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THE SPECIFICITY OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASES IN GREEN PLANTS, *EUGLENA* AND *OCHROMONAS**

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Abstract—Crude extracts of species of several groups of green plants were examined to determine the specificity of the glyceraldehyde-3-phosphate dehydrogenases The results are consistent with the view that in all green plants both the NAD- and the NADP-dependent activities of chloroplasts are catalyzed by one enzyme which is different from the NAD-linked cytoplasmic enzyme In the photosynthetic flagellates, *Euglena gracilis* and *Ochromonas danica*, on the other hand, the NADP-activity does not appear to be associated with any NAD-linked activity, although it cannot be excluded that a separate NAD-dependent enzyme may also occur in chloroplasts of these species

INTRODUCTION

KINETIC investigations[1-3] have demonstrated that in pea and spinach leaves NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH)† activity (E.C 1 2.1.13) is catalyzed by an enzyme which is about equally active with NAD and is, therefore, responsible for the previously recognized NAD-dependent activity located in chloroplasts [4,5] Purification and electrophoretic studies on extracts from various organs of higher plants[6,7] have confirmed the impossibility of completely separating the two activities On the other hand, a cytoplasmic GAPDH enzyme absolutely specific for NAD can be selectively precipitated with ammonium sulphate, at least in extracts of pea[2] and spinach leaves.[3]

NADP-dependent GAPDH activities have been found to be associated with the carbon reduction cycle of all eucaryotic plants capable of photosynthesis, of all Cyanophyceae examined and of the photosynthetic bacterium, *Rhodospirillum rubrum* [8,9] However, in

* Second part of a series of papers entitled 'D-glyceraldehyde-3-phosphate dehydrogenase in photosynthetic cells'

† Abbreviations GAPDH, glyceraldehyde-3-phosphate dehydrogenase = D-glyceraldehyde-3-phosphate NADP oxidoreductase (phosphorylating), E C 1 2 1 13, PN = pyridine nucleotide (s), G_3P, glyceraldehyde-3-phosphoric acid, NAD(P) enzyme = pyridine nucleotide aspecific enzyme, K' = apparent Michaelis constant

[1] B A MELANDRI, A BACCARINI and P PUPILLO, *Biochem Biophys Res Commun* **33,** 160 (1968)
[2] B A MELANDRI, P PUPILLO and A BACCARINI, *Biochim Biophys Acta* **220,** 178 (1970)
[3] P PUPILLO, G GIULIANI PICCARI and B. A MELANDRI, in preparation
[4] M LOSADA, A W TREBST and D I ARNON, *J Biol Chem* **235,** 832 (1960)
[5] U HEBER, N G PON and M HEBER, *Plant Physiol* **38,** 355 (1963)
[6] H G LEBHERZ and W J RUTTER, *Science* **157,** 1198 (1967)
[7] M D SCHULMAN and M GIBBS, *Plant Physiol* **43,** 1805 (1968)
[8] R C FULLER and M GIBBS, *Plant Physiol* **34,** 324 (1959)
[9] R M SMILLIE and R C FULLER, *Biochem Biophys Res Commun* **3,** 368 (1960)

the only case investigated in some detail, the enzyme of the blue-green alga *Anabaena variabilis* was also found to be active with NAD [10]

It was therefore of interest to investigate whether similar unspecific pyridine nucleotide GAPDH enzymes were present in members of several other plant taxa, to establish whether no 'pure' NADP-linked enzyme exists, and to try to find a clue to the metabolic role, if any, of the puzzling NAD-dependent activity of chloroplasts The technique employed[1] was based on the kinetic behaviour of enzymes active with two distinct substrates (NAD and NADP in this instance) which both compete for a single catalytic site [11] If, however, a mixture of a NADP enzyme and of a NAD-specific enzyme is being studied, as appears to be the case with extracts of higher plants, the relative amounts of both enzymes can be predicted, provided that equality of the kinetic parameters of the two NAD activities is assumed [2] Although the latter assumption was found to be not completely true in work with spinach extracts,[3] the apparent Michaelis constants for NAD^+ in both enzymes were nevertheless close, suggesting that the approach described is likely to yield results approximating to the real situation That the competitive behaviour of the coenzymes is actually due to reciprocal inhibitory effects by NADP on a NAD-specific chloroplast enzyme and *vice versa* has been shown to be highly unlikely in work with spinach extracts [3]

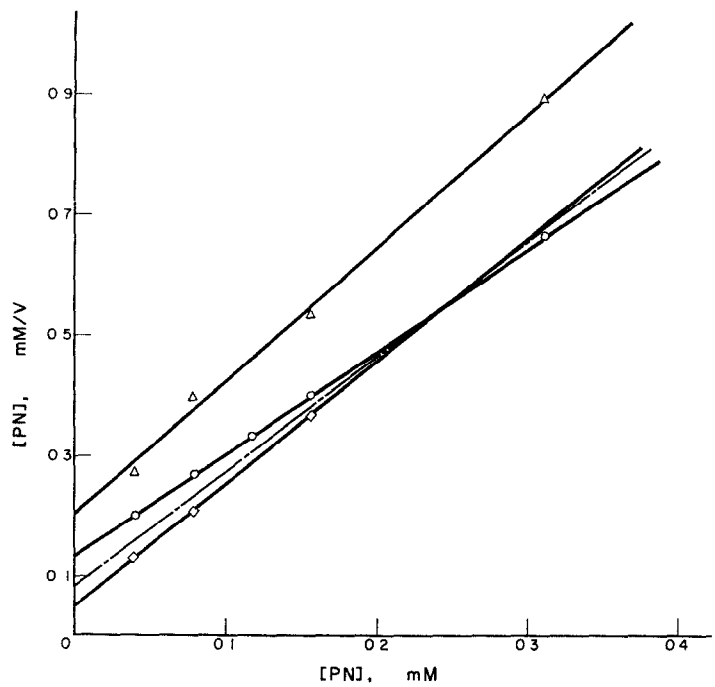

FIG 1 LACK OF ADDITIVITY OF THE NAD AND NADP ACTIVITIES IN A CRUDE EXTRACT OF *Medicago sativa*, HARVESTED UNDER PHOTOSYNTHESIS CONDITIONS

Assay, 10 μl of extract were equivalent to 4 84 μg of chlorophyll △——△——△, NADH-dependent activity, ○——○——○, NADPH-dependent activity, ◇——◇——◇, overall activity in presence of NADH and NADPH in equal concentrations, — — — —, theoretical plot for a single enzyme having the observed apparent kinetic parameters and active both with NADH and NADPH $K'_{NADPH} = 79\ \mu M$, $K'_{NADH} = 92\ \mu M$ $V = \Delta A_{340}/min$

[10] W. HOOD and N G CARR, *Planta* **86**, 250 (1969)
[11] M. DIXON and E C WEBB, *Enzymes* (2nd edition) Longmans, Chapter 4, London (1964)

RESULTS

The data obtained with extracts of fully developed alfalfa (*Medicago sativa*) leaves and shown in Fig 1 agree with earlier evidence[1] that in extracts of photosynthetic organs, harvested after long exposure to the light, NADH and NADPH appear to compete totally for the same enzyme site, that is, the 'competitive fraction'[2] is approximately one The presence of a second minor NAD-specific activity can hardly be detected under these conditions, probably due to the light-promoted activation of the NADP activity[12] and to the limits of sensitivity of the technique

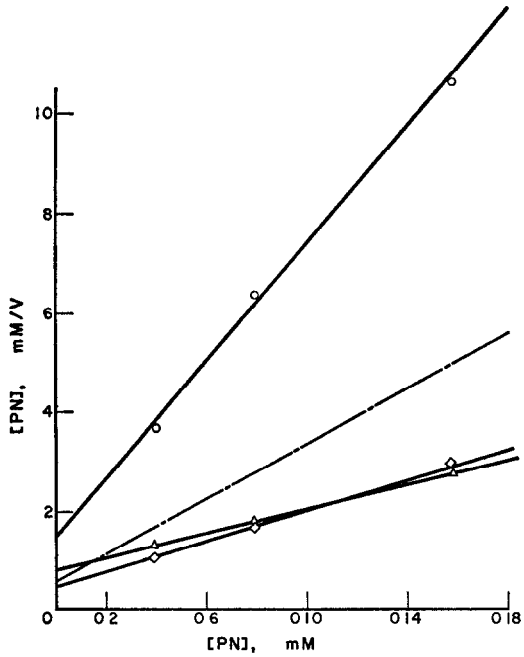

FIG 2 PARTIAL ADDITIVITY IN A CRUDE EXTRACT OF *Ginkgo biloba* UNEXPANDED LEAVES
Symbols as in Fig 1, reductive assay Extract equivalent to 0 64 μg of chlorophyll $K'_{NADPH} = 26$ μM, $K'_{NADH} = 64$ μM The ratio of NADPH to NADH maximum apparent activities was 0 25 in these undeveloped leaves

On the other hand, in extract of very young leaves of *Ginkgo biloba*, the greening and expansion of which were still incomplete, tested in the same assay system (viz in the sense of NADPH oxidation), the NAD activity exceeded the NADP-dependent one (Fig 2) and the 'competitive fraction' was 0 43 No further study of the possible increase of the NAD(P) enzyme during development was made, as no activity could be detected in crude extracts of older *Ginkgo* leaves

A semiquantitative measure of the relative activities of the two GAPDH enzymes of plant cells may also be obtained by carrying out assays first with nearly saturating concentrations of either NAD or NADP alone and then in the presence of both coenzymes simultaneously If the activity in presence of both coenzymes is significantly lower than the sum of the

[12] H ZIEGLER and I ZIEGLER, *Planta* **65**, 369 (1965).

TABLE 1 LACK OF ADDITIVITY OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ACTIVITIES OF SOME GREEN PLANTS* (μmoles/mg chlf /min)

	$NADP^+$	NAD^+	$NADP^+ + NAD^+$	Additivity† ratio
Scilla amoena (Monocotyledonae, Liliaceae)	908	656	1038	0 66
Sesleria coerulea (Monocotyledonae, Gramineae)	1010	624	858	0 52
Dryopteris filix-mas (Pterophyta, Polypodiaceae)	57	84	85	0 60
Hymenostylium recurvirostre (Musci, Pottiaceae)	126	108	168	0 72
Chara zeylanica (Chlorophyta, Characeae)	326	358	503	0 73

* The assay contained NAD^+ or $NADP^+$ separately or in equimolar mixture, 0 25–0 30 mM each, and G-3P 0 55–0 62 mM Other reagents, cofactors and conditions described in legend of Fig 3

† Ratio of overall activity in presence of NAD^+ and $NADP^+$ together to the sum of the separate NAD^+ and $NADP^+$-dependent activities

separate activities, this must mean either reciprocal inhibition between the coenzymes or, more likely,[3] the existence of at least one unspecific pyridine nucleotide enzyme in the

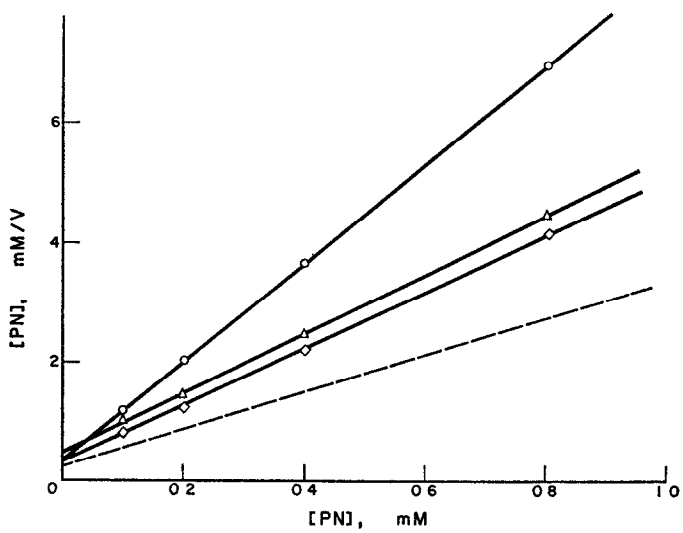

FIG. 3 PARTIAL ADDITIVITY OF THE NAD AND NADP ACTIVITIES IN AN EXTRACT OF *Chlamydomonas reinhardi* CELLS

Oxidative assay 10 μl of extract were equivalent to 61 μg of protein, G_3P 0 94 mM. The symbols are the same as in Fig 1, but they indicate now the activities measured as reduction of NAD^+, $NADP^+$, or both --------, calculated plot for the overall activity in presence of two enzymes, one specific for NAD and the other specific for NADP $K'_{NADP+} = 40\ \mu M$, $K'_{NAD+} = 95\ \mu M$

extract. A number of green plants were tested in this way, and in Table 1 results are given for two monocotyledons, a fern, a moss and a multicellular green alga In all cases the activity in presence of the two coenzymes was much less than the sum of the single activities, the ratio varying between 52 and 73% of the latter (Table 1)

Chlamydomonas reinhardi is a unicellular photosynthetic flagellate, a supposedly primitive member of the Chlorophyceae The data in Fig. 3 clearly demonstrate that in this instance also the rule of partial additivity obtains The calculated value of 0 63 for the competitive fraction is not low, considering that no short-term light-dependent increase of the NADP activity[12] occurs in this species It seems, therefore, that in photosynthetic cells of all green plants a NAD(P) enzyme and at least one NAD-linked enzyme occur, several lines of evidence

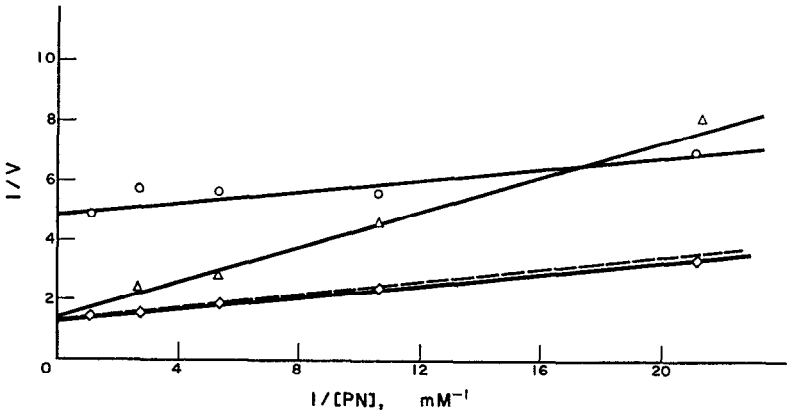

FIG 4 COMPLETE ADDITIVITY OF NAD AND NADP ACTIVITIES IN AN EXTRACT OF GREEN *Euglena gracilis* CELLS

Lineweaver–Burke plot Symbols as in Fig 3, oxidative assay Extract equivalent to 15 μg of chlorophyll or to 226 μg of protein, G_3P 0 62 mM $K'_{NADP^+} = 21$ μM, $K'_{NAD^+} = 202$ μM

concur to indicate that the NAD(P) enzyme is always located in chloroplasts, while the NAD-specific enzyme is cytoplasmic [2,3,5,9,13,14]

However, in the case of *Euglena gracilis* (Euglenophyta) the results of experiments with crude extracts and with preparations partially purified by ammonium sulphate and acetone fractionation demonstrate that the NADP activity is catalyzed by a completely NADP-specific enzyme (Fig 4) No evidence of competition effects could be also found in extracts of *Ochromonas danica* (Chrysophyta) (Fig 5)

Although the numerical values of the Michaelis constants of these enzymes are not to be regarded as absolute, for reasons discussed elsewhere,[2] they were quite reproducible under carefully controlled conditions The apparent constants for NAD^+ in 40–60% ammonium sulphate precipitates were always markedly higher than those for $NADP^+$, and both groups of data were of the same order in all organisms tested (Table 2) It has also been shown[3] that the spinach chloroplast GAPDH has comparable K' values under similar conditions, both in chloroplast crude extracts and in purified preparations

[13] J I MEGO and A T JAGENDORF, *Biochim Biophys Acta* **53**, 237 (1961)
[14] A BACCARINI and B A MELANDRI, *Physiol Plantarum* **23**, 444 (1970)

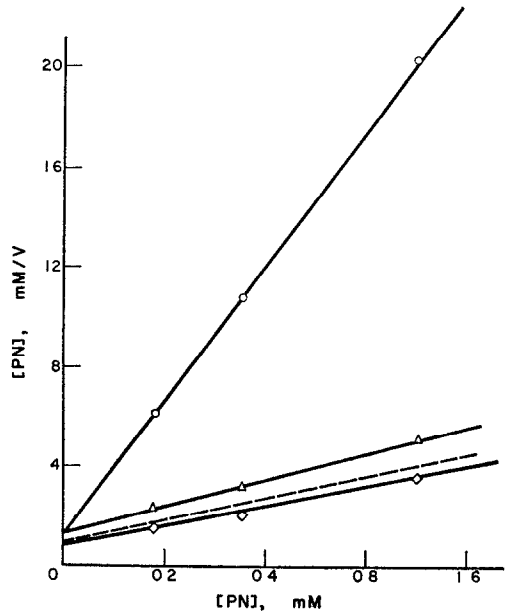

FIG 5 COMPLETE ADDITIVITY OF THE GAPDH ACTIVITIES IN AN EXTRACT OF *Ochromonas danica*
Symbols as in Fig 3, oxidative assay Extract equivalent to 1 52 μg of chlorophyll or to 193 μg of protein, G_3P 0 61 mM $K'_{NADP^+} = 47$ μM, $K'_{NAD^+} = 267$ μM
In the experiment shown the activity in presence of both coenzymes appeared to exceed the sum of the separate activities with NAD^+ or $NADP^+$, but this was not consistently observed

In contrast, the K' for NAD^+ in frozen and thawed *Euglena* enzyme preparations were somewhat lower and agreed with values obtained with partially purified preparations and crude extracts of dark-grown cells It is concluded that the NAD-specific enzyme of *Euglena*, which is resistant to ageing and to freezing–thawing and occurs both in light-grown and dark

TABLE 2 APPARENT MICHAELIS CONSTANTS FOR PYRIDINE NUCLEOTIDES OF SOME GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASES*

	K' for NADP* (μM)	K' for NAD* (μM)
Nasturtium creticum (Dicotyledonae)	34 ± 6 (5)	169 ± 22 (3)
Chlamydomonas reinhardi (Chlorophyta)	25 ± 4 (5)	218 ± 31 (4)
Euglena gracilis (Euglenophyta), light-grown cells	21 ± 3 (3)	209 ± 38 (4)
Euglena gracilis, dark-grown cells	—	76 ± 5 (3)
Euglena gracilis, frozen enzyme†	—	84 ± 6 (3)

* Values obtained with enzyme preparations resuspended from the 40–60% ammonium sulphate precipitates Conditions as in note (*) of Table 1 Number of experiments in brackets
† Ammonium sulphate fractions stored frozen at −15° for at least 3 weeks

[15] G BRAWERMAN and N KONIGSBERG, *Biochim Biophys Acta* **43**, 374 (1960)

adapted cells, is the cytoplasmic form (on the other hand, the instability of the NADP activity of this organism is well documented see Ref 15), the cytoplasmic GAPDH of spinach has comparable K' values [3] It is not understood why the K' for NAD^+ in fresh 40–60% ammonium sulphate fractions or crude extracts of green *Euglena* is higher than in frozen–thawed preparations or in extracts of etiolated cells (see below)

DISCUSSION

The state of the glyceraldehyde-3-phosphate dehydrogenase enzymes in photosynthetic cells has been partly elucidated in recent years [1–3,7] In spinach leaves the kinetic evidence is consistent with the notion of only one chloroplast NAD(P) enzyme[3] and at least one cytoplasmic NAD enzyme, the two differ markedly under some respects [2,3] In this work evidence is presented that a similar situation exists in all green plants, and, furthermore, that the enzymes of such distantly related organisms as *Euglena*, *Chlamydomonas* and *Nasturtium* have very similar kinetic properties These conclusions are of consequence to some earlier suggestions concerning aspects of the behaviour of the NAD-dependent activity under different physiological conditions It had been proposed, for example, that in a *C reinhardi* mutant, *y*-2, grown in the dark, the NAD-linked enzyme may convert to the NADP-requiring form *in vivo* at the start of the illumination[16] and that in higher plants a similar conversion may account for the enhancement of the NADP activity in the light [17,18] On the basis of the recognition that the NAD activity of chloroplasts depends on the same enzyme catalyzing the NADP activity, such mechanisms are difficult to envisage, as the only NAD-linked activity available for the interconversion would remain the cytoplasmic fraction, which is known to be thoroughly compartmentalized [3,5]

On the other hand, in two unicellular organisms, *E gracilis* (Euglenophyta) and *O danica* (Chrysophyta), complete additivity of the NAD and NADP activities is observed, which is direct evidence for the occurrence of a completely NADP-specific enzyme in the chloroplasts The finding of a NAD activity associated with the NADP enzyme only in some plant groups is somewhat unexpected In *A variabilis* (Cyanophyceae) the existence of a single unspecific pyridine nucleotide GAPDH having properties generally similar to the corresponding higher plant enzymes is well documented,[10] and it is surmised that in such procaryotic organisms this enzyme is active in both the glycolytic and the carbon reduction pathway. The now widely accepted hypothesis of a phylogenetic relationship between Cyanophyceae and chloroplasts[19] could then readily explain also the occurrence of a NAD activity associated with the chloroplast enzyme in higher plants It is difficult, however, to believe that such relatively high affinity of the enzyme for NAD is of no physiological significance, and the claims of a lowered NAD-dependent activity in chloroplasts exposed to light[17] are possibly indicative of some kind of regulation

Should a metabolic role for the NAD activity of higher plant chloroplasts be established, for example related to dark metabolism, then the absence of such activity in some algal groups, if confirmed, would be rationalized Indeed, both *Euglena* and *Ochromonas* are versatile facultative heterotrophs which can lose their photosynthetic apparatus after several

[16] G. A Hudock and R C Fuller, *Plant Physiol* **40**, 1205 (1965)
[17] H. Ziegler, I Ziegler and H J Schmidt-Clausen, *Planta* **81**, 181 (1968)
[18] B Muller, H Ziegler and I Ziegler, *Europ J Biochem* **9**, 101 (1969)
[19] L. Margulis, *Science* **161**, 1020 (1968)

generations of culture in the dark It may be postulated that their adaptation to continuous darkness and to organotrophic life is so good as to allow them to dispense altogether with the need of a 'dark' chloroplast metabolism and even with possessing chloroplasts at all under these conditions

On the other hand, the possibility cannot be discounted that *Euglena* and/or *Ochromonas* chloroplasts contain a further separate NAD-dependent GAPDH enzyme The observation that K' values for NAD^+ in crude extract of *Euglena* are higher than in partially purified and frozen-thawed preparations is possibly circumstantial evidence favouring this view. If this really were the case, an interesting example of enzyme evolution would come to light.

EXPERIMENTAL

Materials The whole plants or leaves used as source of enzyme were collected in daylight from the outdoor plots at the Botanic Garden of Bologna University

Euglena gracilis strain Z (Indiana University Algae Culture Collection) was grown in Fernbach bottles on the complete mineral medium of Boger and San Pietro[20] at a temperature of 24 ± 1° on a rotary shaker, a mixture of air and 5% CO_2 was bubbled into the bottles and the light (5300 lx at the level of the cultures) was continuously provided through a batch of eight fluorescent lamps To the cultures grown in the dark 0 5% EtOH was added as carbon source, no air being bubbled The cells were grown for at least 20 generations and then a sample of 5 ml (5–10 × 10^6 cells) was inoculated into fresh medium After 5 days the cells were harvested

Chlamydomonas reinhardi wild type (137c), +, obtained from Dr Robert Togasaki of Indiana University, Bloomington, was grown in minimal medium[21] under the same conditions as described for *Euglena*

Ochromonas danica (Indiana University Algae Culture Collection) was cultured in a medium containing proteosepeptone 1 g/l yeast extract 1 g/l , glucose 1 g/l , thiamine 0 1 mg/l biotin 1 μg/l Vitamin B_{12} 2 μg/l The cells were grown for 3 days at 20° without shaking under fluorescent light (1600 lx) and then harvested

Preparation of extracts The buffer used throughout was Tris-Cl 0 05 M pH 8 5, containing 10 mM EDTA and 10 mM 2-mercaptoethanol *Euglena*, *Chlamydomonas* or *Ochromonas* cells in exponential phase were harvested at 2000 *g* for 10 min in the cold, washed twice in H_2O and finally collected in the same way The cells were resuspended in a suitable volume of buffer and broken in an Aminco French Press at 100 atm In the case of *Euglena* and *Ochromonas* a small amount of physiological substrate (NADPH) was immediately added to the broken cell suspension to a final concentration of 0 5 mM

The filaments of *Chara* and the green parts of *Hymenostylium* were collected, thoroughly washed with tap water and distilled water and triturated in a chilled mortar in presence of quartz sand (about half their fresh weight) and buffer Plant leaves were similarly ground in a chilled mortar but without sand

In all cases the slurries were filtered through 8 layers of cheesecloth, samples were taken for chlorophyll and protein and the extracts were centrifuged for 15 min at 15,000 *g* in the cold The supernatants were directly used as enzyme solutions

Assay of activities The enzyme activities were measured essentially as described elsewhere[2] with minor modifications In the 'reductive' assay, a cell contained in a final volume of 1 20 ml Tris-Cl, pH 7 5, 100 μmoles, 2-mercaptoethanol, 12 μmoles, 3-phosphoglyceric acid phosphokinase, 20 μg, $MgSO_4$, 12 μmoles, ATP, 1 32 μmoles, plant extract, and varying amounts of reduced PN (pyridine nucleotide) The mixture was equilibrated for 2 min at 25° and the reaction was started by addition of 7 μmoles of 3-phosphoglyceric acid The rate of PN oxidation was measured at 340 nm in a recording spectrophotometer against a blank containing Tris, extract and reduced PN In the 'oxidative' assay, a cell contained in 1 20 ml Tris-Cl, pH 8 5, 40 μmoles, NaF, 24 μmoles, Na_2HAsO_4, 21 4 μmoles, 2-mercaptoethanol, 12 μmoles, plant extract, and oxidized PN The reaction was started by addition of G-3P and the initial rate measured

Chlorophyll was determined according to Arnon,[22] in the case of *Ochromonas* the specific extinction coefficients of MacKinney[23] were directly applied Protein was determined by a biuret method [14]

[20] P BOGER and A SAN PIETRO, *Zeitschr Pflanzenphysiol* **58**, 70 (1967)
[21] D S GORMAN and R P LEVINE, *Proc Natl Acad Sci U S* **54**, 1665 (1965)
[22] D I ARNON, *Plant Physiol* **24**, 1 (1949)
[23] H H STRAIN and W A. SVEC, in *The Chlorophylls* (edited by L P VERNON and G R SEELY), p 51, Academic Press, New York (1966)

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Key Word Index—Green plants, *Euglena gracilis*, *Ochromonas danica*; glyceraldehyde-3-phosphate dehydrogenases, NAD- and NADP-dehydrogenases