FLUORESCENCE-SPECTRUM CURVES OF CHLOROPHYLLS, PHEOPHYTINS, PHYCOERYTHRINS, PHYCOCYANINS AND HYPERICIN[1]

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Any quantitative investigations of fluorescence phenomena more or less presuppose some information as to the spectral emission curve of the substances under investigation. Knowledge of the shape of the spectral emission curves of pure substances is necessary to various degrees of accuracy for the identification of pigments by fluorescence, for the detection of fluorescent impurities in mixtures, for the appropriate selection of filters or photocells to measure the fluorescence intensity, and for planning experiments so as to avoid reabsorption of the fluorescence within the sample. Reliable curves for the pure substances are particularly necessary for the analysis of the fluorescence spectra of complex fluorescent mixtures or of living cells. The fluorescent properties of chlorophyll have been investigated from many points of view since the fluorescence of chlorophyll was discovered by Brewster in 1834. Stokes, who discovered the basic principles of fluorescence, also found that chlorophyll fluoresces in leaves. In spite of the widespread occurrence in nature of chlorophyll and its scientific importance we know of only one investigation, Zscheile and Harris (32), reporting the precisely measured fluorescence spectra of pure chlorophylls a and b. The fluorescence spectrum of bacteriochlorophyll in solution has been measured by Vermeulen, Wassink, and Reman (27) and in the intact bacteria by Duysens (9). The present paper revises slightly the calculations upon which Zscheile and Harris based their curves and presents precise curves for other plant pigments, particularly those concerned in the process of photosynthesis.

The yield of chlorophyll fluorescence in polar solvents has recently been found by Livingston and Forster (18) to be 25 %, in contrast to the older accepted value of 10 %. The low fluorescence in pure non-polar solvents and the enhancement of its intensity by traces of polar solvents has been studied by Livingston, Watson, and McArdle (19). Various aspects of chlorophyll fluorescence have been reviewed by Rabinowitch (22), De Ment (5), Pringsheim (21), and Förster (10). Fluorescence spectroscopy provides a very sensitive method for the identification and quantitative determination of the various chlorophylls and other fluorescent substances in plant extracts (13, 14, 15, 23, 29, 30).

Fluorescence spectroscopy of photosynthetic pigments in live plants has been used in studying energy transfer from one pigment to another by Dutton, Manning and Duggar (7), Wassink and Kersten (31), van Norman, French and Macdowall (26), French and Young (12), and Duysens (8, 9). The interpretation of the fluorescence spectra of live plants containing several fluorescent pigments and the use of these spectra in the study of energy transfer depend upon a knowledge of the fluorescence spectra of the individual pigments. Most of the previous investigators of the spectral characteristics of the photosynthetic pigments used visual or photographic observation of the wavelength position of the peaks. Much valuable exploratory work of this type was done by Dhéré and his collaborators (6). Visual and photographic methods are reasonably adequate for measuring the wavelength of major emission bands but are poor for the lesser maxima and often entirely inadequate for those bands that show only as a shoulder on a more intense band. Careful photometry of photographic fluorescence spectra, even when combined with the best calibration procedures, is less precise than the direct photoelectric spectrophotometric measurement of fluorescence curves except when the fluorescence is so weak that only prolonged photographic exposures can detect it. Furthermore, photographic and visual procedures may show distorted positions of the wavelength of the peaks due to the characteristics of the photographic emulsion or of the human eye.

Considering the utility and significance of precise fluorescence curves of biologically important pigments, it is surprising that so few of them have been measured. The chance to make such measurements arose through fortunate coincidences: the pure chlorophylls were on hand through the work of Smith and Benitez (23), the pure phycobilins were prepared for another investigation by Blinks and Airth at the Hopkins Marine Station, and the equipment was on hand from previous work (11, 12, 15, 28, 29, 30).

APPARATUS AND PROCEDURE

The equipment used in these measurements was that of French and Young, modified by Virgin. It has not yet been described in detail, although some of the components are here discussed and previously a block diagram of the apparatus was presented (11). In brief, the apparatus works as follows: The exciting light comes from a high-pressure mercury lamp used with a large grating monochromator or, alternatively, from a medium-pressure mercury lamp with filters. Provision is made for measuring by a thermopile the intensity with which the sample is irradiated. The sample is placed in a holder illuminated on its face at an angle and some of the fluorescent light emitted from the illuminated face is collected by a spherical mirror and passed through another large-grating monochromator to an RCA 6217 photomultiplier. Both monochromators are ordinarily used with a total spectral slit width of 10 mμ. The light from

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the mercury lamp run on the 60-cycle power line coming in 120-cycle pulses produces voltage pulses of this frequency from the photomultiplier. After AC amplification this voltage is attenuated by a potentiometer driven by a cam linked to the monochromator's wavelength drive. This cam introduces corrections for change in sensitivity of the photomultiplier with wavelength, etc. The partially corrected signal then goes to a photoelectric curve follower which compensates for the small though highly reproducible residual errors in the cam. The 120-cycle signal is selected from random noise by a tuned amplifier, rectified, and recorded on a Brown millivolt recorder whose strip chart paper is driven by the monochromator's wavelength drive motor through selsyn coupling. The linear wavelength scale on the recording paper is such that the 1/3″ lines represent 5 mμ. The cam is shaped and the curve on the photoelectric follower drawn so that the apparatus plots correctly the energy distribution for the light from a standard tungsten lamp reflected from magnesium oxide. Filters are used to remove from the fluorescence emission stray light of wavelengths shorter than 570 mμ. The usable range extends from 570 to 770 mμ with this photomultiplier tube. This range was chosen as being suitable for the fluorescence of photosynthetic plant pigments and allows the use of incident light from 560 mμ to shorter wavelengths. Fluorescence excitation spectra may be measured with this equipment from 400 to 560 mμ. Without the correcting mechanism and the stray light filters the equipment has been used for measuring uncorrected fluorescence spectra from about 430 to 770 mμ. In general the intensity measurements appear to be correct within a few percent. We have not attempted to make absolute measurements of fluorescence intensity or yield.

Several different types of sample cells were used, for the most part a 1-cm rectangular Beckman cell which had on its face a slit 1 mm wide between two parallel strips of aluminum foil cemented to the glass. The slit served to restrict most of the fluorescent light effectively emitted from the cell to a layer 1 or 2 mm in depth. This effect is achieved because the fluorescent light is collected only from the volume of the solution in which the incident and emergent beams cross each other. This active volume is restricted to that near the face of the cell by the slit in the aluminum foil. Thus most of the emitted light has to pass through only a thin layer of solution which avoids the reabsorption of the fluorescent light by the solution. Another arrangement used for smaller samples was a Lucite block milled to a depth of 0.5 mm and covered by a microscope slide. With this cell a covering slit was unnecessary due to the thinness of the layer. Cells of adjustable thickness similar to those used in infrared work were also tried but were found to be less convenient. All of the measurements were carried out in redistilled solvents which were neither dried nor deoxygenated.

It is possible to correct for the distortion introduced by the reabsorption of fluorescent light within the solution by equations given by Förster (10) and Duysens (9). In practice, however, we find it more convenient to make measurements with successive dilutions in approximate steps of two with a given solution until two curves of identical shape are obtained. In this way one is sure of avoiding reabsorption distortion and the uncertainties involved in the calculation are avoided. The curves here reported are considered to be free of reabsorption distortion. The linearity of the recording system was checked by reducing the deflection with calibrated screens at the peak or at other significant wavelengths of the fluorescence curve.

The absence of stray light, particularly of the exciting wavelength, in the fluorescence spectra was demonstrated by substituting magnesium oxide for the sample and recording the output of the instrument without changing the conditions of measurement such as incident light intensity and amplification. Adequate freedom from stray light was obtained under the conditions used for recording all the curves reported here because of their relatively intense fluorescence. With plant materials containing very small amounts of pigments, this stray light must occasionally be measured and subtracted. The incident wavelength was usually 436 or 405 mμ. With pigments that absorbed more strongly elsewhere other exciting wavelengths were used, e.g., for bacteriopheophytin 525 mμ and for hypericin and some phycobilins 546 mμ.

Results and Discussion

Chlorophylls and Pheophytins: The fluorescence of chlorophylls a and b in ether has been measured with high precision by Zscheile and Harris (31). Dr. L. N. M. Duysens has kindly called our attention to a minor error in the calibration upon which Zscheile and Harris' fluorescence curves are based. The relative response of their photocell to different wavelengths was measured by comparing the photocell response to the thermopile response when both were placed alternately behind the slit of the monochromator. This procedure calibrates correctly the photocell response to different wavelengths. But there are two factors which prevent this type of calibration from giving a precise measure of the energy distribution of the fluorescent light. In the first place, the slit width in terms of millimicrons isolated is not constant throughout the spectrum. Thus at longer wavelengths a larger wavelength region is admitted to the photocell. In regard to the curves of Zscheile and Harris, figure 4, it is stated that the monochromator slits were 0.2 mm wide. This slit width isolated spectral regions of 9.2, 10.2, 12.0, and 13.8 mμ at 630, 660, 660, 710 and 760 mμ respectively. Since the amount of energy transmitted is proportional to the band width isolated by the monochromator, these figures have been used to apply the necessary correction for the variable band width. This correction reduces the height of the 730 fluorescence peak of chlorophyll a to the 664 mμ peak by the factor $103.5/123.5 = 0.833$. A second possible source of trouble lies in the varia-

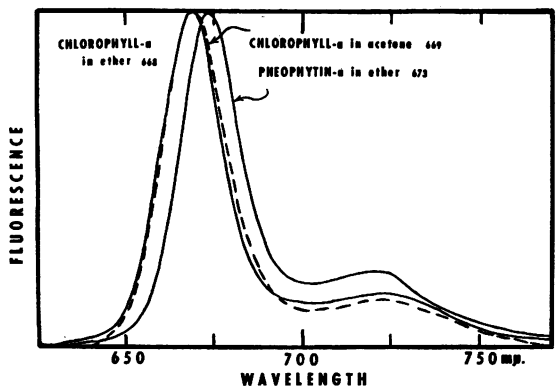

FIG. 1. The fluorescence spectra of chlorophyll a and pheophytin a.

tion of transmission of the monochromator with wavelength, due largely to the variable reflection loss for different wavelengths. Since the total wavelength range covered is small and the necessary data unavailable, we have neglected this minor correction. The curves by Zscheile and Harris recomputed in this way have been published elsewhere in comparison with our chlorophyll a and b curves (23).

The fluorescence spectra of the chlorophylls are shown in figures 1 to 5. These measurements were made with the highly purified chlorophylls prepared by Smith and Benitez (23, 24). Most of the measurements were made in ether, although a few curves in acetone are included. The fluorescence maximum for chlorophyll a dissolved in ether was found by us to be 668 mμ, by Livingston, Watson and McArdle (19) 666 mμ, and by Zscheile and Harris (32) 664.5 mμ. The difference in the positions of the fluorescence peaks found by us and by Zscheile and Harris is consistent with the difference in the absorption peaks also observed by the two groups. The discrepancies while small are outside the experimental errors of the wavelength measurement. They probably indicated differences in the isomeric composition of the chlorophyll or in complex formation with the solvents.

The fluorescence spectrum of pheophytin c in ether shown in figure 3 is quite different from that of pheophytins a, b, and d, whose fluorescence spectra more closely resemble those of the corresponding chlorophylls. The strange shape of this curve may be due to the possibility of some effect of acid upon chlorophyll c other than the mere removal of magnesium. It is, however, similar in shape to the spectrum of protopheophytin shown in figure 5. This protopheophytin curve may be less reliable than the others in this paper because the material was an unpurified acid-acetone extract of the inner seed coats of squash. Acetone extracts of similar seeds, however, showed light absorption in the orange and red part of the spectrum to be due only to protochlorophyll, so it is highly likely that no other orange or red fluorescing substances other than protopheophytin were present in this extract.

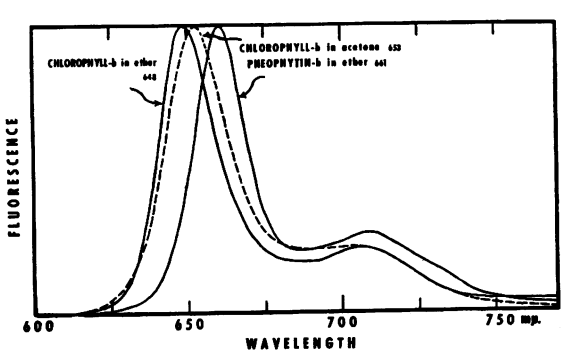

FIG. 2. The fluorescence spectra of chlorophyll b and pheophytin b.

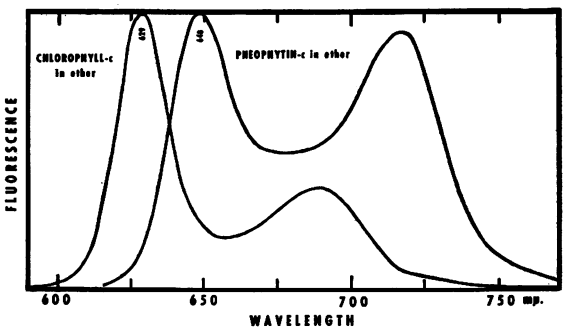

FIG. 3. The fluorescence spectra of chlorophyll c and pheophytin c.

In figure 6 is given the fluorescence of purified bacteriopheophytin in ether and the spectrum of a green pigment found in partially purified preparations of bacteriochlorophyll. The separation of this green pigment has been described by Smith and Benitez (23) and by Holt and Jacobs (17). This green pigment is responsible for the peak at about 700 mμ in the fluorescence curve (measured with wide slits) for bacteriochlorophyll reported by Vermeulen et al (27).

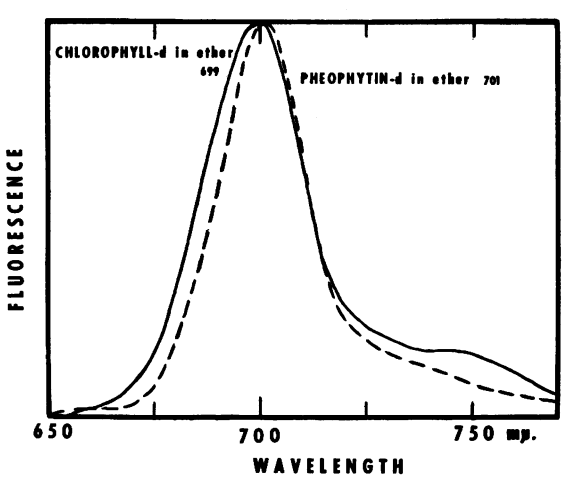

FIG. 4. The fluorescence spectra of chlorophyll d and pheophytin d.

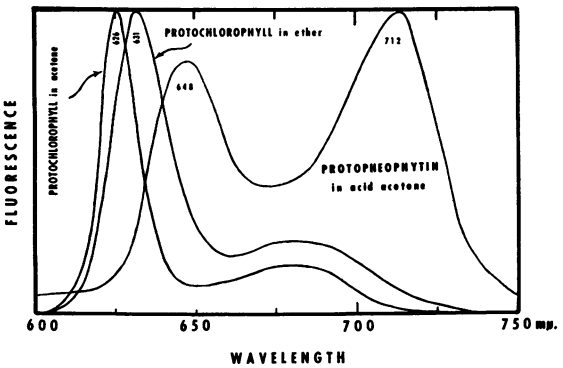

FIG. 5. The fluorescence spectra of protochlorophyll from barley and of protopheophytin in an acid acetone extract of a squash seed coat. The latter curve was made in collaboration with Dr. Robert Hill.

Purified preparations of bacteriochlorophyll do not show this fluorescence peak at 687 mμ. Since the main peak of bacteriochlorophyll comes at a longer wavelength than 770 mμ, we were not able to measure it.

PHYCOBILINS: All phycobilin pigments were isolated from *Porphyra naiadum*. They were separated by column chromatography using the method of Swingle and Tiselius (25) with the omission of sucrose, which is necessary only to carry out a frontal analysis. In addition one sample of phycoerythrin was obtained by crystallization. The absorption spectra of phycocyanin and allophycocyanin, each of which had been rechromatographed once, and of crystalline phycoerythrin are shown in figure 7. The preparation and properties of these pigments have been described by Airth (1). It is apparent from these spectra that the phycocyanin was slightly contaminated by phycoerythrin and that the allophycocyanin contained considerable phycocyanin. About 10 % of the total light absorbed by the allophycocyanin solution at 546 mμ was due to phycocyanin. Blinks has tentatively designated the phycoerythrin from *P. naiadum* as B-phycoerythrin. The allophycocyanin from several species of red algae, which was isolated and described by Haxo, O'h Eocha and Norris (16) had absorption maxima at 650 mμ; whereas the allophycocyanin described here has its maximum absorption at 654 mμ (pH 5 to 7). The phycocyanin from *Porphyra naiadum* has the absorption characteristics of Cyanophyta phycocyanin even though this alga belongs to the division Rhodophyta.

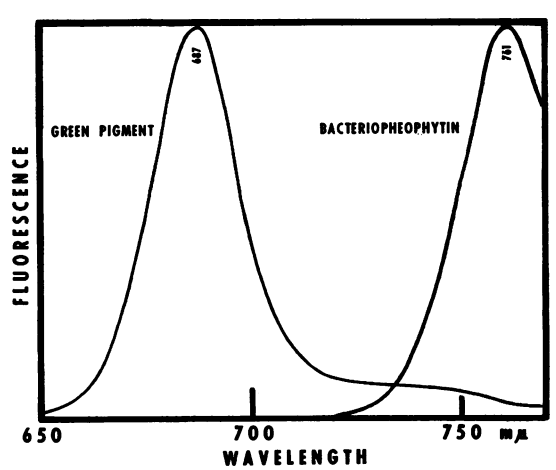

FIG. 6. The fluorescence spectra of bacteriopheophytin and of the green pigment found in preparations of bacteriochlorophyll before final purification. This green pigment is probably acetyl chlorophyll a, which is also called bacterioviridin.

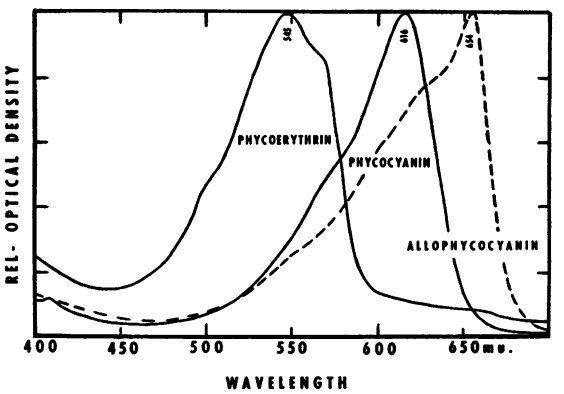

FIG. 7. The relative absorption spectra of the phycobilin pigments isolated by Airth (1) from *Porphyra naiadum*. The phycoerythrin was obtained by crystallization and the others by chromatography. These solutions were used for the fluorescence spectra of figure 8.

After determining the absorption spectra, the same phycobilin solutions were used for the fluorescence spectrum measurements presented in figure 8. For the chromatographically isolated compounds the pH was 5.0; in the case of crystalline phycoerythrin the pH was 7.0. The absorption spectra of the two phycoerythrins were similar except that the minor band at about 565 mμ was higher in the chromatographically isolated phycoerythrin. The height ratios

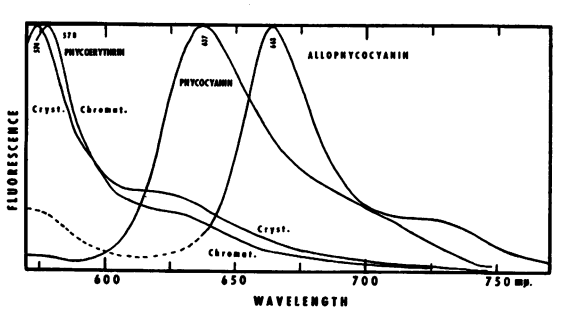

FIG. 8. The fluorescence spectra of the phycobilins from *Porphyra naiadum*. A phycoerythrin preparation purified by crystallization gave a fluorescence peak at 574 mμ while that prepared by chromatography had its peak at 578 mμ like most of the other phycoerythrins that we have measured.

at 565 and 545 mμ of the crystalline phycoerythrin was 0.87 while that for the chromatographic phycoerythrin was 0.93. Furthermore, chromatographic phycoerythrin always contained a phycocyanin impurity which was impossible to remove. In this sample of phycoerythrin the phycocyanin impurity absorbed about 1 % as much at 546 mμ as did the phycoerythrin itself. The fluorescence maximum at 637 mμ for phycocyanin is considerably less than that deduced from the spectrum of the intact algae by French and Young (12) in Porphyridium.

Duysens (9, fig. 6.2) has given the fluorescence and absorption spectra of Oscillatoria phycocyanin. Both absorption and fluorescence spectra for the partially purified phycoerythrin from Porphyridium have been published (11). Corresponding curves for the pure C-phycoerythrin from Porphyra, prepared by Blinks, are also included in (11). We know of no other fluorescence spectrum of allophycocyanin. Absorption curves of phycoerythrin from different sources may show one, two or three peaks; the fluorescence spectra of all phycoerythrins are, however, nearly identical.

HYPERICIN: The absorption spectrum, and photodynamic action of hypericin which sensitizes animals to light has been given by Blum (3). Its fluorescence has been qualitatively investigated by Dhéré (6). Its synthesis has been achieved by Brockman and Muxfeldt (4), and its absorption spectrum measured by Pace and Mackinney (20).

The yellow flowers of *Hypericum perforatum* in the intact state show no fluorescence. When they are immersed in methanol a non-fluorescent yellow pigment comes out. If this solution is allowed to stand for a day or so it darkens and the characteristic fluorescence of hypericin appears. The preparation used was obtained by crushing about twenty flowers with a glass rod and allowing the brei to stand, after which it was extracted with 95 % ethanol. After about two hours the clear red extract was washed several times with small portions of petroleum ether in a separatory funnel to remove the chlorophyll pigments, a small amount of water being added to separate the two phases. The red pigment solution was then evaporated to about 10 ml and an equal volume of absolute alcohol was added. This gave a crude preparation containing hypericin and perhaps also pseudohypericin. Its fluorescence curve is given in figure 9.

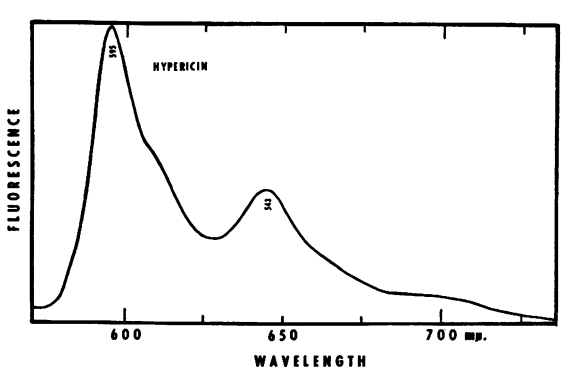

FIG. 9. The fluorescence spectrum of a partially purified ethanol extract of the petals of *Hypericum perforatum*.

SUMMARY

The spectral curves of fluorescence emissions for the following pigments have been measured and are presented in this paper: chlorophylls a, b, c, d, and protochloropyll and their corresponding pheophytins; bacteriopheophytin; phycoerythrin, phycocyanin, allophycocyanin; and hypericin.

Numerous discussions with Professor Lawrence R. Blinks and the use of the phycobilin preparations from his laboratory which made one phase of this work possible are gratefully acknowledged. Dr. L. N. M. Duysens was a member of the laboratory group during most of the time that these measurements were being made and we are indebted to him for many helpful comments. Earlier fluorescence curves for many of the substances reported here were measured by Dr. Violet Koski Young, who also collaborated in the development of the procedures. Improvements in the equipment after she left as well as the greater purity of some of the compounds later available, however, led us to base this paper mainly on the more recent data. The electronic components were designed, revised, and tested by Messrs. B. G. Ryland, H. C. Patton, Jr., D. R. Schuech and G. H. Towner. The mechanical parts of the monochromator were constructed by Mr. Frank Schuster. We wish to thank all of these men for the continued reliability of the apparatus over a six-year period.

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UTILIZATION OF BETAINE AS A METHYL GROUP DONOR IN TOBACCO[1,2]

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The methyl groups of choline were shown to serve as precursors for the N-methyl group of nicotine in three-month-old photosynthesizing tobacco plants (3). On the other hand, choline yielded no methyl groups to hordenine in barley seedlings (9), to ricinine in etiolated castor bean seedlings (7) or to protopine in young Dicentra hybrids (11). It was suggested from these studies that the success or failure of choline to be a methyl group precursor in different species might be related to the presence or absence of a choline oxidase which would catalyze the oxidation of choline to betaine. If the oxidation of choline to betaine were first necessary for methylation to occur, then betaine might act as a methyl group donor in plants which could not use choline. Sribney and Kirkwood (12) recently tested this possibility and found that in barley seedlings betaine methyl groups served as precursors for the methyl groups of N-methyl tyramine and hordenine. However, in sprouting castor beans, betaine failed to supply methyl groups to ricinine (12). The question, therefore, as to whether choline is first oxidized to betaine before it can serve as a methyl donor in higher plants is not completely answered.

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[2] The data in this paper were taken from a thesis presented by C. S. Sato as partial fulfillment of the requirements for the Ph.D. degree.

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