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ISOLATION PROCEDURES AFFECTING THE RETENTION OF WATER-SOLUBLE NITROGEN BY SPINACH CHLOROPLASTS IN AQUEOUS MEDIA

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SUMMARY

Optimum conditions were determined for the retention of protein nitrogen and chlorophyll in isolated spinach chloroplasts. Chloroplasts isolated in buffered 0.3 M sucrose remain intact if maintained at high concentration or if treated with 1–10% glutaraldehyde for 5 min. EDTA in the isolation medium was not bound or adsorbed to any extent to chloroplasts during isolation. Large nitrogen losses occurred when the chloroplasts were diluted in sucrose buffer. Virtually all the nitrogen of concentrated chloroplast preparations could be precipitated by 80% acetone.

INTRODUCTION

Methods for the isolation of chloroplasts in aqueous media result in the loss of water-soluble protein[1,2] while nonaqueous isolation media extract the chlorophyll of chloroplasts and make their microscopic identification difficult. However, aqueous isolation procedures have been improved since SPENCER AND WILDMAN[3] observed highly refractile chloroplasts and expanded chloroplasts with visible grana in conventional chloroplast suspensions. LEECH[4] has recently separated these two types of chloroplasts on a gradient and found that the refractile chloroplasts are intact while the expanded chloroplasts have lost their outer membrane and stroma. She established that the protein to chlorophyll ratio of the expanded chloroplasts was much lower than that of intact chloroplasts. Subsequently she reported two types of expanded chloroplasts, both types being characterized by having lost their outer membranes but one type differing from the other in having retained its stroma[5].

This paper reports experiments undertaken to determine optimum conditions for the retention of nitrogen and chlorophyll in spinach chloroplasts during isolation.

MATERIALS AND METHODS

Plant material

Spinach plants were grown as previously described[6]. Plants were harvested 14–21 days after being transferred to nutrient cultures.

Chloroplast isolation

Leaf tissue (5 g) was sliced with razor blades in 10 ml of isolation medium (0.3 M sucrose–0.05 M potassium phosphate buffer–0.01 M EDTA (pH 8.5)) as described by

SPENCER AND WILDMAN[3]. The homogenate was filtered through 4 layers of fine cloth to remove the cell debris and the filtrate centrifuged at 1000 × *g* for 5 min at 4° to sediment the chloroplasts. The residue was suspended in 2 ml of suspension medium (0.3 M sucrose–0.05 M potassium phosphate buffer (pH 7.3)) and recentrifuged. The residue was suspended in 2 ml of suspension medium.

Nitrogen precipitation from a chloroplast suspension by 80% acetone

Aliquots (0.1 ml) of chloroplast suspension were diluted to a range of volumes with acetone (final acetone concn. 80%). The suspensions were centrifuged at 10 000 × *g* for 10 min at 4°. The residue was suspended in 3 ml of 80% acetone and recentrifuged at 10 000 × *g* for 10 min.

Nitrogen fixation by glutaraldehyde

Chloroplasts were isolated from 30 g of leaf tissue in 60 ml of isolation medium, filtered and divided into 10-ml aliquots. The aliquots were diluted with continuous stirring to 20 ml with solutions of glutaraldehyde to final concns. of 0, 0.01, 0.1, 1.0 and 10% glutaraldehyde.

The suspensions were immediately centrifuged at 1000 × *g* for 5 min. The residues were washed by suspending in 2.5 ml water and centrifuging at 30 000 × *g* for 10 min 3 times.

Chemical analyses

Chlorophyll was measured spectrophotometrically[7]. Nitrogen was measured by Markham distillation after acid digestion.

RESULTS

Nitrogen precipitated by 80% acetone

The proportion of the total nitrogen precipitated by 80% acetone is dependent on the concentration of the chloroplast suspension in the acetone (Table I).

Effect of EDTA on the nitrogen to chlorophyll ratio of the chloroplasts

In replicated experiments it was shown that the nitrogen to chlorophyll ratio was not changed by the omission of EDTA from the isolation medium indicating that EDTA, which is rich in nitrogen, was not bound to any extent to the chloroplasts.

Nitrogen fixation by glutaraldehyde

The effect of a range of glutaraldehyde concentrations on the loss of nitrogen and chlorophyll from chloroplast suspensions in two separate experiments is shown in Table II. Untreated chloroplasts suspended in water disrupted, necessitating high-speed centrifugation to sediment the fragments. Chloroplasts treated with 0.01% glutaraldehyde and suspended once in water were sedimented at 1000 × *g* but on subsequent suspension in water they fragmented and behaved as untreated chloroplasts and required high-speed centrifugation to sediment the fragments. Similar large quantities of nitrogen were lost from chloroplasts treated with 0.01% glutaraldehyde. The amounts of chlorophyll that were lost from these plastids was much less so that the nitrogen to chlorophyll ratio of the chloroplasts was approximately half the initial ratio. Chloroplasts treated with 0.1–10% glutaraldehyde and suspended once in water were identical in appearance to the original chloroplast preparation when examined at

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TABLE I

NITROGEN PRECIPITATED FROM A CHLOROPLAST SUSPENSION BY 80% ACETONE

Each value represents the mean of replicates.

Volume of chloroplast suspension (ml)	*Suspension volume (ml)*	*Nitrogen sedimented (μg)*	*Total nitrogen precipitated (% initial nitrogen of suspension)*
0.2	0.2	966	100
0.2	1	982	102
0.2	2	908	95
0.2	3	884	92
0.2	4	829	86
0.2	8	819	85

TABLE II

EFFECT OF GLUTARALDEHYDE CONCENTRATIONS ON RETENTION OF CHLOROPLAST NITROGEN

Values are given for 2 separate experiments. Each value is the mean of 2 replicate measurements.

Glutaraldehyde concn. in diluted (1 ×) suspensions	*Final chlorophyll per sample (μg)*		*Final nitrogen per sample (μg)*		*Nitrogen to chlorophyll ratio of final pellet*		*Final chlorophyll concn. (% initial)*		*Final nitrogen (% initial)*	
	1	*2*	*1*	*2*	*1*	*2*	*1*	*2*	*1*	*2*
0	851	979	1027	708	1.21	0.72	100	89	61	38
0.01	792	989	952	987	1.21	1.00	93	90	57	53
0.10	816	915	1335	1463	1.64	1.60	96	83	80	78
1.00	661	928	1498	1678	2.26	1.81	78	84	89	90
10.00	664	965	1357	1637	2.11	1.70	76	87	81	88
Initial values of non-diluted suspensions	848	1103	1676	1868	2.38	1.68				

TABLE III

EFFECT OF DILUTION ON CHLOROPHYLL LOSS FROM CHLOROPLAST SUSPENSIONS

Dilution ratio	*Final chlorophyll per sample* (*μg*)	*Final chlorophyll* (% *initial*)
1:0	242	100
1:1	243	100
1:2	251	103
1:5	239	99
1:10	227	93
1:20	206	85
1:50	206	85
1:100	154	64
Initial value	243	

TABLE IV

CHLOROPHYLL AND NITROGEN LOSS FROM DILUTE SUSPENSIONS OF SPINACH CHLOROPLASTS

Treatment	*Chlorophyll in final pellet* (*μg*)	*Nitrogen in final pellet* (*μg*)	*Nitrogen to chlorophyll ratio in final pellet*	*Final chlorophyll* (% *initial*)	*Final nitrogen* (% *initial*)
Initial pellet	174	331	1.90	100	100
Pellet suspended 4 times in isolation medium (pH 8.5); dilution 1:12	133	160	1.20	76	48
Pellet suspended 4 times in suspension medium (pH 7.3); dilution 1:12	144	165	1.15	83	50
Homogenate fixed* and pellet suspended 4 times in water**; dilution 1:12	145	320	2.21	83	97

* Fixed in 3% glutaraldehyde for 10 min.
** Precipitated at 1000 × *g*.

magnifications of 1250 × with phase-contrast objectives. These chloroplasts were sedimented by low-speed centrifugation after subsequent suspensions in water. Chloroplasts treated with 1.0 and 10% concentrations of glutaraldehyde lost similar and small proportions of both their nitrogen and chlorophyll so that their final nitrogen to chlorophyll ratio was similar to the initial value.

Effect of chloroplast dilution on chlorophyll loss

When chloroplast suspensions were prepared as described and kept at 4° the chloroplasts remained intact for several days. If, however, the chloroplast suspensions were diluted 10-fold with suspension medium large numbers of fragments could be observed after 3 h. Addition of 1 mg per ml of bovine serum albumin reduced the rate of fragmentation. The effect of dilution of chloroplast suspensions in buffered 0.3 M sucrose on chlorophyll loss is shown in Table III. After dilution the suspensions were centrifuged at low speed so that any chloroplasts disrupted on dilution would not be sedimented. Dilutions of 1:10 or greater resulted in the loss of chlorophyll. Continued resuspension and recentrifugation of chloroplasts diluted 1:12 resulted in a larger loss of nitrogen than chlorophyll (Table IV) so that the final nitrogen to chlorophyll ratio was half the initial ratio and was the same at pH 7.3 and 8.5. Glutaraldehyde treatment prevented the loss of nitrogen from the diluted suspension and the final nitrogen to chlorophyll ratio of the glutaraldehyde-treated suspension was the same as the initial pellet.

DISCUSSION

It is considered that the nitrogenous compounds of chloroplasts precipitated by 80% acetone (Table I) would be largely composed of protein[1]. Spinach chloroplasts are able to synthesize proteins[8] hence amino acids would be expected in chloroplast preparations. Failure to detect the small molecular weight nitrogenous compounds in the chloroplast preparation may be due to their low concentration or to their rapid removal during chloroplast isolation. Although the possibility exists that small molecular weight nitrogenous compounds were bound during isolation the main nitrogenous compound in the isolation medium (EDTA) was shown to have no effect on the nitrogen to chlorophyll ratio of the final chloroplast pellet.

Chloroplasts suspended in water swell and disrupt, resulting in chloroplast fragments which were only sedimented by high-speed centrifugation. All the chlorophyll was sedimented with the water-insoluble fraction and the nitrogen to chlorophyll ratio of this fraction was half that of the original chloroplast suspension (Table II). Half the chloroplast protein was therefore water soluble. It is of interest that HEBER AND TYSKIEWICZ[9] who used nonaqueous isolation methods to retain all the chloroplast protein observed a similar ratio of water-soluble to water-insoluble protein in spinach chloroplasts.

The nitrogen to chlorophyll ratio of chloroplasts treated with 1–10% glutaraldehyde was not altered by suspension in water (Tables II and IV). Glutaraldehyde is known to produce fixation of protein *in situ*[10].

Chloroplasts suspended in aqueous media retain the water-soluble protein fraction if isolated by gentle procedures and kept at high concentration (greater than 50 μg chlorophyll per ml but preferably 500 μg chlorophyll per ml) or if fixed for 5 min

with concentrations of 1–10% glutaraldehyde. We consider that with these precautions aqueous media can be used to prepare chloroplast suspensions for measurements of both the chlorophyll and nitrogen content of chloroplasts.

REFERENCES

1 J. T. O. KIRK, in J. T. O. KIRK AND R. A. E. TILNEY-BASSETT, *The Plastids; their Chemistry, Structure, Growth and Inheritance*, Freeman and Co., London, 1967, p. 5.
2 J. B. THOMAS, in W. RUHLAND, *Encyclopedia of Plant Physiology*, Vol. V/1, Springer-Verlag, Berlin, 1960, p. 511.
3 D. SPENCER AND S. G. WILDMAN, *Biochemistry*, 3 (1964) 954.
4 R. M. LEECH, in T. W. GOODWIN, *Biochemistry of Chloroplasts*, Vol. 1, Academic Press, New York, 1966, p. 65.
5 S. M. RIDLEY AND R. M. LEECH, *Planta*, 83 (1968) 20.
6 D. E. BOTTRILL, J. V. POSSINGHAM AND P. E. F. KRIEDEMANN, *Plant Soil*, in the press.
7 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
8 D. SPENCER, *Arch. Biochem. Biophys.*, 111 (1965) 381.
9 V. HEBER AND E. TYSZKIEWICZ, *J. Exptl. Botany*, 13 (1962) 185.
10 D. D. SABATINI, K. G. BENSCH AND R. J. BARRNETT, *J. Cell Biol.*, 17 (1963) 19.