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PURIFICATION OF DIHYDROPORPHYRINS FOR SPECIFIC ACTIVITY DETERMINATION BY THIN-LAYER CHROMATOGRAPHY*

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SUMMARY

Chromatography on thin layers of confectioner's (icing) sugar can be used to purify ^{14}C-labeled chlorophylls *a* and *b* for specific activity determinations. With chlorophylls from plants containing lutein, a pre-development of the plate with 20% diethyl ether in light petroleum ensures adequate separation of the lutein from the chlorophyll *a* on subsequent development with 0.5% 2-propanol in light petroleum. The specific activities of ^{14}C-labeled chlorophylls *a* and *b* purified by the outlined procedure agree, within the analytical error, with those obtained for identical samples purified by chromatography on columns of confectioner's sugar. The radiochemical criterion that the specific activity of the ^{14}C-labeled chlorophyll *a* or *b* must agree with that of the corresponding pheophytin is met.

INTRODUCTION

A number of recent papers describe the use of thin-layer chromatography (TLC) in the separation and purification of porphyrins, dihydroporphyrins, and their methyl esters[1-6]. This technique has been found to be suitable for the purification of a variety of porphyrins, their esters, and the dihydroporphyrins from the leaves of higher plants in quantities ranging from a few micrograms to a few milligrams. In our studies of chlorophyll biogenesis in which we administer precursors specifically labeled with ^{14}C to various higher plants[§§], we have been in the past restricted to the use of

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§§ The biosynthetic pathway to heme is now a classic case in biochemistry and is reviewed in ref. 11. The pathway to chlorophyll now seems complete in terms of porphyrin precursors[11-14] and its relationship to the heme pathway in certain steps (*i.e.* up to the protoporphine IX step in biosynthesis) has been inferred mainly through the use of crude cell enzyme preparations of plant tissue. This evidence does not, however, constitute unequivocal proof that the two pathways are identical. We hope to demonstrate this relationship adequately through the use of specifically labeled ^{14}C-precursors and the tracing of their incorporation into the various portions of the chlorophyll molecule as WITTENBERG AND SHEMIN (*J. Biol. Chem.*, 192 (1951) 315) did for heme

large quantities of labeled chlorophylls mainly because of our use of column chromatography to isolate and purify the ^{14}C-labeled pigments[7]. The convenience and rapidity of TLC seemed an excellent means by which to carry out our studies on a smaller scale and in a much shorter time. The question as to whether or not the radiochemical purity of the dihydroporphyrins purified by this technique could be assured, was unanswered. Other questions to be answered involved the choice of the adsorbent and the developing solvent(s) to be used.

In an earlier paper dealing with the radiochemical purity of ^{14}C-labeled chlorophylls, PERKINS AND ROBERTS[8] described two main criteria for establishing the radiopurity of ^{14}C-labeled dihydroporphyrins, namely that the ratio of the absorbance at the Soret maximum to that of the absorbance at the red maximum be in the ranges given in the earlier paper, and that constant and identical specific activities of the chlorophylls and their respective pheophytins be obtained. Either one of these criteria alone did not constitute a basis for claiming radiopurity of a given ^{14}C-labeled chlorophyll (or other dihydroporphyrin).

In this paper, data are presented demonstrating that chromatography on thin layers of icing sugar can be used to yield chlorophylls *a* and *b* and pheophytins *a* and *b* that are radiochemically pure by these criteria. These purifications can be achieved much more rapidly and economically by the method described here than by chromatography on sucrose columns: within one day of their initial extraction from the plants, it is possible to obtain chlorophylls *a* and *b*, the specific activities of which do not change on repeated further purification and whose specific activities are identical to those of the pheophytins *a* and *b* prepared therefrom and subsequently further purified.

METHODS AND RESULTS

The sucrose thin-layer plates were made through the application to glass plates of a slurry of confectioner's sucrose (containing 3 % (w/w) cornstarch–Domino 10-X Confectioner's sugar) in reagent acetone. The slurry was prepared by blending 100 g of sugar in 130 ml of acetone at low speed in a blender for about 5–10 min. This amount of slurry produced four to five 20 × 20 cm plates 0.50 to 0.75 mm in thickness. For best results, the plates were air-dried for 2 h before use. The plates could be prepared as much as one week in advance of their use if stored in a desiccated cabinet. It was not necessary (or even desirable) to "activate" the plates before use as is often done with silica gel plates.

Dihydroporphyrins were best applied to the plates in dried light petroleum solutions, although acetone and diethyl ether could be used. To avoid disturbing the surface of the sugar layer, the pigment solutions were applied using a 5 c.c. tuberculin syringe into the Luer-Lok of which had been inserted a 2.5-cm length of cotton string of such a diameter as to ensure a snug fit. Approximately 0.6 cm of the string protruded from the barrel of the syringe and acted as a wick to dispense the pigment solution gently onto the surface of the plate after the syringe had been filled through the top (following removal of the plunger) with a pipette of appropriate size. Since the string was relatively easily dislodged, as little pressure as possible was used on the plunger in delivering the pigments onto the thin-layer plate. If the pigments precipitated during application, or if quantitative recovery of a sample was required, a diethyl ether rinse

was used. With this device, the solutions could be applied to the plate as bands (using a guide mounted above the surface of the plate) or as spots. To obviate photodecomposition or oxidation of the pigments, the plates were exposed to as little light or air as possible during pigment application and were placed in the developing tank immediately after the sample application was completed. Room illumination was maintained at a maximum of 2 ft.-c at bench level.

The plates were developed by ascending chromatography using solvents and techniques used previously with sucrose columns[7]. Two solvent systems were used successively for the thin-layer plate development of initial crude extracts of plant pigments: (I) 20% diethyl ether in light petroleum to separate the lutein from the chlorophyll *a*[7] and, following a brief drying in air, (II) 0.5% 2-propanol in light petroleum (boiling range 35–60°) to separate the chlorophylls *a* and *b*. The time for complete development in each solvent was approximately 15–30 min. Unlike the procedure developed for purification of these pigments by column chromatography[7], the time of development with either solvent was not critical when TLC was used. Typical results obtained with these methods and materials are shown in Fig. 1. In the later stages of purification of chlorophyll *b* and pheophytin *b*, the use of 1% 2-propanol in light petroleum gave increased separation of these components. The latter solvent was detrimental to the purification of pheophytin *a* samples owing to the excessive band broadening that occurred when it was used. In purifying pheophytins *a* and *b* prepared from purified chlorophylls *a* and *b*, respectively, pre-development with solvent system I was not necessary; again pheophytin *b* was purified using 1.0% 2-propanol in light petroleum.

Pigments were recovered from the plates by scraping the bands off with a new razor blade followed by suspension of the icing sugar in diethyl ether. The sugar was then removed by dissolution in water. The ether solution was washed thoroughly and dried over anhydrous Na_2SO_4 for rechromatography or specific activity determination.

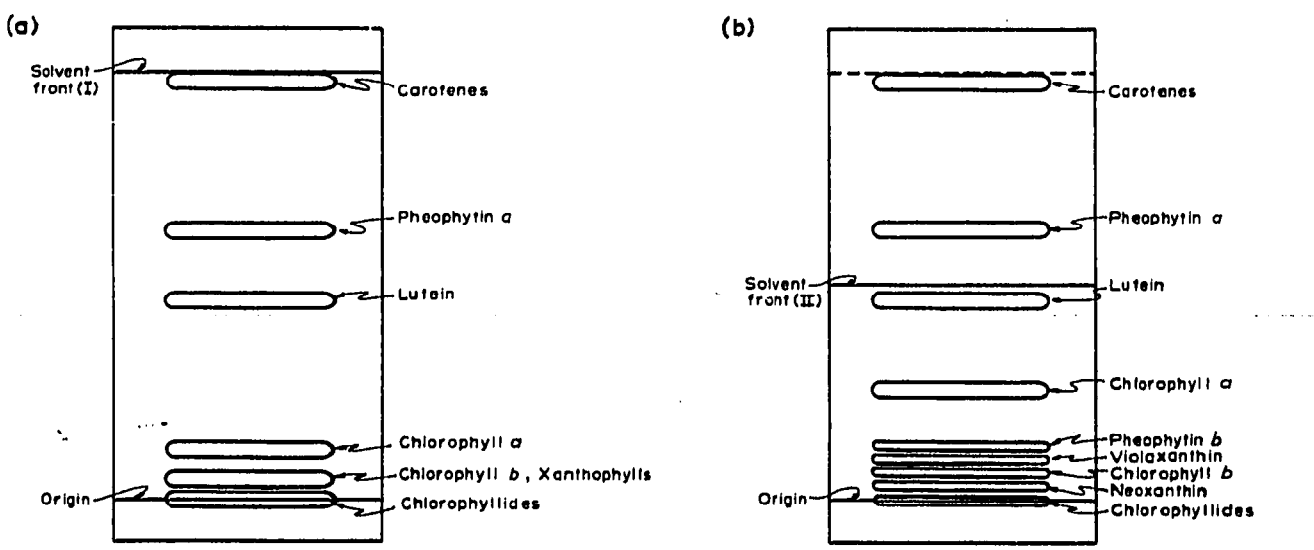

Fig. 1. Typical results of sucrose TLC of leaf pigment extracts. (a) Crude pigment extract developed initially with 20% diethyl ether in light petroleum (solvent I). (b) Pigment distribution on the sucrose thin-layer plate from (a) above after a second development with 0.5% 2-propanol in light petroleum (solvent II).

The data in Table I represent a comparison of the purification of these pigments by TLC on sucrose with that by column chromatography (CC) on sucrose. Shown are the "blue to red" ratios and the specific activities of ^{14}C-labeled chlorophylls *a* and *b* (obtained from wheat plants fed [1-^{14}C]acetate) and of the pheophytins derived therefrom. Following initial extraction from the [1-^{14}C]acetate-fed leaves and exhaustive extraction with 80% aqueous methanol, the light petroleum solution of the crude pigments was divided into two portions: the first was purified by CC on icing sugar[7] and the second by TLC on icing sugar as described.

TABLE I

COMPARISON OF THE OPTICAL PURITY (DIETHYL ETHER SOLUTIONS) AND SPECIFIC ACTIVITIES OF ^{14}C-LABELED CHLOROPHYLLS *a* AND *b* (AND THE PHEOPHYTINS OBTAINED THEREFROM) PURIFIED BY TLC ON SUCROSE, WITH THOSE FOR IDENTICAL SAMPLES PURIFIED BY CC ON SUCROSE

Pigment	*No. of times chromatographed*	*"Blue to red" ratio*[a]		*Specific activity (d.p.m./mg C) × 10^{-4}*	
		CC	*TLC*	*CC*	*TLC*
Chlorophyll *a*	1	1.30	1.26	1.91	2.01
	2	1.29	1.28	1.97	1.93
	3	1.29	1.30	—	—
Pheophytin *a*	1	2.01	2.04	1.85	1.92
	2	2.00	2.04	1.91	1.90
	3	2.02	2.04	1.91	1.89
Chlorophyll *b*	1	2.79	2.79	1.65	1.83
	2	2.83	2.81	1.61	1.61
	3	2.82	—	—	1.64
Pheophytin *b*	1	5.00	5.26	1.71	1.69
	2	—	5.03	—	1.70

[a] Ratio of the absorbance at the Soret maximum to that at the "red" maximum.

The absorbancies (adjusted to between 0.2 and 0.8 at the Soret maximum) of pigment solutions in diethyl ether were measured using a Cary Model 14 recording spectrophotometer. The extinction coefficients of SMITH AND BENITEZ[9] were used in computing the concentration of a pigment. The radioactivity determinations were made by plating, in triplicate, 100 to 200 μl aliquots of the diethyl ether solution on 2-in. ringed copper planchets that had been specially selected for work with a low background counter. The planchets were counted in a Beckman "Lowbeta II" proportional counter having a background of approximately 1.0 c.p.m. and a measured efficiency of approximately 24%. Sufficient counts were accumulated for each sample as to ensure a counting error of less than 1.5% at the 95% confidence level.

The data in Table I demonstrate that ^{14}C-labeled chlorophylls *a* and *b* separated and purified by TLC are as pure spectroscopically and radiochemically as are those separated by CC. We have also routinely obtained by TLC on sucrose pure (spectroscopically and radiochemically) ^{14}C-labeled methyl pheophorbide *a* and methyl pyropheophorbide *a* (derived from pure pheophytin *a*) using 0.5% isopropanol as the developer.

DISCUSSION

Using the procedure described here, we have not experienced the severe degradation STRAIN AND SVEC[10] have suggested can occur on TLC of the chlorophylls. We have routinely observed less than 5% degradation (determined by using visible absorption spectrophotometry) of the pigment in the initial stages of purification. This value becomes significantly lower as the compound is purified and is quite comparable to the amount of degradation observed in CC. It is likely that the use of low light levels in the laboratory, the rapid handling of the plates during the pigment application stage, and the use of small developing tanks (relative to the total plate areas being developed therein), and the prompt recovery of the pigment from the plate following development (thus ensuring minimal exposure of the dried and purified pigment to the air) contribute to the small amount of degradation observed in our laboratory.

We have routinely used the procedure outlined for a period of more than three years and have repeatedly verified these results. We therefore suggest that for the small-scale (*e.g.* milligram, or smaller, quantities) preparation of ^{14}C-labeled chlorophylls, pheophytins, and other derivative dihydroporphyrin esters this procedure is the method of choice.

REFERENCES*

1 J. E. FALK, *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam, 1964.
2 M. DOSS AND U. BODE, *J. Chromatog.*, 35 (1968) 248.
3 L. W. SMITH, R. W. BREIDENBACH AND D. RUBENSTEIN, *Science*, 148 (1965) 508.
4 M. F. BACON, *Biochem. J.*, 101 (1966) 34c.
5 C. J. SELISKAR, *Anal. Biochem.*, 17 (1966) 175.
6 H. H. STRAIN, J. SHERMAN AND M. GRANDOLFO, *Anal. Biochem.*, 24 (1968) 54.
7 H. J. PERKINS AND D. W. A. ROBERTS, *Biochim. Biophys. Acta*, 58 (1962) 486.
8 H. J. PERKINS AND D. W. A. ROBERTS, *Biochim. Biophys. Acta*, 79 (1964) 20.
9 J. H. C. SMITH AND A. BENITEZ, in K. PAECH AND M. V. TRACEY (Editors), *Modern Methods of Plant Analysis*, Springer-Verlag, Berlin, 4 (1955) 142.
10 H. H. STRAIN AND W. A. SVEC, in L. P. VERNON AND G. R. SEELEY (Editors), *The Chlorophylls*, Academic Press, New York, 1966, pp. 21–66.
11 L. BOGORAD, in L. P. VERNON AND G. R. SEELEY (Editors), *The Chlorophylls*, Academic Press, New York, 1966, pp. 481–510.
12 S. ARONOFF AND R. K. ELLSWORTH, *Photosynthetica*, 2 (1968) 288.
13 R. K. ELLSWORTH AND S. ARONOFF, *Arch. Biochem. Biophys.*, 125 (1968) 369.
14 R. K. ELLSWORTH AND S. ARONOFF, *Arch. Biochem. Biophys.*, 130 (1969) 374.

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* *Note added in proof.* B. COLMAN AND W. VISHNIAK (*Biochim. Biophys. Acta*, 82 (1964) 616) used similar systems on saccharose. V. S. SAAKOV (*Biofizika*, 8 (1963) 123) examined the purity of labeled porphyrins using paper chromatography.