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The Purity of Chloroplasts Isolated in Non-aqueous Media

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ABSTRACT

By measuring the relative amounts of high-molecular-weight ribonucleic acids in chloroplasts and in cytoplasm reliable values were obtained for the purity of chloroplasts isolated in non-aqueous media from leaves of tobacco (*Nicotiana tabacum*, var. White Burley), broad bean (*Vicia faba*, var. White Fan), and tomato plants (*Lycopersicon esculentum*, var. Money Maker). Measurements of pyruvate kinase activity, previously used to test chloroplast purity, agreed well with the results of ribosomal ribonucleic-acid analysis for the bean and tomato leaves. The purest chloroplast fractions from tobacco leaves always contained more pyruvate kinase than could be accounted for on the basis of the cytoplasmic contamination measured by the nucleic-acid analysis. Some pyruvate kinase may therefore be present in the chloroplasts in tobacco leaves.

The purest chloroplasts obtained from any of the three species still contained 11 per cent of the cytoplasm even after severe mechanical treatments designed to remove cytoplasm adhering to the surface of the plastids. Chloroplast fractions obtained by the usual non-aqueous techniques always contained at least 15 per cent of the cytoplasm.

INTRODUCTION

The non-aqueous procedure for leaf fractionation (Stocking, 1959; Heber, 1960) prevents redistribution of water-soluble compounds between cell components. Chloroplast fractions separated by this method were reported to contain less than 10 per cent of the total cytoplasm (Heber, 1960; Santarius and Stocking, 1969). Bird, Keys, and Whittingham (1971) found that chloroplast fractions isolated from tobacco leaves always contained more than 10 per cent of the total pyruvate-kinase activity. The activity of this enzyme has been frequently used to assess cytoplasmic contamination of isolated chloroplasts (Heber, Pon, and Heber, 1963; Santarius and Stocking, 1969; Heber, Hallier, and Hudson, 1967; Ritenour, Joy, Bunning, and Hageman, 1967). In the present study we have used the relative amounts of chloroplast and cytoplasmic ribosomal RNA (rRNA) (Ingle, 1968; Dyer, Miller, and Greenwood, 1971) to measure purity of fractions isolated in non-aqueous media. Chloroplast fractions were always contaminated by more than 10 per cent of cytoplasm. Various methods were used in attempts to remove the cytoplasm from chloroplast fractions.

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MATERIALS AND METHODS

Broad bean seed (*Vicia faba*, var. White Fan) was supplied by Carters Tested Seeds Limited, Raynes Park, London, S.W. 20. Tomato plants (*Lycopersicon esculentum*) were of commercial origin and seed for tobacco (*Nicotiana tabacum*, var. White Burley) was from plants grown in a glasshouse. Plants were kept in darkness for 48 h before use. Leaf material was freeze-dried either as a powder (Keys, 1968) or as pieces 1 cm^2 in area (Bird, Porter, and Stocking, 1965). The techniques used for non-aqueous fractionation were described by Stocking (1959), Bird *et al.* (1965), and Keys (1968).

Freeze-dried powders, and the dry fractions resulting from non-aqueous fractionations, were extracted at 0–5 °C with buffered medium containing 30 mM tris, 10 mM cysteine hydrochloride, and 10 mM sodium diethyldithiocarbamate. Solid material was removed by centrifugation and a portion of the supernatant liquid assayed immediately for pyruvate-kinase activity by the method of Miller and Evans (1957) with a correction for phosphatase activity. A further portion of the supernatant liquid was mixed with four volumes of saturated ammonium sulphate solution (pH 7·0). The precipitate that formed was dissolved in phosphate buffer (pH 7·5) and dialysed overnight against the same buffer. The dialysed liquid was assayed for glutamate dehydrogenase (NAD) activity by the method of Bulen (1956). Chlorophyll was measured by the method of Arnon (1949).

Nucleic acids were extracted by the method of Parish and Kirby (1966) using an aqueous solution of sodium 4-aminosalicylate and sodium tri-*iso*propylnaphthalene sulphonate and deproteinized by mixing with a phenol-cresol solution. The nucleic acids were fractionated in 2·4 per cent gels as described by Loening (1967, 1969) using buffers containing tris, NaH_2PO_4, and EDTA. Nucleic acids in the gels were measured spectrophotometrically at 260 nm. Cytoplasmic contamination of the chloroplast fractions was calculated from measurements of the relative amounts of cytoplasmic rRNA (component with M. wt. $1{\cdot}3\times10^6$) and chloroplast rRNA (component with M. wt. $0{\cdot}56\times10^6$) in the fractions (Dyer *et al.*, 1971). The cytoplasmic rRNA found with the chloroplasts in a non-aqueous fraction has been expressed ('cytoplasmic contamination') as a percentage of cytoplasmic rRNA associated with the same number of chloroplasts in the whole leaf.

RESULTS

We have found that both the yield and purity of chloroplast fractions obtained from freeze-dried leaf powder improved when the particle size of the powder was decreased. Thus when a suspension of leaf powder in a mixture of carbon tetrachloride and hexane was treated for 10 min with an ultrasonic disintegrator the yield of chloroplast fraction was increased from 80 to 190 mg per g of leaf powder. The chlorophyll in the fraction was increased from 3·34 to 4·21 per cent of the dry weight and the cytoplasmic contamination calculated from measurements of pyruvate kinase activity (Heber *et al.*, 1963) was decreased from 42 to 23 per cent. The ultrasonic treatment destroyed 18 per cent of the pyruvate kinase activity in the tissue suspension. It follows that estimations of cytoplasmic contamination from pyruvate-kinase activities must be inaccurate. A series of experiments was undertaken with the object of isolating chloroplasts with as little contamination as possible. Pyruvate kinase activity, chloroplast, and cytoplasmic rRNA were measured and used as independent criteria of purity. Mitochondrial contamination was assessed by the glutamate dehydrogenase (NAD) activity of the fractions (Leech and Kirk, 1968). Table 1 shows the application of these analyses to fractions obtained from a tobacco-leaf powder. Contamination by cytoplasm of the purest chloroplasts (buoyant in mixtures of hexane and carbon tetrachloride with densities from 1·26 to 1·28) was calculated to be 22 per cent from the pyruvate kinase activity and 14·4 per cent from rRNA determination. Contamination by 5·5 per cent mitochondria was

calculated from glutamate dehydrogenase activity. Following similar fractionation of bean leaf the purest chloroplast fraction was buoyant at densities between 1·24 and 1·26 and contained 15·9 per cent contamination by cytoplasm calculated from pyruvate kinase activities; 15·7 per cent calculated from rRNA analyses. For tomato leaf the best fraction was buoyant between densities 1·28 and 1·32 and contained 19·6 per cent cytoplasmic contamination calculated from pyruvate kinase activities and 19·4 per cent calculated from rRNA content. These chloroplast

TABLE 1. *The pyruvate kinase, glutamate dehydrogenase* (NAD) *activities and cytoplasmic contamination of fractions from tobacco leaves separated in non-aqueous media*

Before centrifugation the freeze-dried powder was suspended in non-aqueous medium of density 1·24 and treated with an ultrasonic disintegrator for a total of 9 min at 0 °C. The suspension was centrifuged (20 000 g 15 min, 0 °C) and the fraction of the particles remaining in suspension was recovered (density < 1·24). The residue was resuspended in non-aqueous medium density 1·26 and this suspension centrifuged and the fraction of particles that remained suspended was recovered. Successive fractions were recovered in this manner until the residue left was more dense than 1·36.

Fraction (density of CCl_4/C_6H_{14} mixtures between which fractions were buoyant)	Dry wt. of fraction (mg per g whole tissue)	Chlorophyll (μg per mg fraction)	Pyruvate kinase activity	Glutamate dehydrogenase (NAD)activity	'Cytoplasmic contamination'* measured by RNA (%)
			(nmoles substrate transformed in 15 min per mg fraction)		
< 1·24	24·8	43·4	55 (19)†	..	17·0
1·24–1·26	104·8	44·6	61 (20)	7·7 (3·5)‡	14·5
1·26–1·28	122·0	39·9	61 (22)	10·8 (5.5)	14·4
1·28–1·32	62·3	31·8	83 (38)	72·7 (46)	23·8
1·32–1·36	47·6	16·9	102 (89)	143·4 (171)	70·2
> 1·36	592·3	1·4	88 (922)	80·0 (1152)	902·0
Whole tissue	1000·0	15·6	97 (100)	70·6 (100)	100

* See Materials and Methods.

† Figures in brackets represent cytoplasmic contamination calculated from pyruvate kinase activity.

‡ Figures in brackets represent contamination by mitochondria calculated from glutamate dehydrogenase (NAD) activities.

fractions contained close to 6 per cent of the mitochondria. Pyruvate kinase activities were particularly high in chloroplast fractions from tobacco leaves and cytoplasmic contamination calculated from these activities always gave higher values than those calculated from rRNA measurements. In further experiments we isolated from tobacco leaves a chloroplast fraction buoyant between densities 1·24 and 1·28 in carbon tetrachloride and hexane mixtures and corresponding therefore to the purest fractions shown in Table 1. This fraction was treated with an ultrasonic disintegrator for 3 min and the chloroplast fraction with density 1·24–1·28 reisolated. This purified fraction was resuspended in medium (d. 1·24) and treated again for 3 min with the ultrasonic disintegrator. The fraction with density 1·24–1·28 was again isolated. After this procedure was repeated twice more

the chloroplast fraction obtained was estimated, after rRNA analysis, to contain 11 per cent cytoplasmic contamination. In a similar experiment the purest fraction contained 13 per cent cytoplasmic contamination (rRNA) but 24 per cent of the total pyruvate kinase in the leaf. In another experiment treatment with the ultrasonic disintegrator was replaced by passing the chloroplast fraction, four times through a modified French Press (Millbank and Neale, 1969) at 20 000 lbf/in^2. The chloroplast fraction obtained had 13 per cent cytoplasmic contamination (rRNA) but more than 20 per cent of the total pyruvate kinase in the leaf.

Other chloroplast fractions were dried and strongly compressed into pellets. These were ground in an agate mortar. Particles isolated from the powder, which were buoyant at densities between 1·24 and 1·28 still had a cytoplasmic rRNA content equivalent to 15 per cent contamination.

Chloroplast fractions were also obtained in non-aqueous media to which substances had been added which might facilitate removal of cytoplasm adhering to the chloroplasts. Addition to the carbon tetrachloride and hexane mixtures of 2 per cent acetic acid, ethanol, methoxyethanol, phenol, Tween 20, acetone, diethyl ether, Triton X100, chloroform, diethylamine, ethyl acetate, 8-hydroxyquinoline, or 1, 1, 1-trifluoro-3-(2-thenoyl)-acetone was not beneficial and often led to leaching of chlorophyll.

Chloroplast fractions obtained from pieces of tobacco leaf by the method of Bird *et al.* (1965) did not contain less pyruvate kinase or cytoplasmic rRNA than similar fractions obtained from freeze-dried powder.

DISCUSSION

The relative amounts of cytoplasmic and chloroplast high-molecular-weight rRNAs in leaf-tissue fractions provides a reliable basis for determining cytoplasmic contamination of chloroplast fractions. Not only are both types of rRNA more stable than pyruvate kinase, but it is known with certainty that one type of rRNA is present exclusively in the chloroplasts and a different type of rRNA is present exclusively in the cytoplasm (Dyer *et al.*, 1971). By measuring the amounts of both types of rRNA present in the chloroplast fractions and in the tissue before fractionation the extent to which the chloroplasts have been freed from cytoplasm can be calculated. We consider that these data give a more accurate picture of cytoplasmic contamination of non-aqueous chloroplasts than was previously available.

For chloroplast fractions from bean and tomato leaves cytoplasmic contamination calculated from measurements of the distribution of pyruvate-kinase activity gave values very similar to those calculated from rRNA distribution. The purest chloroplast fractions from leaves of tobacco had a cytoplasmic contamination of 23 per cent calculated from measurements of pyruvate-kinase activity but only 13 per cent when calculated from rRNA measurements. This difference may be attributed to the presence in chloroplasts of tobacco leaves of 10 per cent of the total pyruvate-kinase activity.

Pyruvate kinase may not be absent from the chloroplasts of other species. The enzyme is labile and accurate measurements of its activity are made with difficulty.

For example, it was necessary to extract the enzyme into a medium designed to minimize *o*-diphenoloxidase (EC 1. 10. 3. 1) activity, to assay the enzyme soon after extraction, and to allow in the assay for a phosphatase which interfered. Furthermore, pyruvate kinase was inactivated during mechanical disruption of freeze-dried material so that its apparent distribution was altered.

We conclude that the purest chloroplasts isolated in non-aqueous media still have between 10 and 15 per cent of the cytoplasm adhering to them. It has not been possible for us to remove this cytoplasm from the chloroplasts by various mechanical and chemical means.

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