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IS DIRECT SPECTROPHOTOMETRIC DETERMINATION OF CHLOROPHYLL IN PIGMENT EXTRACTS OF TISSUES UNDER DIFFERENT PHYSIOLOGICAL CONDITIONS VALID?

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Direct spectrophotometric determination of chlorophyll content from methanol extracts of young and senescent leaves of Thunbergia grandiflora showed 29 and 16%, respectively, of mature leaves. Spectrophotometric estimation in the same extracts after separation of chlorophyll by thin layer chromatography or by direct fluorometric determination showed only 15 and 0.5% of mature leaves, indicating an error of 4 to 40 fold. In the above wide range of absorbance (0.5 to 100%) the lack of linearity between absorption and concentration resulted in additional error. Bound chlorophyll remaining in the pellet, not extractable by any solvent system, was also reported to vary depending on the physiological conditions of the tissue (Plant Physiol. 51, 660-666). Therefore, it is pointed out that the aforementioned errors observed in direct spectrophotometric determinations could be checked by monitoring simultaneously the cell or plastid suspensions in vivo by fluorometry without any extraction.

INTRODUCTION

Ever since it was shown that chlorophylls a and b fluoresce independently of one another in mixtures and that the concentrations of these components in acetone mixtures of pure chlorophylls a and b could be determined from the fluorescence of such mixtures (1), fluorometric methods (a) for the estimation of chlorophylls (2), chlorophyllides and pheophytins (2, 3) in pigment mixtures, (b) for monitoring the synthesis of the intermediates in the chlorophyll biosynthetic pathway (4, 5) and (c) for determining Chl a/Chl b ratio (6) have been introduced. Spectrophotometric method for the determination of chlorophyll is invariably used in most of the laboratories, either to monitor changes

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in the pigment contents under various physiological conditions or to express the photosynthetic efficiency on the basis of chlorophyll content, because of the readily available instrumentation and formulae. In the course of our investigation of changes in the pigment contents in aging Thunbergia grandiflora leaves in vivo, spectrophotometric determinations showed significantly large amounts of chlorophyll in methanol extracts of fully senescent and young leaves even though the extracts appeared completely yellow and brown. Estimation of chlorophyll in these extracts by spectrophotometry after separation by thin layer chromatography, or by direct spectrofluorometric monitoring showed very low amounts of chlorophyll, indicating an error of 4 to 40 fold by direct spectrophotometric determination caused by the interference of higher concentrations of accessory pigments and other phenolic compounds. Since the chlorophyll content varied 250 fold in leaves depending on its physiological state, the linearity between absorption and chlorophyll content was examined by diluting the same extract to similar concentrations. Even without the interference of other compounds, different dilutions resulted in different values for the same extract and dilution to 250 fold resulted in an error of 518%. It was also pointed out recently that the fraction of bound chlorophyll varies depending on the physiological conditions of the tissue (7). Therefore, it is pointed out in this article that for accurate determination of chlorophyll content of tissues under different physiological conditions or for any meaningful expression of the photosynthetic efficiency on the basis of chlorophyll content, the concentration of the pigment should be monitored simultaneously by fluorometric method in vivo without any extraction. On the other hand, if spectrophotometry alone is used, it should be corrected for (a) the interference of other compounds, (b) the bound chlorophyll that remains in the pellet and (c) the error caused by samples having different absorbance.

MATERIALS AND METHODS

The unicellular green alga Scenedesmus obliquus D_3 (Gaffron) was grown in Kessler's liquid culture medium as reported earlier (8). Leaves of Thunbergia grandiflora Roxb. at different phases of growth (young, developing, mature, senescing and senescent) were collected from field grown plants under controlled conditions in the botanical garden. Chlorophyll content was determined according to MacKinney (9) after extraction with hot (absolute) methanol and measur-

ing the absorbance in a Beckman DU spectrophotometer. Chlorophylls were purified from accessory pigments and phenolic compounds by thin layer chromatography, following the method of Singleton et al. (10) using the solvent system acetone: petroleum ether (1:3, v/v). Fluorescence emission was monitored in algal cells or pigment extracts after excitation with broad band blue light (400-520 nm, Corning 5113) as reported earlier (11). The photomultiplier (Hamamatzu R375) placed 90° to the excitation beam was protected by an interference filter (λ max 690 nm, Half Band width 12 nm, Schott). The signal from the photomultiplier was directly displayed on an oscilloscope or a servo recorder. For spectrofluorometric determinations algal cells were suspended in 0.1 M phosphate buffer, pH 7.0 with a final chlorophyll concentration of 0.5 µg/ml to avoid self absorption. The pigment extracts were also suitably diluted.

RESULTS AND DISCUSSION

In order to investigate the changes in the pigment contents in aging Thunbergia grandiflora leaves in vivo, leaves at different stages of development were selected and their pigments were extracted in hot methanol. The methanol extracts were used either as such for both spectrophotometric and fluorometric analysis or purified by thin layer chromatography. Typical spectrophotometric measurements of the methanol extracts from different leaves showed highest amount of chlorophyll in mature leaves and lower amounts in young and senescent leaves (Table 1). Although the senescent leaves were completely yellow and their methanol extract did not show even the slightest greenish color, the calculated chlorophyll value was about 16% of that in mature leaves (Table 1). Similarly, the methanol extracts of young leaves had more brownish red color due to the presence of phenolic compounds. In order to check the extent of artifact caused by such accessory pigments and phenolic compounds, the methanol extracts were purified by thin layer chromatography and only the chlorophylls were recovered. The TLC purified fractions obtained from the mature and developing leaves did not show much variation whereas in young and senescing leaves much decrease in the chlorophyll content was observed. In young leaves error up to 40% was found to be caused by the presence of high levels of phenolic compounds. Surprisingly, in completely senescent leaves, presence of high levels of carotenes and xanthophylls gave a 40 fold higher value. These results clearly indicate that the reliability of the spectrophotometric measurements in estimating the concentration of substances depends on their purity and Table 1 shows the extent of artifact that could be caused

TABLE 1

Samples	By Absorption mg chlorophyll/g fresh wt		Fluorescence intensity Relative units
	Methanol extract	TLC purified	
Young	0.69 (29.0)	0.40 (17.3)	6.0 (14.0)
Developing	1.86 (78.2)	1.64 (70.7)	30.0 (69.8)
Mature	2.38 (100.0)	2.32 (100.0)	43.0 (100.0)
Senescing	0.59 (24.8)	0.38 (16.4)	5.6 (13.0)
Senescent	0.38 (16.0)	0.01 (0.4)	0.2 (0.5)

Chlorophyll content in leaves of Thunbergia grandiflora at different stages of growth and aging, determined by spectrophotometric and fluorometric methods. Pigments were extracted in hot (absolute) methanol and the absorbance was measured in a Beckman DU spectrophotometer with suitable corrections for turbidity at 740 nm. For fluorometric determinations, methanol extracts were directly used after suitable dilutions. Figures in parentheses are percentage with reference to their respective maxima.

in chlorophyll determination by other colored compounds. On the other hand, when fluorometric method was used, for methanol extracts without any purification, values corresponding to those obtained in the samples after TLC separation were found. This is due to the fact that there is little contribution of the accessory pigments and phenolic compounds in fluorescence emission. The only effect of these compounds on the fluorescence emission, i.e. the reduction of the actinic light intensity by way of absorption, was eliminated by using dilute samples.

Since the chlorophyll content of the leaves varied 250 fold (Table 1) at different stages, the linearity between absorption and Chl content was examined in this range of absorption. Five-day-old Scenedesmus obliquus cells contained maximum chlorophyll and less accessory pigments (12) and we found this ideal for this study. One ml of algal cell suspension was extracted in hot methanol, diluted to several fold and absorbance was measured in a Beckman DU spectrophotometer. The chlorophyll content per ml culture varied depending on the dilution factor and at the pigment range observed in Table 1 the error was 518% (Table 2). Since the ratio of Chl to accessory pigments or other compounds is the same in all these dilutions, we should have obtained essentially

TABLE 2

Dilution factor	A_{650}	A_{665}	Chlorophyll content (µg)	
			per ml extract	per ml cells
1	0.73	1.30	23.82	23.82 (100)
2	0.42	0.79	13.87	27.74 (116)
4	0.24	0.41	7.51	30.04 (126)
8	0.13	0.23	4.11	32.88 (138)
16	0.07	0.12	2.27	36.32 (152)
32	0.04	0.07	1.30	41.60 (174)
64	0.025	0.045	0.82	52.48 (220)
120	0.015	0.030	0.50	64.00 (268)
256	0.015	0.025	0.48	123.39 (518)

Relation between absorbance and chlorophyll concentration. Pigments were extracted from one ml of 5-day-old Scenedesmus obliquus cells in hot (absolute) methanol and the clarified extract was diluted to several fold with absolute methanol. The absorbance was measured in a Beckman DU spectrophotometer. Figures in parentheses are percentage change of chlorophyll content per ml cells from the original stock solution.

the same chlorophyll content per ml culture and the error is probably caused by the limitation of spectrophotometric instrumentation. Similar variation in chlorophyll content is bound to occur in studies on chlorophyll development, greening, aging, etc. While diluting the extract of mature leaves to match the absorbance of the extracts of the senescent leaves would result in high values (because spectrophotometers do not have linearity in this range) it is also difficult to equalize the absorbance of dilute extracts of senescent leaves to that of mature leaves. In contrast to the spectrophotometric method, fluorometric analysis was found to be suitable for extracts with lower chlorophyll concentrations (Fig 1). Good linearity was observed both in the algal cell suspensions and pigment extracts up to 1.0 µg/ml (Fig 1). Increase in the chlorophyll content above 1.0 µg/ml resulted in quenching of the fluorescence by self adsorption. The minimum detectability by this method is about 1.0 ng/ml which is about 1000 fold more sensitive than the usual spectrophotometric method and this could be improved further by increasing the sensitivity of the detector system.

A fraction of chlorophyll remains bound to the membranes and this is not extractable by any solvent system; and this varies from 20 to 60% of the total

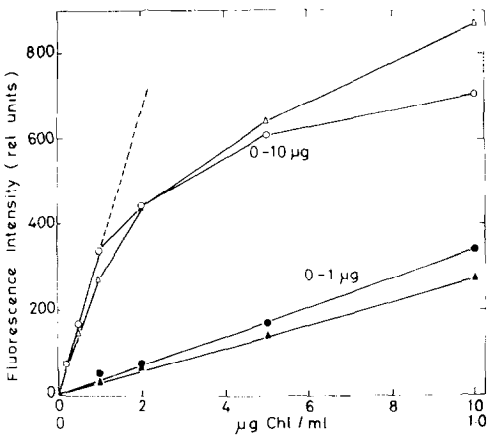

Fig 1. Relative fluorescence intensity as a function of chlorophyll concentration in Scenedesmus obliquus cells (▲-▲ , Δ-Δ) and pigment extracts (●-●, o-o). Fluorescence emission was monitored after excitation with broad band blue light (400-520 nm) and the photomultiplier was protected by an interference filter (λ max 690 nm). For further details see Materials and Methods.

chlorophyll depending on the physiological state of the tissue (7). Therefore, it is suggested that for accurate determination of chlorophyll content, it should be measured in whole cells or plastid suspensions in vivo without any extraction. While it is generally known that fluorometry is more sensitive, we point out there that the errors observed in spectrophotometry could be cross checked and corrected by monitoring the pigments simultaneously by fluorometry.

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REFERENCES

1. Goodwin, R. H. (1947) Anal. Chem. 19, 789.

2. White, R. C., Jones, I. D., Gibbs, E., and Butler, L. S. (1972) J. Agr. Food Chem. 20, 773-778.

3. Bazzaz, M. B., and Rebeiz, C. A. (1979) Photochem. Photobiol. 30, 709-721.

4. Rebeiz, C. A., Mattheis, J. R., Smith, B. B., Rebeiz, C. C., and Dayton, C. F. (1975) Arch. Biochem. Biophys. 171, 549-567.

5. Smith, B. B., and Rebeiz, C. A. (1977) Photochem. Photobiol. 26, 527-532.

6. Boardman, N. K., and Thorne, S. W. (1971) Biochim. Biophys. Acta 253, 222-231.

7. Rebeiz, C. A., Crane, J. C., Nishijima, C., and Rebeiz, C. C. (1973) Plant Physiol. 51, 660-666.

8. Daniell, H., Kulandaivelu, G., and Chandrasingh, V. (1980) Z. Naturforsch. 35C, 136-138.

9. MacKinney, G. (1941) J. Biol. Chem. 140, 315-322.

10. Singleton, W. S., Gray, M. S., Brown, M. K., and White, J. L. (1965) J. Am. Oil Chem. Soc. 42, 53.

11. Kulandaivelu, G., and Daniell, H. (1980) Physiol. Plant. 48, 385-388.

12. Daniell, H., and Kulandaivelu, G. (1981) in G. Akoyunoglou (ed.), Proc. Vth Int. Cong. Photosynth., in press.