been deter-

[3] The purified chlorophyll solutions and the unpurified leaf extracts gave identical absorption curves in the region from 6000 A. to 7400 A. We are indebted to Dr. K. S. Gibson and H. J. Keegan of the Colorimetry Section, National Bureau of Standards, for the determination of these curves with the General Electric recording spectrophotometer.

mined that additions to the standard chlorophyll solutions of carotene or xanthophyll in amounts up to a hundred times that of the chlorophyll, do not influence the transmission in the spectral region employed.

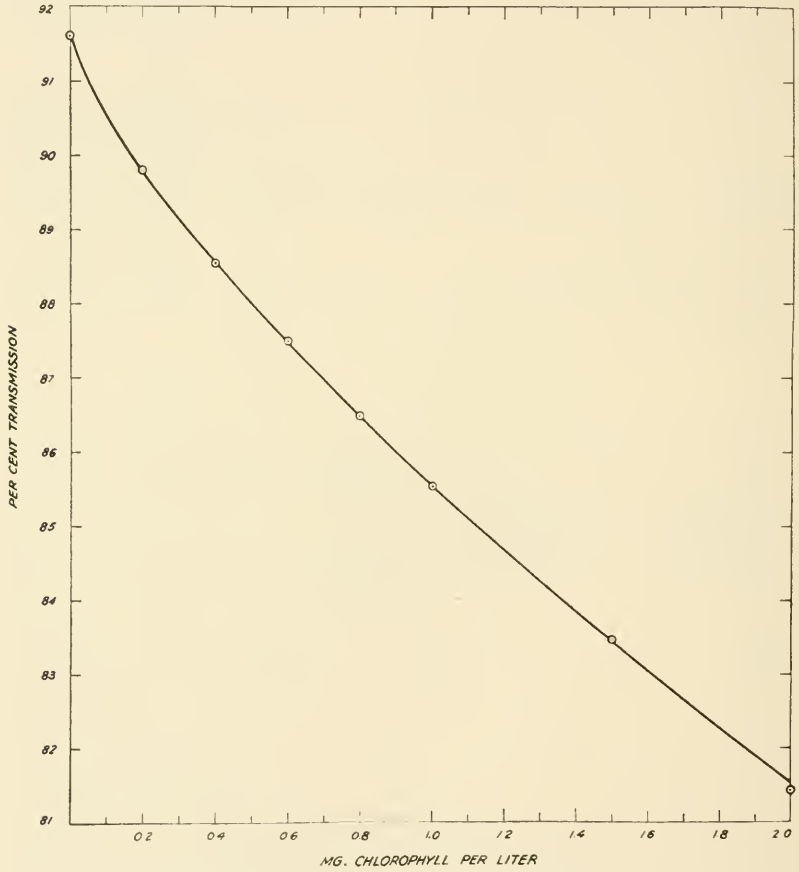


Fig. 2.—Calibration curve showing percentage transmission plotted against milligrams of chlorophyll per liter.

Once this curve has been established the continued use of standard chlorophyll solutions is eliminated. The transmission data obtained from unknown chlorophyll solutions are compared with the calibration curve and the concentrations read directly. The calibration curve used is an empirical one for the particular filters, absorption cell, and solvent employed.

In determining the percentage transmission of a chlorophyll solution the galvanometer deflections with and without the absorption cell and solution in position are observed alternately. The percentage transmission obtained from the average data is used in reading the concentration of chlorophyll from the calibration curve.

About 5 to 10 minutes are required to make the transmission determinations. With the 5-cm. absorption cell the sensitivity is 0.1 microgram (1/10,000 milligram) of chlorophyll. One square centimeter of leaf is sufficient for duplicate determinations which check within 2 to 3 percent. This method is independent of any visual comparisons of intensity or color.

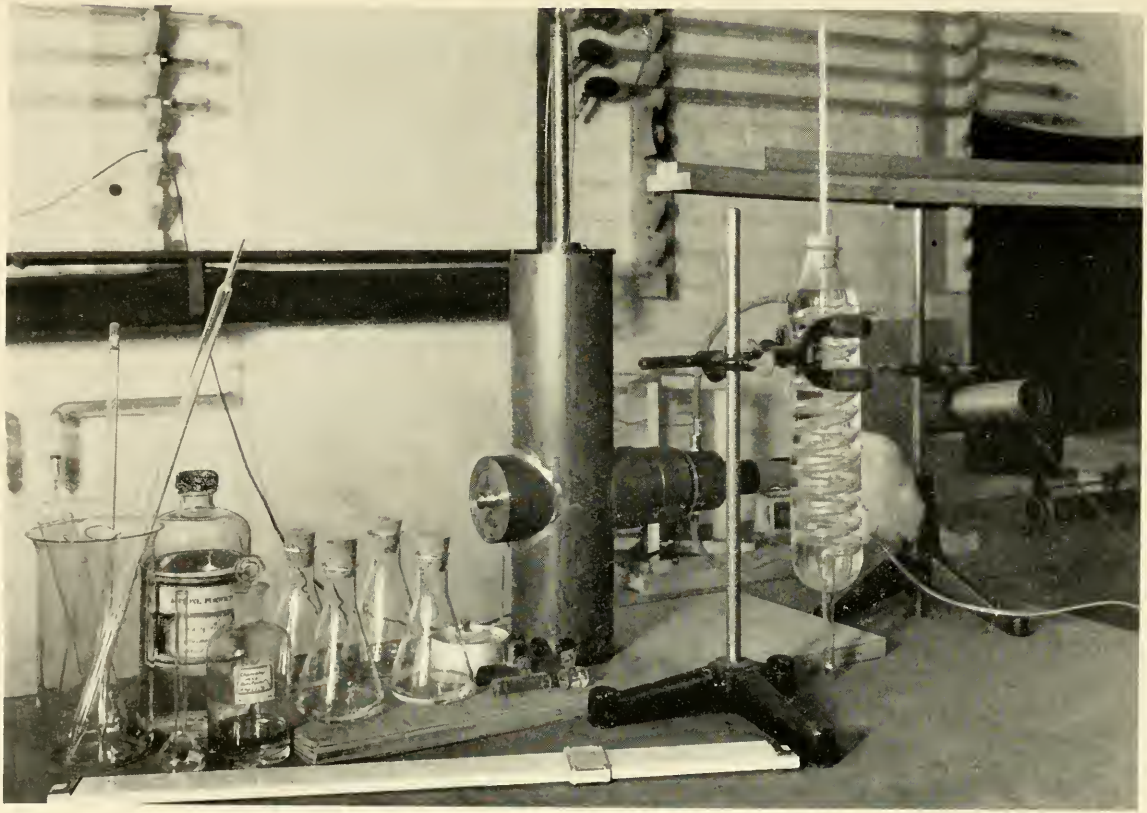
In a study of the influence of the extraction technique on the results, it has been found that preliminary killing of the leaves by immersion in boiling water, or the addition of calcium carbonate during the grinding does not affect the amount of chlorophyll extracted.

Furthermore, in these studies made with barley leaves it was found that the presence of light during the short extraction period is negligible. The chlorophyll content of the extract remains unaltered during at least 2 weeks storage in the refrigerator (about 4° C.).

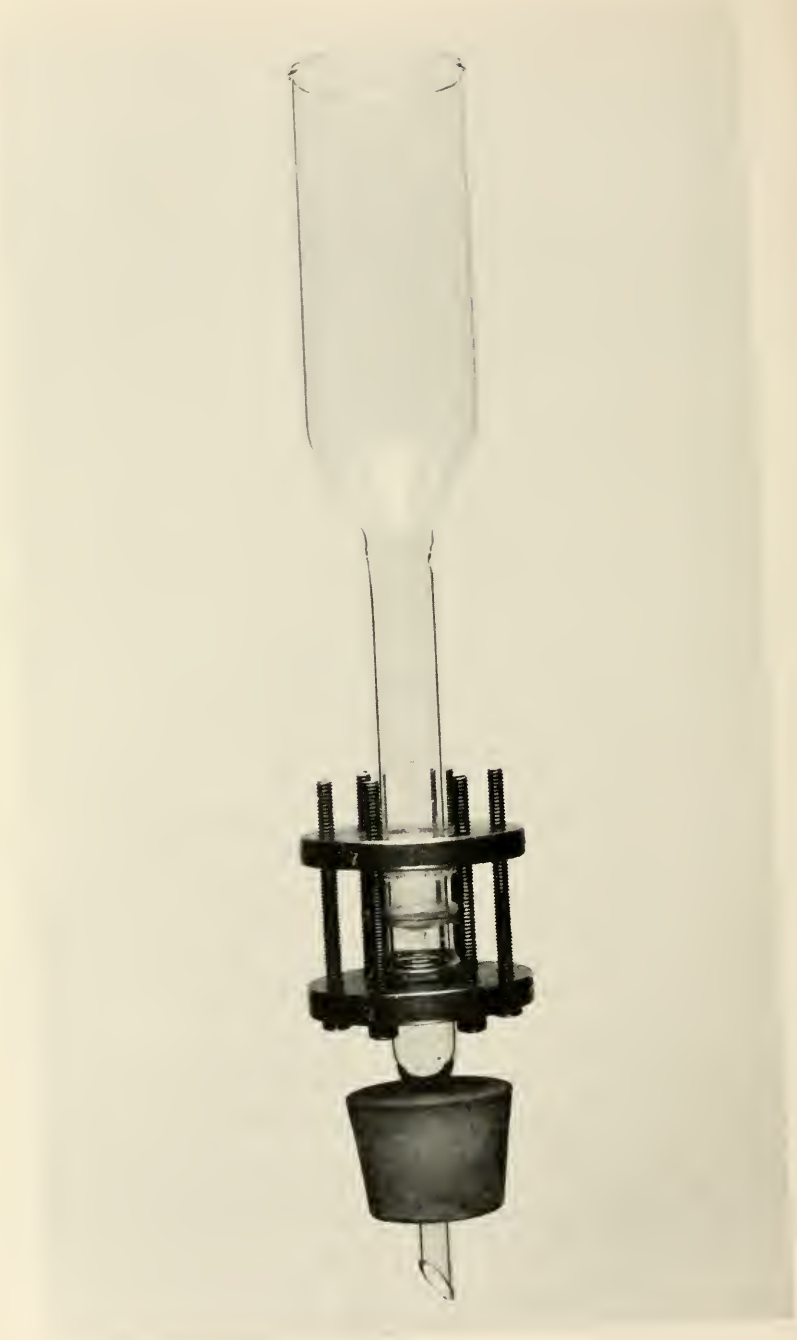
SUMMARY

The method of determining small amounts of chlorophyll herein described is based on the transmission of light in the region of the red absorption band of a solution of chlorophyll in acetone. The transmitted energy is determined by means of a galvanometer and a vacuum thermocouple of extremely high sensitivity. The percentage transmission of the acetone extracts of plant material is then compared with a calibration curve constructed from data obtained with solutions of purified chlorophyll.

This method eliminates the constant use of standard chlorophyll solutions and is not influenced by the presence of carotenoid pigments in the extract. Furthermore, it is unaffected by minor fluctuations in the light intensity, and errors involved in subjective intensity and color comparisons are avoided.



GENERAL VIEW OF APPARATUS USED FOR THE DETERMINATION OF SMALL AMOUNTS OF CHLOROPHYLL



SPECIAL TYPE OF FILTER USED FOR REMOVAL OF SUSPENDED MATERIAL FROM CHLOROPHYLL SOLUTIONS

(Over-all length, 26 cm.)