Fluorometric Estimation of Chlorophylls, Chlorophyllides, Pheophytins, and Pheophorbides in Mixtures

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Procedures have been described for the estimation of any one or more of the eight component groups of pigments comprised of chlorophylls a and b, chlorophyllides a and b, pheophytins a and b, and pheophorbides a and b in solution in diethyl ether or in 80% acetone. Estimation is based on calculations derived from readings made with three primary and one or two secondary filters. Depending upon the filter systems used, the identity and concentration may be determined over the concentration range from 0.001 to 0.3 μmol per liter for all pigments excepting pheophytin a and pheophorbide a, which must be present at concentrations three times larger. Fluorometric estimation may be accurately and rapidly made at pigment concentrations of 1×10^{-2} to 1×10^{-3} of those required for spectrophotometric procedures. The instruments required are relatively compact, sturdy, and inexpensive in comparison with spectrophotometric equipment.

It has been established that chlorophylls a and b fluoresce independently of one another in mixtures and that the concentration of these components in acetone mixtures of pure chlorophylls a and b could be determined from the fluoresence of such mixtures when irradiated with blue and violet light (436 and 405 nm) (Goodwin, 1947). A study of the accuracy of the fluorometric method for the estimation of chlorophyll in phytoplankton using a Turner fluorometer equipped with a blue lamp for increased sensitivity has been made. The chlorophyll of phytoplankton was found to be mainly chlorophyll a and fluorometric estimation was based on readings taken before and after acidification with oxalic acid to convert the chlorophyll to pheophytin (Yentsch and Menzel, 1963). Studies have been made of the continuous measurement of *in vivo* chlorophyll concentration of phytoplankton based on observations with a Turner model 111 fluorometer in which the standard photomultiplier (RCA 931A) had been replaced with the somewhat more red-sensitive one (R136) (Lorenzen, 1966).

Spectrophotometric procedures for the estimation of the components of mixtures of chlorophylls a and b and derivatives have been described (Vernon, 1960; White *et al.*, 1963). This report is an investigation of the substitution of fluorometric for spectrophotometric techniques in the estimation of multicomponent mixtures of pigments similar to those of White *et al.* (1963).

EXPERIMENTAL

Pure samples of the a and b forms of chlorophyll, pheophytin, and pheophorbide were prepared as described by Jones *et al.* (1968). The chlorophyllides were prepared by enzymatic hydrolysis of highly purified chlorphyll a and chlorophyll b (Jones *et al.*, 1972). Formation of chlorophyllide was followed by thin-layer chromatography (Jones *et al.*, 1972).

In preliminary studies adsorption on glass surfaces occurred with the chlorophyllides and pheophorbides during dilution and aliquot measurement and was responsible for large errors. Adsorptive losses could be prevented by the addition of oxalic acid at a concentration of 0.001% for diethyl ether and of 0.00005% for 80% acetone solution. Higher oxalic acid concentrations caused error with the chlorophyllides through conversion to the pheophorbides. It was found to be necessary to make up the acidified solvents daily as used to avoid undesirable changes resulting from acidification. In clean-up operations all vessels used in handling and storage of chlorophyllides and pheophorbides were given a preliminary rinse with acetone acidified with a few drops of 6 *N* hydrochloric acid.

The concentration of suitably diluted aliquots of the various pigments in diethyl ether was established by means of a Beckman DK-2A Spectrophotometer based on the molar absorbance coefficients of White *et al.* (1963). Pure aqueous acetone solutions (80%, v/v) of the pigments were obtained by removal of the diethyl ether through vacuumization of aliquots of pigment solutions diluted with pure acetone followed by adjustment with appropriate volumes of pure acetone and water. The molar absorbance coefficient of each pigment in 80% acetone was determined from its absorbance in each solvent and the coefficient in diethyl ether. The coefficients for the chlorophylls and pheophytins agreed closely with those determined by Vernon (1960). Molar absorbance coefficients for the chlorophyllides and pheophorbides in 80% acetone was nearly identical to those for corresponding chlorophylls and pheophytins, a similarity previously reported for these pigments in diethyl ether (White *et al.*, 1963).

A Turner model 111 fluorometer equipped with a blue fluorescent lamp (F4T5) and a high sensitivity door was used. Samples in diethyl ether or 80% acetone were read in round Pyrex cuvettes fitted with polypropylene stoppers. Primary and secondary filters were selected following determination of the excitation and emission spectra of the various pigments by means of the Aminco-Bowman Spectrophotofluorometer. Observations on the Turner fluorometer were made using combinations of series of glass filters by Corning or of interference filters by Bausch & Lomb covering the following approximate ranges—for the excitation filter, 350–460 nm, and for the emission filter, 620–680 nm.

Limited observations were made initially with the fluorometer equipped with the standard photomultiplier tube. The primary filters were narrow band-pass filters with the following approximate wavelength for maximum transmission: CS #5-62, 405 nm; CS #5-74, 430–436 nm; CS #5-75, 460 nm. The secondary filter was a sharp cut filter, CS #2-60.

The major portion of this study was conducted with the fluorometer equipped with the more red-sensitive R136 photo-

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Table I. Filter Identification and Characterization

Filter type and reference no.	Filter position	Control data			
		Peak wavelength		Peak transmittance	Half width
Narrow band-pass interference[a]		Nominal	Actual		
405	Primary	405 nm	402 nm	46%	11 nm
440	Primary	440 nm	442 nm	45%	11 nm
460	Primary	460 nm	465 nm	49%	11 nm
680	Secondary	680 nm	680 nm	40%	10 nm
650	Secondary	650 nm	653 nm	47%	10 nm

	Wavelength at which transmission was	
Sharp cut glass[b]	Greater than 37%	Less than 0.5%
CS #2-60	619 nm	599 nm

[a] Bausch and Lomb, 2nd order interference. [b] Corning.

Table II. Fluorescence Maxima (nm) of Pigments

	Excitation max		Emission max	
Pigment	Diethyl ether	80% acetone	Diethyl ether	80% acetone
Chlorophyll a	420	432	668	674
Chlorophyll b	454	466	649	659
Pheophytin a	400	398	673	676
Pheophytin b	416	437	661	662

are at a concentration of 1.00 μmol per liter. Regression equations were determined from replicated observations. Y intercept values were established for each pigment. Such values were not significantly different from a Y intercept value of 0. The regression lines were linear, indicating that error caused by reabsorption of fluorescence due to thickness of solution layer or to solution concentration was negligible.

Mixtures of known quantities of the purified pigments, chlorophylls a and b and pheophytins a and b, were made up

Table III. Relative Fluorescence Intensity Values[d]

	Filter systems, as designated					
	405[a]		440[b]		460[c]	
Pigment	Ether	Acetone	Ether	Acetone	Ether	Acetone
Chlorophyll a (C_a)	1390	1150	763	405	625	361
Chlorophyll b (C_b)	139	114	1230	508	3490	1793
Pheophytin a (Py_a)	1460	1190	81.2	83.5	102	101
Pheophytin b (Py_b)	417	346	1250	1134	1190	1210

[a] Primary, 405; secondary, 680 + (#2-60); aperture 10×. [b] Primary, 440; secondary, 650 + (#2-60); aperture 3×. [c] Primary, 460; secondary, 650 + (#2-60); aperture 10×. [d] Expressed as calculated dial readings for pigments at concentration of 1.0 μmol per l. Pigment in diethyl ether or in acetone (80%) as indicated.

multiplier tube. The filters used are listed and characterized in Table I. In some experiments the secondary filter CS #2-60 was used alone, the narrow band-pass interference filter being omitted. The omission of the interference secondary increased the sensitivity about ten times but decreased the selectivity. Observations were made of replicated serial dilutions of pure pigments to provide estimates of the relative fluorescence intensity of solutions with filter combinations listed in Table I. The concentrations were adjusted to permit fluorometer readings between 10 and 90, when the range selector regulating the amount of exciting light was set at the 3× or the 10× position. The selection of filters was based on the excitation and fluorescence maxima of the pigments in diethyl ether and 80% aqueous acetone (Table II). The emission peaks agree closely with those reported by French *et al.* (1956).

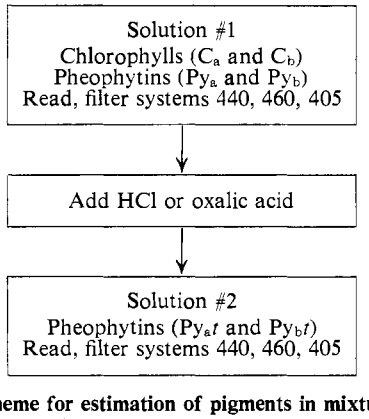

Figure 1. Scheme for estimation of pigments in mixtures in diethyl ether or acetone (80%)

RESULTS AND DISCUSSION

Presented in Table III are relative fluorescence intensity values for the chlorophylls and pheophytins in diethyl ether and 80% acetone solutions, respectively. The intensity values are expressed as calculated dial readings on the Turner 111 fluorometer fitted with indicated filter systems when pigments in diethyl ether and also in 80% aqueous acetone. The ratio of the a to b components was approximately 2:1, respectively. Estimation of the pigments from the mixture was accomplished according to the scheme, as shown in Figure 1. Pigment solution #1 was read with filter systems designated as 440, 460, and 405 (Table II). Pigment solution #2 was formed by acidification of solution #1 but the acid used was

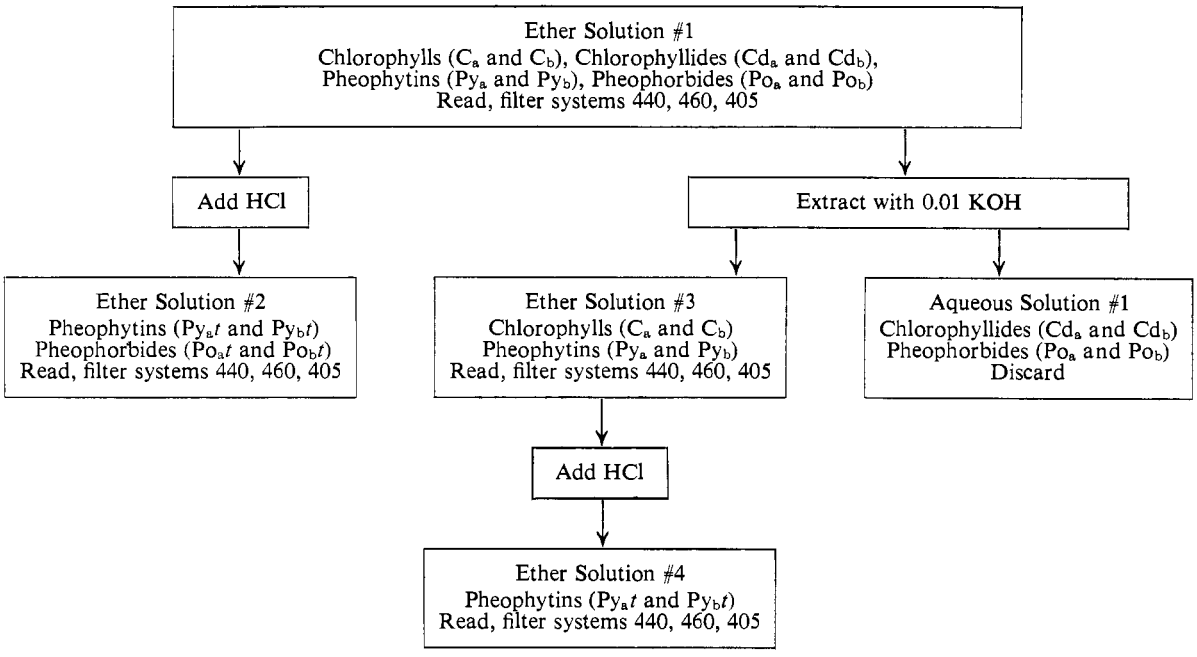

Figure 2. Scheme for estimation of pigments in mixtures in diethyl ether

different, depending upon the solvent. Diethyl ether solutions were acidified by the addition of 0.1 ml of concentrated hydrochloric acid to 50 ml of solution and permitted to stand for 2 hr before reading. When 80% acetone was the solvent the procedure was as follows. To 10 ml of the pigment solution was added 1 ml of 0.5 oxalic acid in 80% acetone. The solution was permitted to stand 2 hr and was then diluted to 25 ml with 80% acetone for reading. Pigment solution #2, whether the solvent was diethyl ether or 80% acetone, was read with each of the filter systems designated as 440, 460, and 405.

Derivation of Equations for Pigment Estimation. Reference to the pigments will be by means of the symbols indicated in Table III and Figures 1 and 2. The symbol F has been used to indicate fluorescence (dial readings) of a sample, with appropriate superscripts and subscripts attached to designate, respectively, the specific filter system used and the specific pigment or pigment mixture in the solution. Filter systems are as characterized in Table III. The symbols C_a, C_b, Py_a, and Py_b represent the pigments chlorophyll a, chlorophyll b, pheophytin a, and pheophytin b, respectively, or their respective concentrations in micromoles per liter. If the pigment solution has been acidified to form pheophytins from chlorophylls, then the letter A is also added as a subscript.

When a solution containing the four pigments chlorophyll a, chlorophyll b, pheophytin a, and pheophytin b, the equations required to estimate each component are developed according to the examples which follow. Chlorophylls a and b are estimated by the changes in fluorescence with filter systems designated as 440 and 460 from readings taken before and after acidification of the pigment solutions. Such fluorescence change is indicated by ΔF which is equal to $F - F_A$. Pheophytin a and b are estimated from fluorescence readings of acidified solutions read with filter systems designated as 440 and 405.

In this study involving solutions of four pigments there has been a verification of an earlier observation (Goodwin, 1947) to the effect that pigments in mixtures fluoresce independently. Accordingly, total fluorescence (dial reading) of a pigment mixture is the sum total of the fluorescence of each component in the mixture. This may be expressed as

$$F^{440} = F_{C_a}{}^{440} + F_{C_b}{}^{440} + F_{Py_a}{}^{440} + F_{Py_b}{}^{440} \quad (1)$$

By substituting the appropriate relative fluorescence intensity values listed in Table III into eq 1, eq 2 is obtained, which for pigments in diethyl ether, is:

$$F^{440} = 763\ C_a + 1230\ C_b + 81.2\ Py_a + 1250\ Py_b \quad (2)$$

Equation 2 indicates the relative contribution of each pigment toward the total fluorescence under the conditions of these observations (specific filter systems, specific instrument, diethyl ether as solvent, etc.). Upon acidification of the solution as specified above, conversion of the chlorophylls may be indicated by eq 3:

$$F_A{}^{440} = F_{Py_at}{}^{440} + F_{Py_bt}{}^{440} \quad (3)$$

where symbol t indicates total pheophytin a and pheophytin b. Because Py_at and Py_bt are, respectively, the sums of the Py_a and the C_a and of the Py_b and C_b initially present, eq 3 may be rewritten as eq 4 for solutions of pigments in diethyl ether:

$$F_A{}^{440} = 81.2\ Py_at + 1250\ Py_bt = 81.2\ (C_a + Py_a) + 1250\ (C_b + Py_b) \quad (4)$$

which may also be expressed as

$$F_A{}^{440} = 81.2\ C_a + 1250\ C_b + 81.2\ Py_a + 1250\ Py_b \quad (5)$$

Subtracting eq 5 from eq 2 we have

$$\Delta F^{440} = F^{440} - F_A{}^{440} = 682\ C_a - 20\ C_b \quad (6)$$

where the symbol Δ represents the change in fluorescence readings for a given filter system following acidification. By similar calculation

$$\Delta F^{460} = 523\ C_a + 2300\ C_b \quad (7)$$

Solving for each component by simultaneous equations

$$C_a = (1.46\ \Delta F^{440} + 0.0127\ \Delta F^{460}) \times 10^{-3}\ \mu\text{mol/l}. \quad (8)$$

and

$$C_b = (0.432\ \Delta F^{460} - 0.331\ \Delta F^{440}) \times 10^{-3}\ \mu\text{mol/l}. \quad (9)$$

Table IV. Comparison of Relative Fluorescence Intensity Values of Phytylated and Corresponding Nonphytylated Pigments[d]

Filter system	Pigment	RFI value	Pigment	RFI value	*t* test	% C.V.
460[a]	Chlorophyll a	614	Chlorophyllide a	609	N.S.	1
440[b]	Chlorophyll a	704	Chlorophyllide a	668	**	1
405[c]	Chlorophyll a	1012	Chlorophyllide a	1005	N.S.	1
460[a]	Chlorophyll b	3143	Chlorophyllide b	2975	**	2
440[b]	Chlorophyll b	989	Chlorophyllide b	1033	N.S.	3
405[c]	Chlorophyll b	91.6	Chlorophyllide b	111	**	2
460[a]	Pheophytin a	86.5	Pheophorbide a	90.9	**	3
440[b]	Pheophytin a	69.8	Pheophorbide a	73.7	*	0.42
405[c]	Pheophytin a	1099	Pheophorbide a	1084	N.S.	3
460[a]	Pheophytin b	1146	Pheophorbide b	1081	**	3
440[b]	Pheophytin b	1091	Pheophorbide b	1023	**	3
405[c]	Pheophytin b	285	Pheophorbide b	298	N.S.	5

[a] Filter system: primary, interference 460; secondary, interference 650 + CS #2-60. [b] Filter system: primary, interference 440; secondary, interference 650 + CS #2-60. [c] Filter system: primary interference 405; secondary, interference 680 + CS #2-60. [d] Expressed as calculated dial readings for pigments at concentration of 1.0 μmol per l. Pigments in diethyl ether.

Table V. Recovery Data from Two Component Mixture of Pure Pigments

Statistics for regression of amount of recovery upon amount added. Photomultiplier tube, R-136. Filter system: primary, interference 460, 440, and 405; secondary, interference 650 or 680 + CS #2-60

Solvent	Pigment	Concentration range[a]	b (slope)	a (intercept)	C.V.
Diethyl ether	C_a	0.037–0.044	1.009	+0.0011	1%
Diethyl ether	C_b	0.011–0.019	0.973	+0.0005	1%
80% acetone	C_a	0.055–0.065	0.682	+0.0184	2%
80% acetone	C_b	0.031–0.043	0.863	+0.0049	1%

[a] Concentration expressed as μmoles per liter.

Substituting values from Table III and solving simultaneously

$$Py_at = (0.698\ F_A^{405} - 0.233\ F_A^{440}) \times 10^{-3}\ \mu mol/l. \quad (10)$$

and

$$Py_bt = (0.815\ F_A^{440} - 0.0453\ F_A^{405}) \times 10^{-3}\ \mu mol/l. \quad (11)$$

Since Py_at or Py_bt is actually the sum of Py_a and C_a or Py_b and C_b initially present

$$Py_a = Py_at - C_a \quad (12)$$

and

$$Py_b = Py_bt - C_b \quad (13)$$

In similar manner, equations may be developed for estimation of chlorophylls a and b and pheophytins a and b separately or in mixtures in solution of aqueous acetone (80%), providing appropriate relative fluorescence intensity values are introduced. The relative fluorescence intensity values which pertained to this study are presented in Table III.

If the original solution (solution #1, Figure 1) contains chlorophylls a and b only, the concentration of each pigment can be calculated from the dial readings with the 405 and 460 filter systems. For a solution of these pigments in diethyl ether the equations are:

$$C_a = (0.733\ F^{405} - 0.0292\ F^{460}) \times 10^{-3}\ \mu mol/l. \quad (14)$$

$$C_b = (0.292\ F^{460} - 0.131\ F^{405}) \times 10^{-3}\ \mu mol/l. \quad (15)$$

Equations 14 and 15 were derived from data in Table III. Similar equations were developed for estimation of chlorophylls a and b only in an 80% acetone solution.

The chlorophyllides and/or pheophorbides may be encountered especially in extracts of processed green plant tissue (vegetables preserved by brining, freezing, or canning). Studies to determine the relative fluorescence intensity values of the chlorophyllides and pheophorbides in diethyl ether and 80% acetone were made by the same procedure used for establishing these values for the chlorophylls and pheophytins. The relative fluorescence intensity values for the nonphytylated pigments in diethyl ether were found to be very similar to their phytylated counterparts, as is shown in Table IV. Because of this similarity they have been considered to be the same. Accordingly, the chlorophyllides and/or pheophorbides, if present with chlorophylls and/or pheophytins in Solution #1 (Figure 1), would be estimated as chlorophylls and/or pheophytins by eq 1–15. The chlorophyllides and/or pheophorbides could be estimated, however, in solutions in diethyl ether but not in 80% acetone by a modification of the scheme in Figure 1, as shown in Figure 2. This scheme in Figure 2 was used for the spectrophotometric estimation of the eight components by White *et al.* (1963).

The authors would make it very clear that the specific relative fluorescence intensity values presented in Table III are peculiar to the conditions of this investigation. Any change of the instrument or of any component of the specific filter systems used would cause the relative intensity fluorescence values to be different than those reported above. It is imperative that each investigator establish relative fluorescence intensity values peculiar to his instrument and filter systems using purified pigments as standards.

Pigment Estimation from Mixtures. Indicated in Tables V and VI are examples of recovery of the various pure pigments when present either in diethyl ether or 80% acetone in two and four pigment component systems, respectively. The concentration ranges to which reference is made are those for the determinations reported in the tables and do not indicate the limits of the ranges over which estimations may be made with a high degree of accuracy. For the observations made in Table VI with the system consisting of the standard photomultiplier tube and the narrow band-pass primary filters neither the relative fluorescence intensity values determined nor the equations required for calculations of the corresponding results have been included in this report. The concentra-

Table VI. Recovery Data from Four Component Mixture of Pure Pigments

Statistics for regression of amount of recovered upon amount added.

Observations made with fluorometer equipped with two different photomultiplier tubes and two different filter systems.

	Diethyl ether solution				80% acetone solution			
Pigment	Concentration range[a]	b (slope)	a (intercept)	% C.V.	Concentration range[a]	b (slope)	a (intercept)	% C.V.
Photomultipler tube, RCA 931A (Standard). Filter system: CS #5-62, CS #5-74, and CS #5-75; secondary, CS #2-60.								
C_a	0.05–0.11	1.008	−0.0026	3	0.07–0.13	0.921	+0.0107	2
C_b	0.03–0.06	1.050	−0.0036	0.5	0.05–0.11	0.967	−0.0005	3
Py_a	0.04–0.11	1.121	−0.0030	7	0.12–0.24	0.988	−0.0057	2
Py_b	0.01–0.06	0.988	+0.0029	7	0.06–0.12	0.982	+0.0028	6
Py_at	0.09–0.22	1.057	−0.0054	4	0.25–0.31	1.067	−0.0239	1
Py_bt	0.04–0.06	1.016	−0.0002	2	0.12–0.18	1.023	−0.0049	2
Photomultiplier tube, R-136. Filter system: Primary, interference 460, 440, and 405; secondary, interference 650 or 680 + CS #2-60.								
C_a	0.012–0.024	0.960	+0.0028	4	0.03–0.04	0.991	+0.0015	6
C_b	0.007–0.015	0.982	+0.0005	2	0.01–0.02	0.823	+0.0031	4
Py_a	0.017–0.034	0.929	+0.0000	4	0.02–0.04	0.536	+0.0133	5
Py_bt	0.012–0.027	0.978	+0.0008	7	0.01–0.02	1.407	−0.0072	4
Py_at	0.04–0.05	0.837	+0.0074	1	0.05–0.07	0.842	+0.0096	2
Py_bt	0.02–0.03	1.180	−0.0035	0.5	0.03–0.04	1.102	−0.0034	2

[a] Concentration expressed in μmoles per liter.

Table VII. Averages of Estimates of True Values

Pigment content of puree of frozen spinach heated at 100°C for various time periods; comparison of spectrophotometric and fluorometric procedures of estimation; pigments expressed as concentration in μmoles per 1.42 g of sample.

		Pigments					
Treatment	Instrument	Py_at	Py_bt	C_a	C_b	Py_a	Py_b
0 hr	Spectrophotometer	36.2	12.1	32.0	9.55	4.2	2.5
	Fluorometer[a]	35.6	12.0	30.8	10.6	4.8	1.4
	Fluorometer[b]	35.6	11.7	31.3	11.7	4.3	−0.1
0.5 hr	Spectrophotometer	35.5	11.3	7.91	4.86	27.6	6.5
	Fluorometer[a]	35.3	11.5	8.04	6.36	27.3	5.3
	Fluorometer[b]	35.6	11.6	7.77	5.83	27.8	5.9
1 hr	Spectrophotometer	34.8	10.3	1.24	2.50	33.5	7.8
	Fluorometer[a]	35.1	10.7	1.55	2.71	33.6	8.0
	Fluorometer[b]	34.9	12.3	−0.3	2.28	34.6	10.0

[a] Filter system, primary interference 460, 440, and 405; secondary, interference 650 or 680 + CS #2-60. [b] Filter system, primary, interference 460, 440, and 405; secondary, CS #2-60.

tion of pigments in this study is approximately one-hundreth of that required for estimation of spectrophotometric means utilizing 1-cm cells.

A comparative study of the fluorometric and spectrophotometric estimation of chlorophylls and derivatives using frozen spinach as a pigment source has been made and is summarized in Table VII. Indicated is the total quantity of pigment in the fractions specified contained in replicated samples (1.42 ± 0.04 g) of a puree made from frozen spinach. Samples in triplicate in closed tubes were extracted by shaking with repeated small aliquots of 80% acetone. The solids were centrifuged down between extractions. The heat treatment shown was given to cause conversion of the chlorophylls to pheophytins.

Pigment estimation was made on centrifuged 80% extracts. The spectrophotometric procedure was that of White *et al.* (1963), utilizing as absorbances those for 80% acetone, determined in this laboratory and indicated in eq 16 to 19 inclusive as follows:

For chlorophylls in 80% acetone

$$C_a = 30.5\ \Delta A^{664.0} - 3.81\ \Delta A^{646.5}\ \mu\text{mol/l.} \quad (16)$$

$$C_b = 36.1\ \Delta A^{646.5} - 10.0\ \Delta A^{646.0}\ \mu\text{mol/l.} \quad (17)$$

For pheophytins in 80% acetone

$$Py_at = 24.5\ A^{665.8} - 7.40\ A^{653.4}\ \mu\text{mol/l.} \quad (18)$$

$$Py_bt = 36.7\ A^{653.4} - 14.6\ A^{665.8}\ \mu\text{mol/l.} \quad (19)$$

Fluorometric analyses were made by the procedure discussed in detail above and by the variation of this procedure mentioned earlier, in which the interference secondary filters were omitted from the filter systems.

The extracts of the samples, made to an initial volume of 25 ml, were subsequently diluted in the order of 4, 400, and 4000 times to permit pigment determination by the three procedures, the spectrophotometric, the fluorometric with secondary interference filters, and the fluorometric without secondary interference filters, respectively. Sources of error common to all methods of estimation were variations associated with routine sampling and extraction operations.

The differential heat treatments decidedly altered the quantity of chlorophylls and pheophytins present, as would be expected, but did not appreciably change the total pigment fractions designated Py_at and Py_bt. The agreement between the three procedures was considered to be good. A more detailed report on the more sensitive alternative fluorometric

technique is to be published later. It is observed that the naturally occurring carotenoids were not a source of appreciable error in the fluorometric methods.

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Further Purification, Fractionation, and Properties of Trypsin Inhibitor Isolated from *Lathyrus sativus*

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The trypsin inhibitor isolated from *Lathyrus sativus* was further purified and fractionated by DEAE cellulose column into five proteins with high trypsin inhibitory activity and a sixth protein with negligible activity. The first five proteins could be eluted in one block at pH 7.0, while the sixth was eluted at pH 3.6. The five-protein fraction IV(A) on dialysis and lyophilization resulted in a yield of 26.2 mg per 100 g of original seed. This fraction, IV(A), showed five distinct protein bands by disc acrylamide gel electrophoresis.

Isolation and partial purification of a specific trypsin inhibitor from *Lathyrus sativus* (L.S.) and some of its properties were reported by Roy and Rao (1971). The partially purified trypsin inhibitor showed a growth depressing activity in rats (Roy, 1972).

The present report describes further purification and properties of this inhibitor.

EXPERIMENTAL

Preparation of Material. The trypsin inhibitor was isolated and partially purified by the method reported earlier (Roy and Rao, 1971) and was designated as Fraction III.

DEAE-Cellulose Chromatography. Columns of 75.5 × 1.5 cm were prepared with DEAE–cellulose (Whatman column chromedia, De 11, medium fibrous powder, normal capacity 1.0 mequiv/g), washed with distilled water before use. The column was first equilibrated for 48 hr with 0.05 *M* phosphate buffer, pH 7.0, at 25°C; 32.6 mg of partially purified inhibitor (Fraction III) dissolved in 5 ml of phosphate buffer, pH 7.0, was loaded on the column, eluted first with 0.05 *M* phosphate buffer of pH 7.0, and then subsequently with phosphate–citrate buffer of pH 3.6. The flow rate was maintained at 45.5 ml/hr and 6.2-ml fractions were collected. Fractionation on the DEAE–cellulose column was repeated at a higher load (62.4 mg) of the inhibitor and the reproducibility of the pattern of elution was confirmed.

Disc Gel Electrophoretic Studies. Disc gel electrophoresis was conducted at pH 8.3 using 7.5% acrylamide system as described by Davis (1964). The effect of urea on the preparation was studied by using 4.6 *M* and 8.0 *M* urea in all the solutions and buffers of pH 8.3. Electrophoresis was carried out by applying 165–200 μg of protein sample, at 5 mA per tube for 1.5 hr, using Bromophenol blue as the indicator for the moving front. After each run, the gels were stained with 1% Amido Black in 10% acetic acid, subsequently destained by repeated washings with 10% acetic acid until the clear protein bands were visible.

Protein Determination. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Test for Carbohydrates. The purified fraction was subjected to hydrolysis by the method of Miyata *et al.* (1970). A 30-mg sample was hydrolyzed with 3 ml of 2 *N* H_2SO_4 by refluxing for 24 hr. The hydrolysate was neutralized with $Ba(OH)_2$ and then centrifuged. The clear supernatant was evaporated to a minimum volume. The presence of carbohydrates in this solution was examined by the Molisch test (Vogel, 1957).

Assay for Enzyme Activity. The inhibitory activity of the fractions was determined by the method described earlier (Kakade *et al.*, 1969; Roy and Rao, 1971). A 2% casein solution in phosphate buffer (0.1 *M*, pH 7.6) was used as substrate, while the enzyme used was trypsin (E. Merck, about 20,000 Fuld-Gross units per gram) (5 mg/ml). The incubation mixture consisted of 0.5 ml of trypsin solution, 2 ml of 2% casein, 1.0 ml of phosphate buffer (pH 7.6, 0.1 *M*), 0.3 ml of HCl (0.001 *M*) solution and 0.2 ml test solution. The total volume was 4 ml in each case. Incubation was carried out at 37°C for 20 min, after which 6.0 ml of 5% TCA solution was added to stop the reaction. Corresponding blanks were run concurrently. In all these experiments, one trypsin unit (TU) was arbitrarily defined as an increase of 0.01 absorbance unit at 280 mμ in 20 min for 10 ml of reaction

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