BBA 45749

NICOTINAMIDE COFACTORS OF INTACT CHLOROPLASTS ISOLATED ON A SUCROSE DENSITY GRADIENT

M. J. HARVEY* AND A. P. BROWN

Department of Biochemistry, University of Birmingham, Birmingham (Great Britain)

(Received July 31st, 1968)

SUMMARY

1. The potassium content of chloroplasts expressed as a potassium–chlorophyll ratio was adopted as a new criterion of chloroplast integrity.

2. Chloroplast fractions from pea seedlings were prepared using a sucrose density gradient and found to contain not less than 80% intact chloroplasts and no recognisable cellular components as contaminants.

3. $NADP^+$, NADPH, NAD^+ and NADH assays on these preparations are reported, and compared with published values.

4. The light-induced reduction of endogenous $NADP^+$ and NAD^+ was followed and similarly the reduction of exogenous $NADP^+$. A NADPH peak occurred during the initial 30-sec illumination, in agreement with data published for illumination of the whole leaf.

5. The observations indicate that these chloroplasts are freely permeable to $NADP^+$ and may accumulate it against a concentration gradient. There is also some evidence to indicate that conversion of exogenous $NADP^+$ into NAD(H) occurs on illumination.

INTRODUCTION

This work forms part of an investigation directed towards a better understanding of the chloroplast as a tissue compartment. Evidence from electron microscopy indicates that the chloroplast is bounded by a limiting membrane that might reasonably be expected to act as a barrier to diffusion; signs of this occur in many reports of photosynthesis.

The membrane appears to be fragile and easily broken during chloroplast isolation, however, and a criterion of chloroplast integrity was required to evaluate isolation procedures. The retention of potassium in chloroplast preparations (expressed as a potassium–chlorophyll ratio) amounts to a simple assay (by flame photometer) that directly tests the retention of the diffusional barrier. The possibility of light-activated potassium movement across the membrane was initially unresolved, but

Abbreviation: DCIP, dichlorophenolindophenol.

* Present address: Biology Department, Brandeis University, Waltham, Boston, Mass., U.S.A.

CROFTS[1] has since shown that under conditions of light-activated ammonia migration, the movement of potassium is negligible.

A number of successful isolations of chloroplasts in an aqueous medium have been reported during this work by FRANKI, BOARDMAN AND WILDMAN[2], HONDA, HONGLADROM AND LATIES[3], LEECH[4], SPENCER AND UNT[5] and WALKER[6] among others. These reported procedures have in common the use of a polyhydric compound such as sucrose or glycerol to maintain the osmotic strength of the medium, replacing the use of electrolytes for this purpose. In non-aqueous isolation techniques, whole leaves are exposed to liquid nitrogen and then freeze-dried. A chloroplast-rich fraction is then prepared by centrifugation in a lipophilic solvent system. The resulting chloroplast fraction shows a much improved retention of water-soluble components compared with preparations from electrolyte-reinforced aqueous media, but a limited ability to reduce dye-stuffs on illumination indicates that changes have occurred beyond mere dehydration. Nonetheless the reports of HEBER AND SANTARIUS[7] and OGREN AND KROGMANN[8] using this method established new standards for the chloroplast content of water-soluble components, including NAD(H) and NADP(H) with which the present work has been compared. The time course of light-activated $NADP^+$ reduction was also reported by HEBER AND SANTARIUS[7] and provides a test of chloroplast performance. Our preparations showed a very similar response to that reported by these authors, and the results suggest that $NADP^+$ from the medium was readily reduced.

A preliminary report of this work has been published by HARVEY AND BROWN[9], and the light-induced uptake and esterification of phosphate has also been examined and briefly reported[10].

MATERIALS AND METHODS

Chemicals and enzymes

Glc-6-*P*, $NADP^+$, NADPH and NADH sodium salts, Glc-6-*P* dehydrogenase (EC 1.1.1.49) and alcohol dehydrogenase (EC 1.1.1.1) were purchased from Boehringer Corporation (London). Phenazine methosulphate and NAD^+ (acid) were purchased from Sigma Chemical Co., St. Louis, Mo.

Assays

Chlorophyll was assayed by the method of ARNON[11].

Protein was assayed by the biuret procedures of GORNALL, BARDAWILL AND DAVID[12] and ITZHAKI AND GILL[13]. Protein was precipitated by methanol and collected by centrifugation at 1720 $\times$ *g* for 8 min.

Potassium was assayed by flame photometry. The sample in 1.0 ml conc. HNO_3 (A.R.) was digested by heating 30 min in a boiling water bath, and then diluted to 2.5 ml or 5.0 ml for assay. Standard solutions of equimolar KCl and NaCl were used to calibrate the flame photometer (Evans Electroselenium, Model No. 100).

Nicotinamide coenzymes were assayed by a slight modification of the method of SLATER, SAWYER AND STRÄULI[15]. For NADP(H) assays the cuvette contained in 2.9 ml volume 1 μmole EDTA, 50 μmoles Tris–HCl (pH 8.0), 10 μmoles Glc-6-*P*, 0.133 μmole dichlorophenolindophenol (DCIP) and 0.66 μmole phenazine methosulphate. For NAD(H) assays the cuvette contained in 2.9 ml volume 50 μmoles

phosphate (pH 7.4), 170 μmoles ethanol, 0.133 μmole DCIP and 0.66 μmole phenazine methosulphate.

NADH and NADPH were extracted from 2.0-ml samples of the incubation system by the method of GLOCK AND MCLEAN[14], using boiling 0.1 M NaOH. A further heating for 1 min in a boiling water bath caused complete destruction of NAD^+ and $NADP^+$ and a 4 % loss of NADH and NADPH. NAD^+ and $NADP^+$ were extracted in 1 M $HClO_4$. Recoveries of 96–104 % of $NADP^+$ and NAD^+ were obtained and complete destruction of NADH and NADPH. The tissue extract was neutralised, the $KClO_4$ was removed by centrifugation, 10 μmoles nicotinamide were added and 2 ml of the extract were added to the assay cuvette. After 5-min equilibration at 20°, 50 μg Glc-6-*P* dehydrogenase in 10 μl (NADP(H) assay) or 300 μg alcohol dehydrogenase in 10 μl (NAD(H) assay) were added, and the drop in absorbance at 600 mμ was followed in a modification (Gilford Instruments) of the Unicam SP 500 spectrophotometer. The change in absorbance was compared with a sample of known cofactor content taken through the same extraction and assay procedure but lacking the tissue extract. A second sample of tissue extract to which a known sample of the appropriate cofactor had been added was also assayed to detect any enzyme inhibition. The assay method was calibrated against cofactor *A* measurements at 340 mμ.

Chloroplast isolation

The cultivation of seedlings of *Pisum sativum* (var. Meteor) has been previously described[16]. Seedlings were harvested at 13 ± 2 days after sowing. Chloroplasts were prepared in an isolation medium consisting of 0.4 M sucrose buffered with 27.5 mM Tris–HCl (pH 7.8). In early experiments the isolation procedure of BROWN[16] was used. For sucrose density gradient experiments a more concentrated chloroplast suspension was required, and leaf tissue was chopped by hand in approximately its own volume of isolation medium at 4°. Cell debris was strained off with nylon fabric (4 layers of 70 strands to 1 inch), and the chloroplast suspension applied to the sucrose gradient.

Illuminated incubations

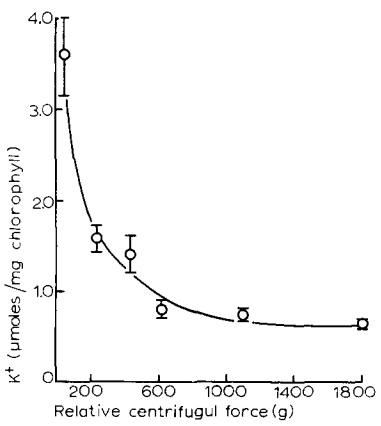
2.5 ml of chloroplast suspension (75 μg/ml chlorophyll) in isolation medium reinforced with $MgCl_2$ (1 mM) were incubated at 22–25°. The light source was a 275-W tungsten lamp (Philips PE 216B/43 photolita) 10 cm from the incubations, with a 10 % (w/v) solution of $CuSO_4 \cdot 5\ H_2O$ interposed as a heat filter (light path, 2.0 cm).

RESULTS

Preliminary tests indicated that the potassium–chlorophyll ratio might be a useful criterion of chloroplast integrity, but in early tests the assays indicated that a loss of potassium occurred during centrifugation (Fig. 1). At a given centrifugal force, the reciprocal of the potassium–chlorophyll ratio was linearly related to centrifugation time (Fig. 2). The plots extrapolated back to zero centrifugation time intercept the axis at 6.4 ± 0.1 μmoles K^+/mg chlorophyll, and in the absence of comparable published information, this value was accepted as the potassium content of the chloroplasts.

The potassium appeared to occur in the chloroplast in a free soluble form (it

could be released by fragmenting the chloroplasts) and was recovered (92–108%) in the 72 500 × *g* supernatant. Chloroplast suspensions could be diluted or washed by resuspension without significant loss of potassium, and incubation in 5 mM KCl solutions followed by washing gave no evidence of potassium adsorption by the organelles. The loss of potassium during the preparative centrifugation of chloroplasts



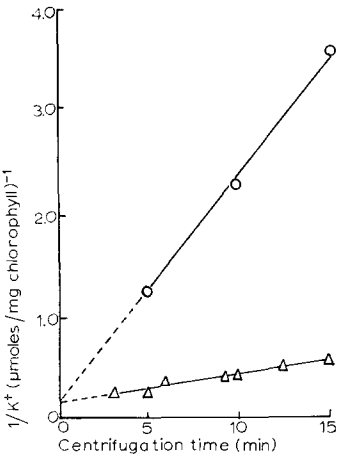


Fig. 1. The effect of centrifugal force (*g*) on the potassium content of chloroplast pellets. Centrifugation time, 5 min. Results are the means of 4 determinations ± S.E.

Fig. 2. Chloroplast potassium content (plotted as reciprocal) related to centrifugation time. △—△, 50 × *g*; ○—○, 620 × *g*. The extrapolated plots intercept the axis at 6.4 ± 0.1 μmoles K^+/mg chlorophyll.

was ascribed to frictional damage, especially in pellet formation, as microscopic examination of resuspended pellets showed chloroplast aggregates, whereas chloroplasts were individually dispersed in non-pelletted suspensions. To overcome this, the use of discontinuous preparative gradients was adopted, following the lead of JAMES AND DAS[17].

The strained leaf homogenate in 0.4 M sucrose was layered on 0.75 M sucrose with a layer of 1.5 M sucrose below this, all three solutions containing 27.5 mM Tris–HCl (pH 7.8). After 20 min centrifugation at 750 × *g* in a swing-out rotor, the chloroplast band recovered from the 0.75–1.5 M sucrose interface was found to have 5.5 ± 0.8 μmoles K^+/mg chlorophyll. A sample of this band suitably diluted and applied to a second identical gradient gave a value of 5.2 μmoles K^+/mg chlorophyll. Further samples examined by light and reverse phase microscopy gave a count of 80% intact and highly refractive chloroplasts (see Fig. 3) with no contamination by cell components. (Bacterial contamination occurs, although in fresh preparations this is low.) LEECH[18] following KAHN AND VON WETTSTEIN[19] considers the halo of highly refractive chloroplasts as a criterion of integrity. We regard such chloroplasts as intact and fully contracted. The remaining 20% in our preparations represents expanded chloroplasts[20,3] which may also still possess an intact limit membrane, but this is uncertain and the preparations are best described as having not less than 80% intact chloroplasts. This procedure of chloroplast preparation, including the use of a discontinuous sucrose gradient, was adopted for the experiments that follow.

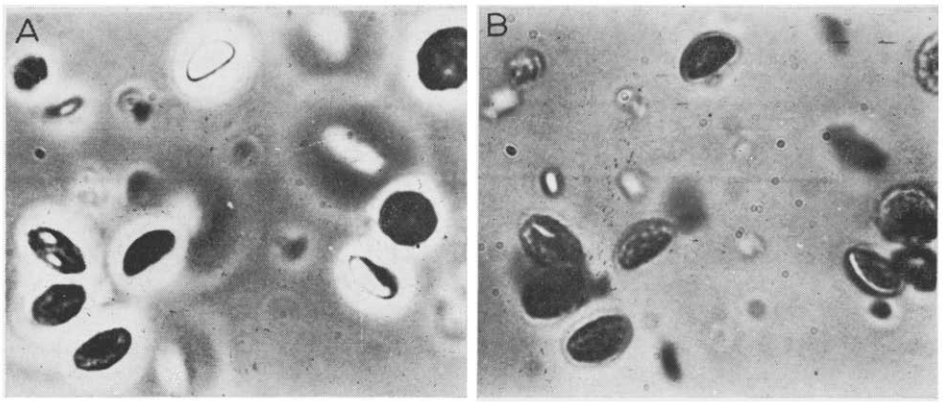


Fig. 3. Chloroplast preparation recovered from the 0.75 M–1.5 M sucrose interface after density gradient centrifugation for 20 min at 750 × *g*. The same field of view is presented, magnification × 1440. (A) Phase contrast; (B) transmitted light.

Nicotinamide cofactor content of leaf tissue and chloroplasts

To test the extraction and assay techniques and to establish a reference level that was independent of chloroplast integrity, the nicotinamide cofactor level of extracts of pea leaf was determined. In Table I the results are compared with published data for leaf tissues of various species. The values given there show a measure of agreement for $NADP^+$, NADPH and total NAD(H), which is regarded as acceptable. In the present work, the NADH content is greater than the published figures, and this produces a different NAD^+/NADH ratio.

TABLE I

NICOTINAMIDE COFACTOR CONTENT OF LEAF TISSUE

	NADP+	*NADPH*	*NAD+*	*NADH*
	Coenzyme (nmole/mg dry wt.)			
Pea (this study)*	0.141 ± 0.019	0.025 ± 0.04	0.200 ± 0.054	0.068 ± 0.007
Spinach and bean (ref. 7)	0.15	0.08	0.50	0.015
Pea (ref. 21)**	0.05	0.005	0.10	0.016
	Coenzyme (nmoles/μmole chlorophyll)			
Pea (this study)	7.35 ± 0.99	1.33 ± 0.23	9.83 ± 2.86	3.52 ± 0.39
Spinach and bean (ref. 8)	10.0	9.0	35.0	1.7

* Results from the present work are means of 3 experiments each with duplicate samples ± S.E.

** These authors used an aqueous isolation technique.

Chloroplast preparations from sucrose density gradients were assayed for cofactor content immediately after preparation, and also after dilution and washing on a second identical sucrose gradient. The results are presented in Table II and compared there with data published for chloroplast fractions derived by non-aqueous procedures and also with data for mitochondria. The figures show that no loss of cofactor has resulted from the second exposure to density gradient centrifugation,

TABLE II

NICOTINAMIDE COFACTOR CONTENT OF CHLOROPLAST PREPARATIONS

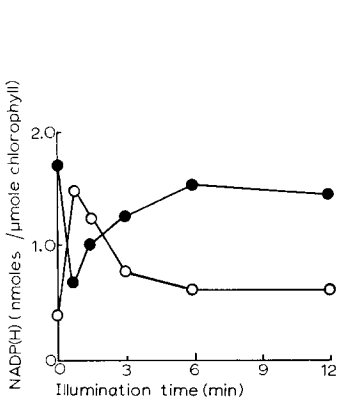
	NADP+	*NADPH*	*NAD+*	*NADH*
	Coenzyme (nmoles/μmole chlorophyll)			
Pea (this study)*	1.23 ± 0.28	0.41 ± 0.06	2.52 ± 0.38	0.75 ± 0.14
Pea (this study)**, washed prep.	1.15 ± 0.22	0.42 ± 0.11	2.40 ± 0.40	0.81 ± 0.17
Bean (ref. 8)	10.5	6	20	1
Spinach and bean (ref. 7)	9.5	3.9	21	0.84
	Coenzyme (nmoles/mg protein)			
Chloroplasts (ref. 7)	$7.0 \cdot 10^{-4}$	$2.25 \cdot 10^{-4}$	$15 \cdot 10^{-4}$	$0.60 \cdot 10^{-4}$
Mitochondria (ref. 21)	$3.5 \cdot 10^{-4}$	$33.5 \cdot 10^{-4}$	$16.7 \cdot 10^{-4}$	$15.8 \cdot 10^{-4}$

* Results from the present work are presented as means of 6–10 determinations ± S.E.
** A chloroplast fraction prepared on a sucrose density gradient (as described in the text) was diluted with isolation medium and then isolated on a second identical sucrose density gradient.

and from this and the potassium–chlorophyll ratios presented above, it is concluded that very little if any loss of cofactor has occurred in the isolation procedure. In comparison with the literature values obtained by non-aqueous procedures, only the NADH value is of the same order of magnitude, and since this probably reflects the higher NADH value for the whole leaf shown in Table I, it is probably a fortuitous coincidence of values. The $NAD^+/NADH$ ratio of 3.15 is very similar to that of 2.94 of Table I. The significance of these results is discussed below.

Effect of light on nicotinamide cofactor reduction

The chloroplast endogenous content of $NADP^+$ and NADPH during a 12-min illumination period is shown in Fig. 4. The expected increase in NADPH and decrease in $NADP^+$ occurred, and the data of Fig. 4 suggest that the peak in NADPH accumulation occurred before the first harvest of the time course at 45 sec. In the following



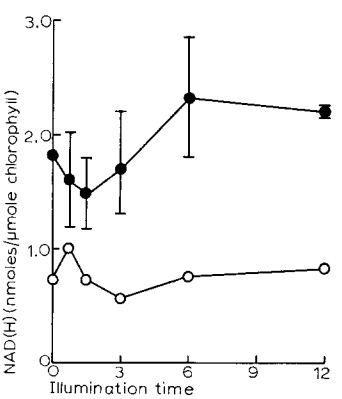


Fig. 4. Light-induced changes in endogenous NADPH (○—○) and $NADP^+$ (●—●) of chloroplasts isolated on the sucrose gradient.

Fig. 5. Light-induced changes in endogenous NADH (○—○) and NAD^+ (●—●) of chloroplasts isolated on the sucrose gradient. Results are the means of 4 determinations ± S.E.

1–5-min period of illumination, the $NADP^+$ and NADPH levels returned to approximately the initial readings. This suggested that the ratio of $NADP^+$ to NADPH would not be excessively sensitive to exposure to low-intensity light during the isolation procedure. In comparison the changes in NAD^+ and NADH levels shown in Fig. 5 were slight and possibly not significant, although the changes in this redox couple are sympathetic to those of the $NADP^+$–NADPH system. On the basis of this evidence, the NAD^+/NADH ratios reported above do not appear to result from exposure to low-intensity light during the isolation.

A higher rate of $NADP^+$ reduction was expected than that shown in Fig. 4. The addition of $NADP^+$ to the medium at three concentrations up to 20 μmoles $NADP^+$/μmole chlorophyll (0.8 μM $NADP^+$) led to rates of reduction (initial 30-sec period) that were proportional to the $NADP^+$ concentration; that is within these limits the $NADP^+$ concentration was not saturating, and the rate of reduction depended upon $NADP^+$ availability. The time course of changes in $NADP^+$ and NADPH at the highest $NADP^+$ level examined is given in Fig. 6. This shows that a peak of NADPH accumulation occurred before the earliest harvest at 45-sec illumination. In further experiments we found a peak in NADPH content occurring within 10–20 sec illumination, in agreement with the observation of HEBER AND SANTARIUS[7].

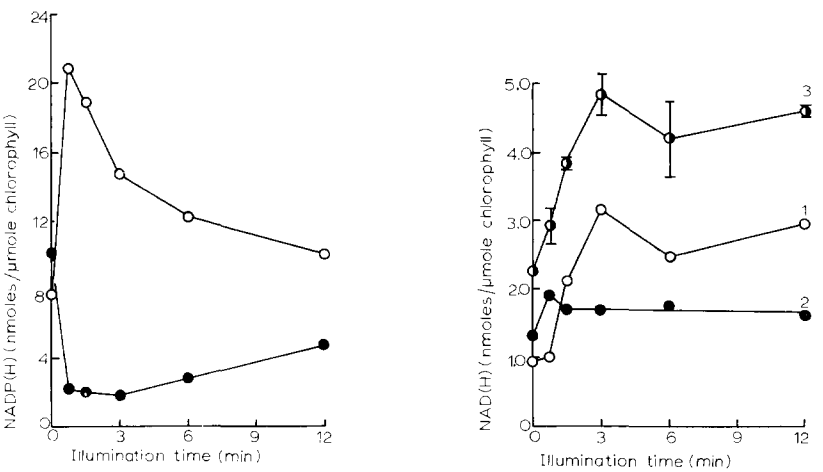


Fig. 6. Light-induced reduction of $NADP^+$ by chloroplasts isolated on a sucrose gradient. Initial concentration, $8 \cdot 10^{-7}$ M $NADP^+$. ○—○, NADPH; ●—●, $NADP^+$.

Fig. 7. Light-induced changes in endogenous NAD(H) of chloroplasts incubated in $NADP^+$. Initial concentration, $8 \cdot 10^{-7}$ M $NADP^+$. Results are the means of 4 determinations ± S.E. Curve 1, NADH; Curve 2, NAD^+; Curve 3, total NAD(H).

In the experiment of Fig. 6, the endogenous levels of NAD^+ and NADH were also assayed and are plotted in Fig. 7. The results show a fairly steady level of NAD^+ but an increase in NADH in the first 3 min producing an increase in total NAD(H). One possible explanation for this is a conversion of NADP(H) into NAD(H), so the data were reexamined, and the nicotinamide cofactor changes tabulated in Table III. This shows that the rise in NAD(H) with time of illumination was accompanied by a fall in NADP(H), thus supporting the suggestion of an interconversion of NADP(H) and NAD(H). Furthermore a lack of symmetry is apparent in Fig. 6 (the recovery of

TABLE III

REDISTRIBUTION OF COENZYMES AFTER ILLUMINATION OF CHLOROPLASTS IN $NADP^+$-ENRICHED MEDIUM

	Coenzyme (nmoles/μmole chlorophyll) *Illumination time (min)*					
	0	*0.75*	*1.5*	*3*	*6*	*12*
NAD^+	1.30	1.90	1.70	1.69	1.74	1.62
NADH	0.94	1.02	2.12	3.14	2.46	2.96
Total NAD(H)	2.24	2.92	3.82	4.83	4.20	4.58
$NADP^+$	10.85	2.29	2.08	1.81	2.87	4.80
NADPH	7.82	20.80	18.90	14.70	12.25	10.00
Total NADP(H)	18.67	23.09	20.98	16.51	15.12	14.80
Total NAD(P)(H)	20.91	26.01	24.80	21.34	19.32	19.38
Recovery (%)	100	124	118	102	93	93

$NADP^+$ does not match that of NADPH), and this could also be due to such an interconversion. FORTI, TOGNOLI AND PARISI[23] have reported a phosphatase showing activity towards NADP(H); BRADBEER AND WALLIS[24] have recently commented on this.

A further experiment was conducted in which chloroplasts were illuminated in a medium reinforced with all nucleotide cofactors to the levels for the cytoplasmic fraction reported by HEBER AND SANTARIUS[7]. The results confirmed the results of Figs. 5–7 concerning the time course of $NADP^+$ and NADPH changes and a later increase in NADH comparable with that of Fig. 7.

DISCUSSION

The potassium–chlorophyll ratios reported refer to one batch of seeds purchased, and no attempt has been made to establish how representative this is. The results encourage the belief that potassium retention by these chloroplast preparations is related to the presence of an intact limiting membrane.

GRAHAM AND COOPER[25] have shown that prior illumination of detached mung bean leaves leads to a decrease in NAD(H) and an increase in NADP(H). It is difficult to make a direct comparison of our values for the pea leaf with their results, which are reported on a g fresh wt. basis, but the ratio NAD(H)/NADP(H) they obtain in the dark is 3.65, and in the light 1.41. The ratios obtained by HEBER AND SANTARIUS[7] (2.82), OGREN AND KROGMANN[8] (1.93) and in the present work (1.62) all fall within this range. (The pea seedlings were harvested from the light with no dark-adaption period, and our ratio is close to that of GRAHAM AND COOPER[25] for illuminated leaves.)

The nicotinamide cofactor content of isolated chloroplasts in this work was so much greater than in previously published reports using aqueous techniques[26,27] that comparison becomes unprofitable. Using non-aqueous techniques, HEBER AND SANTARIUS[7] and OGREN AND KROGMANN[8] obtained good agreement in their results (see Table II), but the present result is only 10–14% of that reported. In part this contrasting level is reflected in the whole-leaf results, which are also lower (30–50%).

Having obtained excellent retention of cofactor in resuspended chloroplasts (Table II, *cf.* lines 1 and 2), we are disinclined to attribute the remaining difference to loss of cofactor in our preparations. It is noteworthy that in our experiments the chlorophyll cofactor ratio of chloroplasts is markedly different from that of the whole leaf, and this perhaps indicates a better fractionation of subcellular components than that achieved in the non-aqueous techniques.

Another point of difference that could possibly be correlated with technique is shown by the $NAD^+/NADH$ ratio. In the present work the ratio was approx. 3 (for leaf, 2.94; for the chloroplast fraction, 3.15), whereas the non-aqueous procedure gave a ratio of 20. The latter figure is so far from a redox balance that the possibility of (non-enzymic) oxidation of NADH in the freeze-drying stage of the non-aqueous procedure seems to merit closer examination.

Membrane permeability of $NADP^+$

HEBER AND SANTARIUS[7] consider that the chloroplast membrane is effectively impermeable to nicotinamide cofactors. This conclusion is based on their contrasting results for cytoplasmic and chloroplastid cofactor contents (especially NADP(H)), indicating compartmentation within the cell. They tested this conclusion by showing that the rate of exogenous $NADP^+$ reduction by chloroplasts isolated in an aqueous sucrose medium was many times greater after treatments designed to rupture the limiting membrane (*e.g.*, hypotonic swelling and sonication). Compartmentation does not preclude membrane permeability, however, and the $NADP^+$ concentration adopted in their tests was approx. 0.6 mM, a thousand times greater than in the present work. At that level the chloroplast might well be permeable to $NADP^+$ and still show a substantially increased rate of $NADP^+$ reduction after rupturing treatments.

In the final experiment reported here, chloroplasts were illuminated in the presence of NAD(H) and NADP(H) in both oxidised and reduced forms. The medium contained 40 nmoles NAD^+ and 2.5 nmoles $NADP^+$ per mg chlorophyll. The time course of NADPH accumulation was similar to that of Fig. 4 and Fig. 6, while the time course of NADH accumulation showed a later peak in agreement with Fig. 6 and a rate of NADH accumulation also similar to that of Fig. 7. This indicates that $NADP^+$, for example, was reduced by a specific mechanism that was not affected by the presence of NAD^+ at a 15-fold greater concentration.

Photosynthetic reduction of endogenous $NADP^+$ occurs in the chloroplast stroma but closely associated with the granal membrane system. The results of Figs. 5–7 and Table III show that exogenous $NADP^+$ was reduced on illumination. It is necessary then to hypothesize the existence of either a specific mechanism for NADPH (internal)/$NADP^+$ (external) transhydrogenation that resides in the limiting membrane of the chloroplast, or alternatively a specific mechanism for the entry of exogenous $NADP^+$. The evidence that illumination in a $NADP^+$-reinforced medium leads to enhanced reduction of endogenous NAD^+ could have various explanations, but it seems both to weaken the hypothesis of a membrane-situated NADPH/$NADP^+$ transhydrogenase and to strengthen that of penetration by $NADP^+$.

The endogenous $NADP^+$ concentration can be estimated from the values in Table II and the water content taken as the difference between chloroplast pellet fresh weight and dry weight[16]. This leads to the value of 16–26 μM $NADP^+$. In the

experiment of Fig. 6, the $NADP^+$ concentration in the medium was 0.8 μM. On the basis of this evidence it appears possible that $NADP^+$ is taken up by the chloroplast against a concentration gradient.

ACKNOWLEDGEMENT

This work has been supported by a research grant from the Agricultural Research Council that is gratefully acknowledged.

REFERENCES

1 A. R. CROFTS, *J. Biol. Chem.*, 242 (1967) 3352.
2 R. I. B. FRANKI, N. K. BOARDMAN AND S. G. WILDMAN, *Biochemistry*, 4 (1965) 865.
3 S. I. HONDA, T. HONGLADAROM AND G. C. LATIES, *J. Exptl. Botany*, 17 (1966) 460.
4 R. M. LEECH, *Biochim. Biophys. Acta*, 79 (1964) 637.
5 D. SPENCER AND H. UNT, *Australian J. Biol. Sci.*, 18 (1965) 197.
6 D. A. WALKER, in T. W. GOODWIN, *Biochemistry of Chloroplasts*, Vol. II, Academic Press, London, 1967, p. 53.
7 U. W. HEBER AND K. A. SANTARIUS, *Biochim. Biophys. Acta*, 109 (1965) 390.
8 W. OGREN AND D. W. KROGMANN, *J. Biol. Chem.*, 240 (1965) 4603
9 M. J. HARVEY AND A. P. BROWN, *Biochem. J.*, 105 (1967) 30 P.
10 M. J. HARVEY AND A. P. BROWN, *Plant Physiol.*, 43 (1968) S-20.
11 D. I. ARNON, *Plant Physiol.*, 24 (1949) 1.
12 A. G. GORNALL, C. J. BARDAVILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
13 R. F. ITZHAKI AND D. M. GILL, *Anal. Biochem.*, 9 (1964) 401.
14 G. E. GLOCK AND P. MCLEAN, *Biochem. J.*, 61 (1955) 397.
15 T. F. SLATER, B. SAWYER AND U. STRÄULI, *Arch. Intern. Physiol. Biochem.*, 72 (1964) 427.
16 A. P. BROWN, *Biochem. J.*, 102 (1967) 791.
17 W. O. JAMES AND V. S. R. DAS, *New Phytologist*, 56 (1957) 325.
18 R. M. LEECH in T. W. GOODWIN, *Biochemistry of Chloroplasts*, Vol. I, Academic Press, London, 1967, p. 65.
19 A. KAHN AND D. VON WETTSTEIN, *J. Ultrastruct. Res.*, 5 (1961) 557.
20 K. NISHIDA, *Plant Cell Physiol.*, 4 (1963) 247.
21 A. L. GREENBAUM, J. B. CLARK AND P. MCLEAN, *Biochem. J.*, 95 (1965) 161.
22 E. C. SLATER, M. J. BAILIE AND J. BOUMAN, in T. W. GOODWIN AND O. LINDBERG, *Biological Structure and Function*, Academic Press, London, 1961, p. 207.
23 G. FORTI, C. TOGNOLI AND B. PARISI, *Biochim. Biophys. Acta*, 62 (1962) 251.
24 J. W. BRADBEER AND M. E. WALLIS, *Biochem. J.*, 108 (1968) 24 P.
25 D. GRAHAM AND J. E. COOPER, *Australian J. Biol. Sci.*, 20 (1967) 319.
26 D. W. KROGMANN, *Arch. Biochem. Biophys.*, 76 (1958) 75.
27 M. L. DAS AND F. L. CRANE, *Biochim. Biophys. Acta*, 48 (1961) 594.