CENTRIFUGALLY ACCELERATED PAPER CHROMATOGRAPHY OF CHLOROPLAST PIGMENTS*

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The techniques of centrifugally accelerated paper chromatography were developed by McDONALD *et al.* in 1957[1]. The combination of two vectors, chromatography and centrifugal acceleration, makes it possible to separate compounds in very short times, for examples in 15 minutes instead of 8 hours. The chlorophylls and carotenoids are very labile compounds which undergo marked decomposition in the lengthy paper chromatography necessary for adequate resolution. It was hoped that this difficulty might be overcome by the use of rapid centrifugally accelerated paper chromatography.

The literature contains separations of water-soluble compounds, such as dyes and amino acids[2], but as yet there is no mention of separation of any other compounds. Therefore, a study was made of the fundamental factors involved in obtaining good separations and reproducible chromatograms of the algal pigments with nonpolar solvents.

EXPERIMENTAL

The centrifugally accelerated chromatograms were made on the chromatofuge which was assembled and operated in the prescribed manner**. All the experiments reported were carried out at 25°. Whatman Nos. 1 and 3 MM circles were used, as well as circles cut from the very thick Whatman No. 17 MM sheets. Since most of the pigments were required for spectroscopic and radioactive measurements or for further chromatography, it was desirable to use the thicker Whatman Nos. 3 MM or 17 MM papers. The pigment extract was placed on a circular origin which must be at least 8 cm in diameter. It was most satisfactory to apply the pigments in ethereal solution, as the ether evaporated quickly; however, other solvents were also feasible. With practice it was possible to make very thin uniform circles of pigment on the origin by holding the paper vertically with one hand and applying the extract with the other; papers were spotted in the cold room with as little light as possible.

The total algal extract which could be placed on such a circular origin on a Whatman No. 3 MM sheet was equivalent to 0.3 to 0.4 mg pigment, *i.e.* the extract obtained from 0.05 ml wet packed algal cells. About three times as much material

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** Labline No. 5060 Chromatofuge, Labline Inc., Chicago, Ill.

could be placed on the circular origin of a Whatman No. 17 MM paper. Since the paper was thick it was necessary to spot the material on *both* sides or else the pigment zones on the underside of the paper after development, would have lower R_F values.

It was also desirable to use a liner for the chromatofuge head. A sheet of Whatman No. 3 MM paper was placed in close contact with the bottom of the head, by cutting holes in the sheet so that the stainless steel points protruded through the liner paper. Some of the solvent was placed on this liner and the machine rotated for 5 min in order to saturate the chamber head with the solvent fumes, before placing the paper to be developed in the chromatofuge. In subsequent runs, about 10 to 20 ml of solvent was poured on to the liner, the paper placed in position and developed. It was not necessary, in the subsequent runs, to rotate the machine for the 5-min period, before the development of the paper.

In the case of petroleum ether–isopropanol solvents, it was necessary to mix the solvent several days before it was required for use. Fresh solvent mixtures used under the same running conditions gave anomalous results (the solvent did not travel nearly as far and the separations obtained were very poor).

RESULTS AND DISCUSSION

First of all it should be pointed out that the chromatograms obtained, despite the velocity of the rotation, are not circular in shape but elliptical, as was reported by McDONALD *et al.*[2] for aqueous solvents. This is not a disadvantage however, since measurement of R_F values from different parts of the ellipse gave values which were within the experimental error ($\pm$ 0.02 R_F units).

It was found that the resolution of the carotenoids and chlorophylls was the same as that obtained in conventional ascending or descending chromatography; the latter methods have been reviewed by ŠESTÁK[3]. That is, any solvent which gave satisfactory resolution in the conventional methods could be used for the centrifugally accelerated chromatograms, although it was necessary to determine suitable running conditions for the chromatofuge.

Most of the factors reported by McDONALD *et al.*[2] for development of the chromatograms with aqueous solvent, were found to be effective in these experiments with nonpolar solvents. In order to obtain reproducible R_F's it is necessary to have a flow rate of solvent such that the perimeter of the wetted area does not increase after the rotor and solvent flow are stopped. It is also necessary to avoid such a high flow rate that flooding of the paper will occur during development, as this causes distortion of the zones.

The placement of the circular origin is important; it must not be less than 7.5 cm in diameter or else some of the pigments will migrate inwards. It was found that an increase of 1.0 cm for the diameter of the circular origin resulted in an increase of about 0.04 for intermediate R_F values. It was not advantageous to increase the diameter beyond 12 cm since under those conditions only spots with R_F values less than 0.3 would be separated.

In general, under the same conditions of nitrogen pressure, velocity of rotation, and with the same delivery jet, different solvents will travel different distances, as might be expected. For example, at 500 r.p.m., 7.5 p.s.i. nitrogen and a flow rate of 2.0 ml/min, toluene traveled 15 cm; petroleum ether (b.p. 75°), 13 cm; petroleum ether (b.p. 30–60°), 6 cm; and petroleum ether–isopropanol (100:2.5) 14 cm in 10 min.

Also with different grades of paper, the distance traveled by the solvent will vary. With a flow rate of 2.5 ml/min of toluene at 500 r.p.m. the results given in Table I were obtained.

TABLE I

	Time of development (min)	*Origin → solvent front (cm)*
Whatman No. 1	10	16
	12.5	17.5
	15	19
Whatman No. 3 MM	10	14
	12.5	15
Whatman No. 17 MM	10	6
	20	14
	25	16

It can be seen that increasing the time results in a nonlinear extension of the solvent front. Obviously when trying out a new grade of paper or a different solvent, it is necessary to make some trial runs with no pigments on the paper to find a satisfactory time.

Yet another variable needs to be considered: the use of the liner paper. This is essential with most solvents, in particular with petroleum ether solvents. Even with the heavier Whatman Nos. 3 MM or 17 MM circles, it is necessary to have a liner.

Table II illustrates the R_F values obtained, using the same solvent and conditions, at different velocities. In general, it is seen that above 450 r.p.m., the R_F decreases as

TABLE II

CHROMATOFUGE R_F VALUES OF ALGAL PIGMENTS

Conditions: Whatman No. 3 MM paper (unwashed). Solvent petroleum ether–isopropanol (100:2.5); flow rate of 2.5 ml/min; 7.5 p.s.i. nitrogen pressure; temp. 25°; liner saturated with 20 ml of solvent; time of development 10 min; circular origin 10 cm. The R_F values reported are the average of 3 separate runs at each velocity quoted.

Speed in r.p.m.	*350*	*400*	*450*	*500*	*550*	*600*	*650*	*750*	*850*	*950*
Carotenes	0.91	0.92	0.94	0.94	0.96	0.95	0.97	0.96	0.97	0.98
Lutein	0.46	0.47	0.56	0.47	0.44	0.42	0.36	0.35	0.32	0.30
Chlorophyll *a*	0.44	0.45	0.47	0.38	0.37	0.30	0.26	0.27	0.23	0.22
Chlorophyll *b*	0.27	0.26	0.26	0.23	0.23	0.16	0.14	0.14	0.11	0.08
Neoxanthin	0.06	0.08	0.10	0.13	0.11	0.11	0.08	0.05	0.05	0.04

Note: As in ascending or descending chromatograms, with petroleum ether–isopropanol (100:2.5), violaxanthin is masked in the bottom of the chlorophyll *a* spot.

the velocity of rotation increases, although for a change of 50 r.p.m. the decrease in the R_F is within the experimental error. Changes however, obtained over the complete range of velocities available (*i.e.* 350 to 950 r.p.m.) are significant. It will be noticed that between 350 and 450 r.p.m. the R_F values remain the same or increase with increasing velocity, while the general pattern in the range 450 to 950 r.p.m. is one of decreasing R_F values. The speed at which the R_F values are a maximum will be termed the "optimum speed"; the "optimum speed" in Table II is, therefore, about 450 r.p.m.

For several other solvents a similar set of runs was made at varying velocities and the same type of pattern was found as that shown in Table II. Instead of listing the whole gamut of R_F values obtained, Table III shows the R_F values at the "optimum

TABLE III

CHROMATOFUGE R_F VALUES OF ALGAL PIGMENTS AT "OPTIMUM SPEED"*

Conditions: Whatman No. 3 MM paper (unwashed); flow rate of 2.5 ml/min; 7.5 p.s.i. nitrogen pressure; liner saturated with 20 ml of solvent (unnecessary for toluene); time of development 10 min; circular origin, 10 cm. R_F values reported are the average of three separate runs at the velocities quoted.

	Solvent: toluene, "optimum speed" 600 r.p.m.		*Solvent: toluene–petroleum ether (4:1), "optimum speed" 500 r.p.m.*	
Compound	*Total extract*	*Carotenoid extract*	*Total extract*	*Carotenoid extract*
Carotenes	0.96	0.97	0.98	0.97
Lutein	0.89	0.90	0.90	0.90
Violaxanthin	0.82	0.80	0.78	0.74
Chlorophyll *a*	0.34	—	0.37	—
Chlorophyll *b*	0.19	—	0.27	—
Neoxanthin	0.04	0.06	0.07	0.07
	Solvent: toluene–petroleum ether (1:1), "optimum speed" 750 r.p.m.		*Solvent: toluene–petroleum ether (1:3), "optimum speed" 500 r.p.m.*	
Carotenes	0.98	0.94	0.98	0.95
Lutein	0.83	0.85	0.75	0.73
Violaxanthin	0.70	0.50	0.48	0.35
Chlorophyll *a*	0.33	—	0.30	—
Chlorophyll *b*	0.22	—	0.19	—
Neoxanthin	0.10	0.07	0.12	0.17

* "Optimum speed" is defined as that velocity of rotation of the chromatofuge where the R_F values of the pigments are a maximum.

speed". It is seen that this "optimum speed" is not constant for different solvents, but seems to lie nearer the lower velocities of rotation.

Comparison of the R_F values listed in Tables II and III to those obtained in the conventional ascending or descending chromatography, shows the similarity obtained with the same solvents. In all cases carotenes run right at the solvent front. Solvents with a higher proportion of toluene are useful for the separation of the carotenoids; on the other hand, the chlorophyll *a* and *b* travel further in pertoleum ether–isopropanol

solvents. Figs. 1 and 2 show the width of the pigment zones obtained; it is seen that the bands are very narrow and well-defined. They are less than one-half as wide as those which result from descending or ascending chromatograms, that have been run the same distance as the chromatofuge chromatograms.

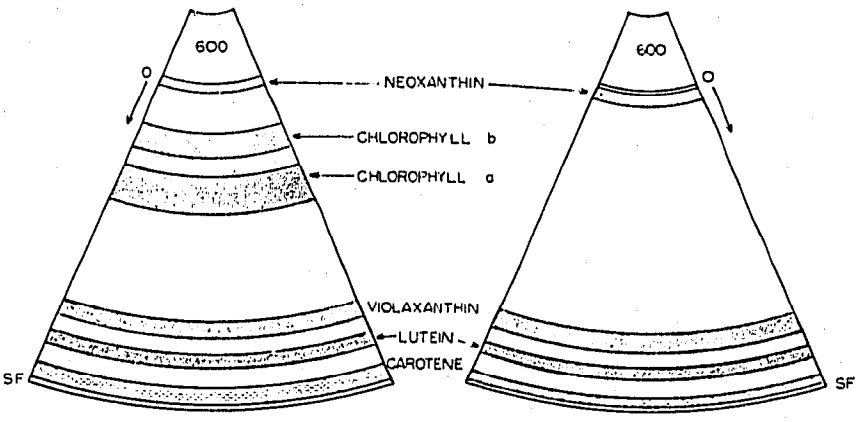

Fig. 1. Sectors of centrifugally accelerated paper chromatograms of the total pigment and carotenoid extracts from *Chlorella* using toluene as the solvent at 600 r.p.m. Time of development, 12.5 min; flow rate of 2.5 ml/min; origin → solvent front, 17 cm; Whatman No. 3 MM paper.

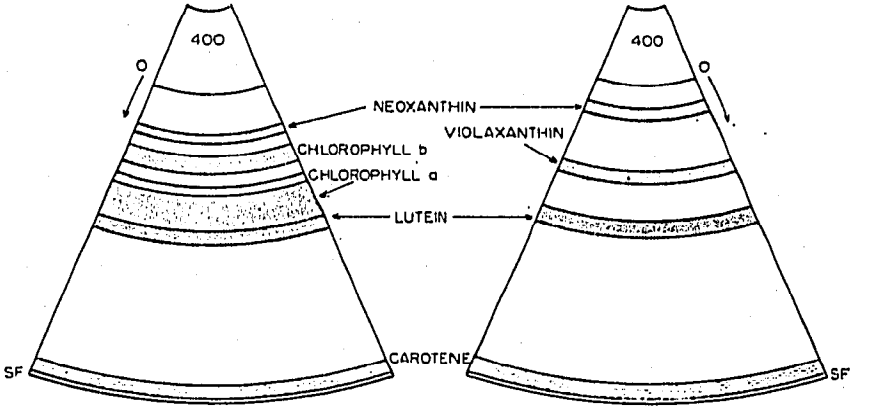

Fig. 2. Sectors of centrifugally accelerated paper chromatograms of the total pigment and carotenoid extracts from *Chlorella*, using petroleum ether–isopropanol (100:2.5) as the solvent at 400 r.p.m. Time of development, 10 min; flow rate of 2.5 ml/min; origin → solvent front, 17 cm; Whatman No. 3 MM paper.

In the hopes of obtaining better separation of chlorophyll *a* and *b*, reverse phase chromatography was tried. The methanol used as the solvent for the development of the paraffin oil-impregnated paper showed a great tendency to flood the papers. A flow rate of 1.25 ml/min with a 4 p.s.i. of nitrogen pressure was necessary, which meant at least 15 min development time. The results obtained were disappointing; the bands were diffused and streaking in to one another. All the pigments other than the carotene which stayed right at the origin had R_F values greater than 0.6, which meant that they were all crowded together despite the variety of conditions tried.

The quantitative recovery of pigments from a typical chromatogram was checked. About 88 % of the pigments placed on the origin were recovered, which is

much better than ascending chromatography where the average recovery was about 70 %[4]. Since the chromatograms take 10 to 15 min instead of 2 to 3 h to be developed, there is less time for decomposition of the pigments to occur. The pigment zones are easily eluted with ether and less time is needed to obtain colorless paper, hence there is a smaller loss in this operation than on the ascending or descending chromatograms. It would probably be advantageous to operate the chromatofuge at a lower temperature than 25°; it was not possible to do this with our machine.

SUMMARY

This paper presents the application of centrifugally accelerated paper chromatography for the separation of chloroplast pigments. It was found that similar results to conventional paper chromatographic methods were obtained. This technique appears to be very promising for the separation and recovery of the labile chlorophylls and carotenoids because of the narrowness and compactness of the pigment zones and the rapid development time. The pigments were easily eluted from the paper and a greater recovery of the pigments was obtained than from the usual paper chromatograms.

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