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Paper Chromatographic Characterization of Chlorophylls

ARNE JENSEN and OLAV AASMUNDRUD

Norwegian Institute of Seaweed Research and Department of Biochemistry, The Technical University of Norway, Trondheim, Norway

Two simple and convenient paper chromatographic systems for the separation of chlorophylls, and one system for the separation of their phæophytins have been worked out. Together with the light absorption properties of the compounds mentioned, the R_F-values obtained may be used for a rapid and reliable characterization of micro amounts of the original algal and bacterial chlorophylls.

Both algae and photosynthetic bacteria are known to contain a number of different chlorophylls,[1–3] and in studies on organisms belonging to either of these two groups, a rapid and reliable characterization of the different chlorophylls is often desirable. The technique of paper chromatography has been applied to this problem with some success [4–18]. It has not been established, however, whether all of the algal and bacterial chlorophylls known may be separated by paper chromatography using one or two simple systems. The value of the method as a means of characterizing these pigments is thus not fully known. The present study was undertaken in order to explore further the potentiality of paper chromatography as applied to this field.

The most promising chromatographic techniques with respect to simplicity and selectivity seemed to be the system of Sporer, Freed and Sancier [6] using sucrose impregnated paper and the technique of Angapindu, Silberman, Tantivatana and Kaplan [7] making use of calcium carbonate impregnated paper. However, instead of impregnating the paper with calcium carbonate, we preferred to use Schleicher & Schüll No. 996 filter paper which contains approximately 20 % of calcium carbonate as a filler, and which is commercially available and ready for use without further treatment.

During the present study it was found that another type of commercial filter paper, namely Schleicher & Schüll No. 667, an aluminium oxide-containing paper, was well suited for the separation of the phæophytins of the algal and bacterial chlorophylls.

MATERIALS AND METHODS

Solvents used for extraction, chromatography and spectroscopy, were of analytical grade and were used without further purification.

For chromatography circular papers (Whatman No. 1, 18 cm diameter) were soaked in an 18 % (w/v) aqueous sucrose solution,[8] and excess liquid was pressed out between layers of ordinary filter paper. The moist circular papers were then dried at room temperature and finally activated for 30 min at 105°C immediately before use. The calcium carbonate-containing (No. 996) and aluminium oxide-containing (No. 667) papers were obtained commercially from the filter paper manufacturers Carl Schleicher & Schüll, Kreiss Einbeck, West Germany. The calcium carbonate paper was activated at 105°C for 30 min prior to use, while the aluminium oxide paper was used without activation.

When working with bacterial chlorophylls and bacterial phæophytins all solvents used for extraction, chromatography and spectroscopy were saturated with hydrogen sulphide prior to use,[9] and all operations were carried out in darkness when possible or in dim light.

The chromatographic separations were carried out according to the technique previously described [11]. The solvent system used with the sucrose impregnated paper consisted of 0.5 % (v/v) butanol in petroleum ether (boiling range 60 – 80°C), while 5 % (v/v) acetone plus 1 % (v/v) butanol in petroleum ether was used with the calcium carbonate paper. With the aluminium oxide paper 10 % acetone in petroleum ether was used.

The light absorption spectra in the visible range were determined in a Zeiss PMQ 2 spectrophotometer, using acetone as the solvent.

The isolation and purification of the bacterial chlorophylls and the preparation of their phæophytins will be described elsewhere [12].

Isolation of chlorophylls a and b. Chlorophylls *a* and *b* were isolated from red clover leaves by extracting 5 g of freshly collected leaves with successive portions of methanol in a mortar. To 100 ml of the extract was added 50 ml of diethyl ether, and sufficient aqueous sodium chloride solution to cause separation into two layers. The ether phase was washed three times with 50 ml of distilled water and dried over anhydrous sodium sulphate. The solution was then concentrated to approximately 5 ml *in vacuo* at room temperature, and aliquots (0.05 – 0.1 ml) were applied onto calcium carbonate papers for chromatographic separation.

The grass green and yellow green zones of 10 chromatograms were eluted and pooled into two samples which were rechromatographed separately on thick paper (Schleicher & Schüll No. 2247) impregnated with sucrose. These purified samples of chlorophyll *a* and *b* were used for the determination of the chromatographic and spectroscopic properties of the two chlorophylls.

Preparation of phæophytin a. Approximately 5 g (fresh weight) of the brown seaweed *Fucus serratus* L. was extracted with successive portions of methanol in a mortar, giving a final volume of 100 ml of filtered extract, to which 50 ml petroleum ether and a small amount of an aqueous sodium chloride solution were added. The petroleum ether phase was then separated from the aqueous methanolic layer, washed three times with water and dried over anhydrous sodium sulphate, whereafter the solvent was removed *in vacuo* at room temperature. The residual pigments (mainly chlorophyll *a* and some β-carotene) were dissolved in 20 ml of diethyl ether:methanol (1:1). To this solution 2 ml of concentrated hydrochloric acid was added, and the mixture was shaken for 10 min at room temperature. More ether was then added and the pigment solution washed free of acid with distilled water. After drying over anhydrous sodium sulphate, the ethereal solution was concentrated *in vacuo* at room temperature, and aliquots were applied onto paper chromatograms.

Preparation of phæophytin b. The chlorophyll *b* zones from 10 chromatograms of red clover extract obtained as described above were extracted with diethyl ether:methanol (1:1), and the volume of the pigment solution was made up to 5 ml using the same solvent mixture. To the solution 1 ml of concentrated hydrochloric acid was added, and the resulting phæophytin worked up as described above for phæophytin *a*.

Chlorophyll c. Chlorophyll *c* was prepared according to the method of Strain and Manning [13]. Approximately 5 g (fresh weight) of *Fucus serratus* was extracted with 4 portions of methanol (50 ml each), and 35 ml of water was added to the combined extracts. Chlorophyll *a* and carotene were extracted into 100 ml of petroleum ether by shaking,

and the methanolic phase was further extracted with 3 portions of diethyl ether (100 ml each) after the addition of 160 ml of water to the alcohol. The greater part of the carotenoids together with residual chlorophyll *a* were thus removed. To the remaining aqueous methanolic phase was added 400 ml of 10 % aqueous sodium chloride, and the green pigments (mainly chlorophyll *c*) were extracted with 100 ml of diethyl ether, which removed all coloured matter from the aqueous layer. After drying over anhydrous sodium sulphate and concentration *in vacuo*, at room temperature, aliquots of the pigment solution were subjected to paper chromatography.

Preparation of phæophytin c. The chlorophyll *c* fraction obtained from 5 g of *Fucus serratus* as described above was purified by chromatography on a cellulose column (Schleicher & Schüll No. 123) (15 × 150 mm). Traces of chlorophyll *a* and carotenoids were washed through with 300 ml of 0.5 % (v/v) butanol in petroleum ether, whereafter the chlorophyll *c* zone was eluted with 5 % (v/v) butanol in petroleum ether. The purified pigment was then converted to its phæophytin according to the method of Smith and Benitez,[2] and aliquots of the product were subjected to paper chromatography.

Oxidation of chlorophyll a [14]. Approximately 1 mg of chlorophyll *a* from *Fucus serratus* was dissolved in 3 ml of freshly purified acetone [14] and 0.1 ml portions of aqueous 3 % (w/v) potassium permanganate were added at intervals at room temperature. The course of the reaction was followed by paper chromatography. After 1 h, 0.8 ml of oxidant had been added, and the reaction was stopped by the addition of 100 ml of diethyl ether. Manganese dioxide was removed by filtration, and the ether layer washed thoroughly with distilled water. Paper chromatographic separation of the products gave 4 zones as shown in Table 1.

From spectroscopic and chromatographic data, zone 2 was tentatively identified with fraction A of Holt and Morley,[14] who proved the structure of this compound to be 2-desvinyl-2-formyl chlorophyll *a*.

RESULTS AND DISCUSSION

The R_F-values of the chlorophylls and phæophytins investigated are collected in Table 2 and the absorption spectra in acetone of the compounds are given in Table 3. The identity of the pigments investigated was based on the sources from which they were isolated and on spectroscopic properties. The absorption spectra compare favourably with the data reported in the literature [2,3]. Starting with very small samples of bacterial or algal materials the correct absorption maxima could usually be obtained without rechromatography.

Despite repeated attempts chlorophyll *d* [1] could not be obtained from any of the eight red algae investigated in our laboratory. These were: *Porphyra umbilicalis* I. A. Agardh., *Polysiphonia fastigiata* Grev., *Rhodymenia palmata* Grev., *Furcellaria fastigiata* Lyngb., *Gigartina stellata* Batt., *Delesseria sangui-*

Table 1. Oxidation products of chlorophyll *a*.

Zone	R_F1 [a]	R_F2 [a]	Relative amount	Absorption maxima mμ, in acetone	Tentative identification
1	58	69	++	382, 412, 432, 533, 582, 620, 666	Chlorophyll *a*
2	32	48	+	394, 445, 550, 600, 660, 693	Fraction A [b]
3	21	29	++	393, 432, 550, 595, 630, 690	Fraction B [b]
4	11	17	+++	385, 405, 423, 533, 575, 612, 657	Fraction C [b]

[a] See Table 2 for explanation.
[b] According to the nomenclature of Holt and Morley.[14]

nea Lamour., *Odonthalia dentata* Lyngb. and *Dumontia incrassata* Lamour. Acetone extracts of the above algae, prepared within 1/2 h after harvesting, gave only one chlorophyll zone upon paper chromatography, and the light absorption spectra of the crude extracts showed no shoulder on the long wavelength side of the 666 mμ peak of chlorophyll *a*. Chlorophyll *d* was therefore apparently absent from the species investigated. Previously Holt and Morley [14] have looked in vain for the presence of this pigment in the red algae *Chondrus crispus*, *Gigartina stellata* and *Rhodocorton rothii*.

Holt and Morley [14] have claimed that chlorophyll *d* is identical with 2-desvinyl-2-formyl chlorophyll *a*. The latter compound was therefore synthesized and its paper chromatographic properties studied.

As may be seen from Table 2 all the algal chlorophylls investigated, 2-desvinyl-2-formyl chlorophyll *a* included, could be easily separated from each other both on the sucrose paper and on the calcium carbonate paper. Complete separation of all the bacterial chlorophylls, except for the two chlorobium chlorophylls, was also easily achieved on the calcium carbonate paper, and with some care a similar separation could likewise be obtained on the sucrose paper. The pigment zones were usually sharper and better defined on the calcium carbonate paper. In the case of the bacterial pigments, the better resolution of the zones was obtained on this paper. The main reason for this was the anomalous behaviour of bacteriochlorophyll *b*,[15] which, in contrast to all the other pigments of Table 2, moved faster on the sucrose than on the calcium carbonate paper, thus giving a very satisfactory spreading of R_F-

Table 2. R_F-values of various chlorophylls and phæophytins. All R_F-values in the table have been multiplied by 100.

Compound	R_F1 [a]	R_F2 [b]	R_F3 [c]	Colour on the paper [d]
Chlorophyll *a*	59	70	—	blue green
Chlorophyll *b*	25	44	—	grass green
Chlorophyll *c*	5	0	—	light green
2-desvinyl-2-formyl chlorophyll *a*	32	48	—	blue green
Bacteriochlorophyll *a* [e]	46	58	—	blue
Bacteriochlorophyll *b*	48	40	—	bluish green
Chlorobium chlorophyll-650	~10	~20	—	grass green
Chlorobium chlorophyll-660	~10	~20	—	grass green
Phæophytin *a*	70	85	62	grey, B.R.
Phæophytin *b* [e]	48	63	37	grey, B.R.
Phæophytin *c*	5	0	0	yellow, B.R.
Bacteriophæophytin *a*	62	79	55	red violet, D.B.
Bacteriophæophytin *b*	58	66	48	violet brown, D.B.
Chlorobium phæophytin-650	15–25	40–50	~25	greyish green, B.R.
Chlorobium phæophytin-660	20–30	30–50	~30	greyish green, B.R.

[a] Sucrose impregnated paper, solvent system: 0,5 % butanol in petroleum ether.

[b] Schleicher & Schüll No. 996 paper, solvent system: 5 % acetone plus 1 % butanol in petroleum ether.

[c] Schleicher & Schüll No. 667 paper, solvent system: 10 % acetone in petroleum ether.

[d] The fluorescence in filtered U.V. light is indicated by B.R. = brilliant red, and D.B. = dark blue.

[e] The nomenclature of the bacterial chlorophylls and their derivatives is discussed in Ref.[15]

values on the latter paper. The chlorobium chlorophylls (and their phæophytins), as prepared for the present study, appeared to consist of a complex mixture of pigments. Precise R_F-values for these compounds were therefore not obtained in the solvent systems used. This problem is being further investigated.

The phæophytins which have frequently been used for the characterization especially of bacterial chlorophylls, could as a rule be easily separated on the aluminium oxide paper, an exception being the chlorobium phæophytins which migrated at almost identical rates. Mixtures of algal phæophytins could be well resolved also on the two other types of paper, but the majority of the phæophytins of bacterial origin showed nearly identical R_F-values on the sucrose paper, and were separated only with difficulty on the calcium carbonate paper. It should be mentioned here that the location of the phæophytins on the papers after the chromatographic separation was most conveniently determined in filtered ultraviolet light, making use of the brilliant red or dark blue fluorescence shown by these compounds.

Both chlorophyll *c* and its hydrochloric acid conversion product(s) were much more strongly retained by the adsorbents than were any of the other pigments investigated. The homogeneity of the former compounds could therefore not be tested in the chromatographic systems of the present study.

When care was taken, the R_F-values of the compounds investigated were reproducible to within $\pm$ 0.02 units. The solvent mixtures should be used only once for determination of the R_F-values, as repeated use of the same solvent leads to steadily decreasing mobilities.

The aluminium oxide paper gave somewhat greater variations in the R_F-values than did the other papers, probably because the former paper was not

Table 3. Light adsorption maxima and relative intensities in acetone for various chlorophylls and phæophytins.

Compound	λ_{max} in mμ. Intensities in brackets.						
Chlorophyll *a*	382(48)	412(72)	432(100)	533(5)	582(9)	620(15)	666(63)
Chlorophyll *b*	432(44)	456(100)	545(6)	600(11)	648(37)		
Chlorophyll *c*	395(24)	448(100)	583(7)	633(12)			
2-Desvinyl-2-formyl chlorophyll *a*	394(88)	445(99)	550(12)	600(12)	660(41)	693(100)	
Bacteriochlorophyll *a*	358(100)	390(60)sh	577(29)	773(103)			
Bacteriochlorophyll *b*	368(100)	407(87)	582(26)	794(83)			
Chlorobium chlorophyll-650	406(80)	425(100)	577(10)	605(15)	651(70)		
Chlorobium chlorophyll-660	415(62)	431(100)	624(16)	661(70)			
Phæophytin *a*	410(100)	471(5)	505(12)	533(9)	560(3)	606(8)	683(42)
Phæophytin *b*	415(44)	433(100)	528(8)	555(5)	600(5)	657(17)	
Phæophytin *c*	422(100)	515(8)	575(7)	595(7)	645(3)		
Bacteriophæophytin *a*	357(100)	385(52)	523(28)	675(11)	745(48)		
Bacteriophæophytin *b*	368(100)	397(84)	525(23)	685(10)	775(48)		
Chlorobium phæophytin-650	407(100)	503(11)	533(10)	601(10)	657(44)		
Chlorobium phæophytin-660	410(100)	515(10)	547(15)	608(7)	666(46)		

activated under standardized conditions prior to use. It was found that drying of this paper at 105°C even for short periods of time tended to give too active a material. Storing in desiccators at standardized humidity and temperature may improve the reproducibility of the R_F-values in this case. One might also introduce a reference compound and make use of the relative mobilities.

For a satisfactory characterization of the chlorophyll in photosynthetic bacteria approximately 1—15 mg of dry cell material is needed, while *ca.* 100 mg must be available in the case of the common, marine brown algae.

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