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ISOLATION AND CHARACTERIZATION OF NAD(P)H-DEHYDROGENASES FROM SEEDS OF THE CASTOR BEAN

CORNELIS C. VILJOEN, FRANCOIS CLOETE, DAWIE P. BOTES and HELÉNE KRUGER

National Chemical Research Laboratory, P.O. Box 395, Pretoria, South Africa

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Key Word Index—*Ricinus communis*; Euphorbiaceae; castor bean; seed NAD(P)H-dehydrogenases; isolation; characterization.

Abstract—Two pyridine nucleotide dehydrogenases have been isolated from castor bean seed extracts by a combination of ion exchange chromatography on DEAE-Sepharose and gel permeation chromatography on Sephadex G-200. The enzymes were designated D-I and D-II according to their elution position on DEAE-Sepharose. Both enzymes D-I and D-II are globular proteins which have MWs of 66 000 and 60 000, respectively. Dehydrogenation is observed with both NADH and NADPH as electron donors, while the electron acceptor specificity demonstrates that the enzymes are probably NAD(P)H: quinone oxidoreductases. Successful coupling of dehydrogenase activity with that of peroxidase indicates a possible role of the enzymes in seed germination.

INTRODUCTION

Enzymes catalysing the pyridine nucleotide linked reduction of artificial electron acceptors are present in a wide variety of sources. Such NAD(P)H diaphorases have been demonstrated in animal and microbial sources [1], while this type of activity is also present in chloroplasts, mitochondria, microsomes and soluble fractions obtained from extracts of plant tissue [2]. Diaphorases have been partially purified from the soluble fraction of tobacco roots and wheat germ [2], while concentrated preparations have also been obtained from spinach chloroplasts [3–5] and mung bean seedlings [6].

Reports on the isolation and purification of NAD(P)H-dehydrogenases from plant seeds [7] are relatively scanty. Wosilait and Nason [8] and Wosilait *et al.* [9] characterized a pyridine nucleotide:quinone reductase from dry seeds of *Pisum sativum*, while Ida and Morita [10, 11] isolated a flavoprotein with NADPH diaphorase activity from rice embryo. In seeds of castor bean (*Ricinus communis*) Lord and Beevers [12] noticed ferricyanide-dependent NADH oxidation in the supernatant fraction obtained after homogenizing and centrifuging endosperm tissue. However, no purification of the enzyme(s) was attempted.

The present report describes the isolation from castor bean seeds of enzymes showing NAD(P)H diaphorase activity, and some molecular properties thereof. Based on their substrate specificities and response to certain inhibitors, the enzymes are tentatively classified in the group of pyridine nucleotide:quinone oxidoreductases (EC 1.6.99.2).

Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; PES, phenazine ethosulfate; BZV, benzylviologen; INT, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; AcPyAD, 3-acetylpyridine adenine dinucleotide.

RESULTS

Fractionation of a crude extract of castor bean seeds on DEAE-Sepharose is depicted in Fig. 1. Although linear mixing between buffers A and B was attained, the actual pH gradient produced showed maxima and was rather steep towards the end. However, the separation of active material, designated fractions I and II, respectively, from contaminating substances and from each other was of a satisfactory quality and allowed purification of these dehydrogenases D-I and D-II by chromatography on Sephadex G-200.

Polyacrylamide gel electrophoresis at pH 4.5 and SDS gel electrophoresis established the homogeneity of the two enzymes with regard to charge and MW. Homogeneity could also be inferred from amino acid analyses of the enzymes which analysed to integral numbers of residues. The analyses are summarized in Table 1.

Judged by their electrophoretic mobilities on SDS gels the dehydrogenases have a MW of *ca* 67 000 and 63 000 for D-I and D-II, respectively. When the enzymes were subjected to gel filtration on Sephadex G-200 corresponding values of 66 000 and 60 000 were found. These values were supported by MW calculations from amino acid analyses. The behaviour of the enzymes on SDS gels coupled with the results obtained from CC, seem to indicate that the enzymes are not composed of subunits but exist as single polypeptide chains. However, SDS-gel electrophoresis should also be carried out in a reducing medium, to confirm this result. Table 2 illustrates the estimates for MW together with the $A^{280}_{1\,mg/ml}$ values and some hydrodynamic properties of the enzymes.

Dehydrogenases D-I and D-II show a rather narrow specificity for electron acceptors since only quinone-like substrates are effective. The results of the study are given in Table 3. No activity was observed using NADPH as electron donor and the following electron acceptors: BZV, acridine, INT, cytochrome *c*, AcPyAD, Nile blue,

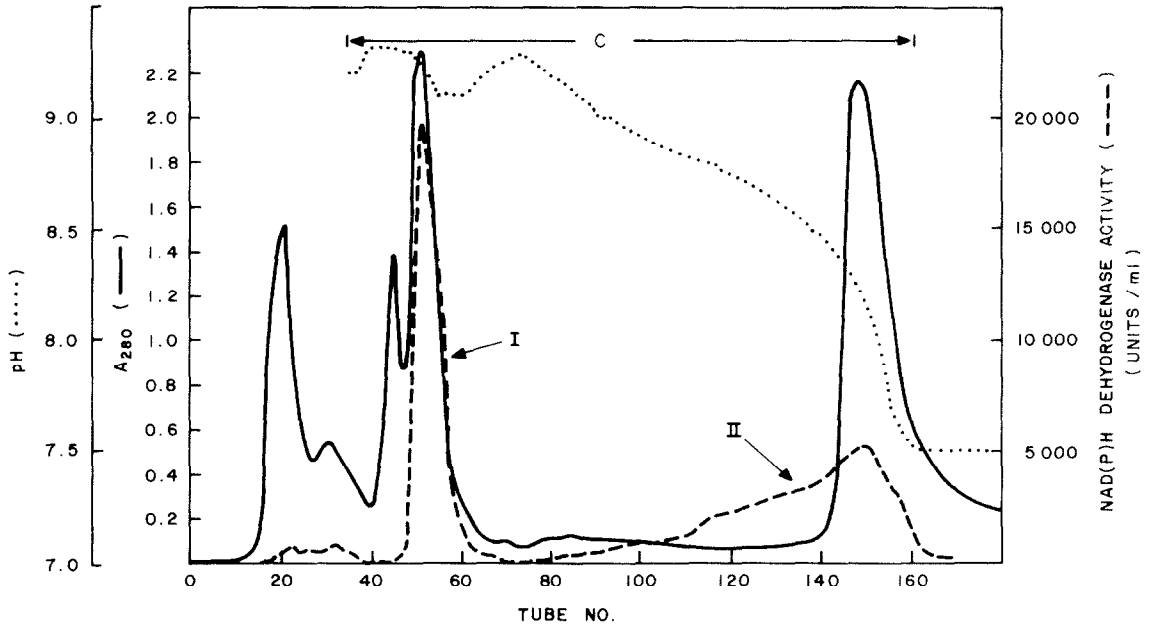


Fig. 1. Chromatography of 3 g crude castor bean extract on DEAE-Sepharose (2.6 × 35 cm). Elution of adsorbed material was affected by a pH gradient from pH 9.5 to 7.5. The gradient was established as described in the Experimental by linear mixing of buffers A and B. Buffer A: 0.025 M 2-aminoethanol–HCl, pH 7.5; Buffer B: 0.025 M Tris–HCl, pH 7.5. The gradient, C, extended over 1.5 l. Fraction volume: 10 ml. NADPH-dehydrogenase activity was determined as described in the Experimental.

phenosafranine, FAD, FMN, acetaldehyde, pyruvate, nitrate and nitrite (for abbreviations, see Experimental). Substitution of NADPH by NADH also produced negative results. Reaction velocity increased upon addition of FMN, FAD or Nile blue to reaction mixtures, as was also observed when methylene blue or phenosafranine was present in assay mixtures. When glycolate or benzylamine was used as electron donor no transfer of electrons to DCPIP was observed.

Both enzymes are inhibited to a similar extent by sulfhydryl reagents such as iodoacetic acid (*ca* 24%) and Ellman's reagent (*ca* 50%) while inhibition by EDTA and thenoyltrifluoroacetone (*ca* 30%) are examples of the effect of chelating agents on the enzymes. 2,4-Dinitrophenol was a potent inhibitor, inhibiting both D-I and D-II by 70%. Enzyme and DCPIP were preincubated with inhibitor (0.11 mM) for 10 min before NADPH was added to start the reaction.

Table 1. Amino acid composition of NAD(P)H-dehydrogenases D-I and D-II

Amino acid	Residues/mol D-I	Residues/mol D-II
Asp	71.77(72)	63.02(63)
Thr	40.01(40)	37.30(37)
Ser	43.02(43)	40.82(41)
Glu	53.76(54)	51.92(52)
Pro	27.03(27)	28.60(29)
Gly	44.71(45)	39.20(39)
Ala	43.89(44)	39.98(40)
Cys*	11.87(12)	11.72(12)
Val	35.87(36)	30.72(31)
Met	7.83(8)	5.96(6)
Ile	36.95(37)	34.82(35)
Leu	53.20(53)	47.04(47)
Tyr	25.12(25)	22.85(23)
Phe	20.97(21)	19.02(19)
Lys	15.70(16)	11.19(11)
His	5.86(6)	6.22(6)
Arg	39.92(40)	36.21(36)
Trp†	12.11(12)	11.76(12)
Total	591	539

*Determined as cysteic acid.

†Determined spectrophotometrically by the method of ref. [26].

Table 2. Molecular parameters for dehydrogenases D-I and D-II

Parameter	D-I	D-II
MW from SDS gel electrophoresis	67 000	63 000
MW from Sephadex gel chromatography	66 070	60 260
MW from amino acid analysis	65 932	60 178
Stokes radius, R_S(Å)*	33	31.5
Frictional ratio, f/f_0*	1.24	1.23
Diffusion coefficient*, $D \times 10^7$ cm^2/sec	6.53	6.83
Partial specific volume $\bar{v}$ (ml/g)	0.728	0.728
$A_{280}^{1\ mg/ml}$	1.55	1.62

*Calculated from gel filtration data.

Table 3. Substrate specificity of dehydrogenases D-I and D-II

Electron acceptor*	Electron donor	Activity† D-I	Activity† D-II
DCPIP	NADPH	4.29	0.49
	NADH	3.54	0.40
$K_3[Fe(CN)_6]$	NADPH	2.51	0.52
	NADH	2.29	0.58
Benzoquinone	NADPH	3.31	0.41
	NADH	3.31	0.34
Chloranil	NADPH	2.50	0.28
DCPIP + FMN	NADPH	9.71	1.11
DCPIP + FAD	NADPH	5.37	0.67
DCPIP + Nile blue	NADPH	5.48	0.81

Measurements were performed at 20° in 0.05 M Tris–HCl, pH 7.5. Concentration of reduced nucleotide in test mixture: NADPH, 0.038 mM; NADH, 0.045 mM.

*Acceptors were present in the following concentrations in reaction mixtures: (i) BZV, $K_3Fe(CN)_6$, INT, acridine, 0.024 mM; (ii) DCPIP, Nile Blue, cytochrome *c*, benzoquinone, chloranil, AcPyAD, PES, acetaldehyde, sodium pyruvate, sodium nitrite, sodium nitrate, sulfite, 0.008 mM; (iii) FAD, 7 μM; FMN, 11 μM. Only positive results are indicated.

†Activity is expressed as μmol acceptor reduced/min·mg enzyme.

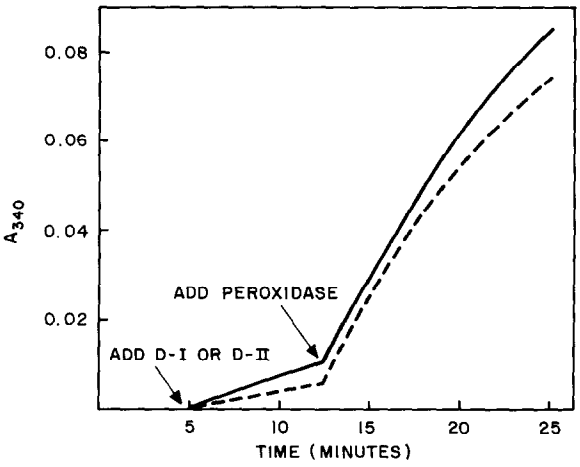


Fig. 2. Coupling of dehydrogenases D-I and D-II with peroxidase. The reaction mixture (1 ml) contained 0.038 mM $NADPH_2$, 0.056 mM hydroquinone and 0.056 mM hydrogen peroxide in 0.05 M Tris–HCl, pH 7.5. At the indicated times enzyme was added as follows and the change in *A* at 340 nm followed: 2 μl enzyme D-I (0.15 mg/ml); 5 μl enzyme D-II (0.15 mg/ml); 5 μl peroxidase (1 mg/ml); (——) reaction mixtures containing D-I; (– – – –) reaction mixtures containing D-II.

Activity of dehydrogenases D-I and D-II was measured in the pH range 5–9.5. The enzymes are active over a wide pH range, activity increasing with increasing pH and showing a broad maximum between pH 7 and 8.5. The alkaline optimum agrees wih the alkaline optima observed for rice kernel NADPH diaphorase [11] (pH9.0), menadione reductase from mung been seedlings [6] (pH 7.8) and chloroplast TPNH diaphorase [3] (pH 9.0), but contrasts with the optimum found for the quinone reductases concentrated from pea seeds [8] (pH 6.5) and spinach leaves [13] (pH 6.8).

Addition of peroxidase to reaction mixtures containing NADPH, hydroquinone, hydrogen peroxide and either D-I or D-II, resulted in the oxidation of NADPH (Fig. 2). No oxidation was observed in the absence of any one of the dehydrogenases. When D-I and D-II were added to assay mixtures in the absence of peroxidase, a slow oxidation of NADPH resulted (See Fig. 2). A similar slow rate of reaction was observed when peroxidase was added in the absence of D-I or D-II, and which may be ascribed to the non-enzymic reduction of NADPH by *p*-quinone formed by peroxidase.

Both fractions lost most of their activity within 1 week at 5° when solutions were prepared in distilled water. Addition of 25% glycerol did not prevent loss of activity even when solutions were kept at −15°. When enzyme was dissolved in 0.05 M sodium phosphate, pH 8, and kept at 5°, enzyme D-II appeared to be stable for at least 1 month while under similar conditions dehydrogenase D-I lost *ca* 30% of its activity after 1 week. Thereafter it seemed to be stable.

When 10 μm dithioerythritol was added to dilute salt solutions of the enzymes a stabilizing effect was noted for the solution of D-I, although this effect appeared to be transient. The enzyme still lost *ca* 15% of its activity after 1 week and then again stabilized.

The absorption spectrum of the enzymes exhibits one maximum at 275 nm and a shoulder at 290 nm, which indicates the presence of tryptophan in agreement with amino acid analyses. The enzymes show a rather low absorption in the visible region which increases steadily from *ca* 400 nm to the UV region.

DISCUSSION

The purification procedure reported here has yielded two fractions from castor bean seed extracts showing NAD(P)H-dehydrogenase activity. These enzyme fractions were homogeneous according to charge, molecular size and amino acid analyses.

The enzymes are colourless proteins and show very similar hydrodynamic properties (Table 2). The value for the partial specific volume (0.728 ml/g) falls within the range normally found for proteins [14] while both D-I and D-II behave as typical globular particles since their f/f_0 values are those characteristic of particles with this molecular shape. The frictional ratios (f/f_0) of D-I and D-II (1.24 and 1.23, respectively) are close to unity indicating a small deviation from the hydrodynamic behaviour of the hypothetical spherical particle for which f_0 is calculated [15].

The enzymes are almost equally active with NADH or NADPH as electron donor which is characteristic of a number of diaphorases [1]. Substitution of benzylamine or glycolate for pyridine nucleotides as possible electron donors produced negative results indicating that D-I and D-II cannot be classified as primary amine or glycolate oxidases. Enzyme activity was noted when the quinoid redox dye, DCPIP, quinones such as *p*-quinone and chloranil and ferricyanide were used as electron acceptors. However, both enzymes are unable to catalyse electron transfer between pyridine nucleotides such as has been

reported for the NADPH diaphorase isolated from rice kernel [11]. Likewise, no reaction was elicited when other possible physiological acceptors such as nitrate, nitrite, sulfite, acetaldehyde, pyruvate, FMN, FAD and cytochrome *c* or artificial acceptors such as BZV, iodonitrotetrazoliumchloride, acridine, indigocarmine, Nile blue or phenosafranine were employed. No cytochrome *c* reductase activity could be obtained even in the presence of FMN or FAD as has been noted for the NADPH diaphorase isolated from spinach leaves [5].

Since assays were carried out under aerobic conditions, molecular oxygen can probably be excluded as electron acceptor as reduced pyridine nucleotide was not oxidized in the absence of any one of the compounds acting as acceptor.

Flavin nucleotides stimulated the reaction velocity of D-I and D-II, in agreement with the findings of Mains *et al.* [16] for the NADH(DCPIP) oxidoreductase from *Bacillus stearothermophilus* and of Thomson and Shapiro [17] for the NADH-quinone oxidoreductase from *Escherichia coli* membranes. However, in those instances a specific flavin nucleotide was required, i.e. FMN and FAD for the first and second cases, respectively. The requirement for flavin in the activation of D-I and D-II points to a mediatory role of this compound in the flow of electrons between electron donor and acceptor. Electron migration is also facilitated by substituting flavin derivatives by chemicals such as methylene blue, Nile blue or phenosafranine, although reaction rates were somewhat lower in the latter cases. These compounds have rather different redox potentials, but all possess a tricyclic system which may accept electrons from NAD(P)H in a similar way as flavins, thus limiting the range of molecules capable of activating the enzymes to those showing the structural characteristics of the above-mentioned activators. This suggestion may be borne out by the fact that indigocarmine, an indole derivative with an almost identical redox potential (-110 mV) as Nile blue (-120 mV), failed to stimulate dehydrogenase reaction rates.

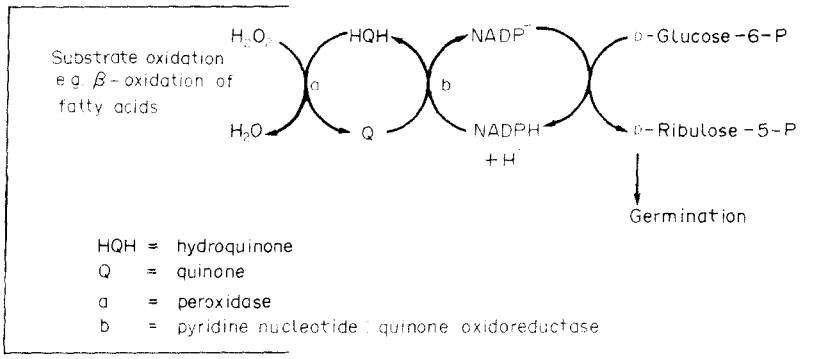
Inhibition studies with thenoyltrifluoroacetone (a powerful metal chelator [18]) and EDTA may indicate that metal ions are necessary for activity of the enzymes, while the presence of one or more essential sulfhydryl groups are demonstrated by inhibition of dehydrogenase activity by Ellman's reagent [19] and iodo-acetic acid. The effective inhibition of enzyme action by 2,4-dinitrophenol is a characteristic also shared by quinone reductases from various sources [9, 13].

Enzymes showing diaphorase activity are usually flavin containing proteins. These prosthetic groups appear to be lacking from dehydrogenases D-I and D-II as can be judged from their spectra which do not disclose the typical absorption bands found for flavin groups in the visible region, i.e. those at *ca* 370 and 450 nm [20]. It is possible that the prosthetic group may be lost during purification of the enzymes and that flavin indeed is part of the active enzymes. However, the fact that the presence of FMN and FAD is not an absolute requirement for enzyme action, seems to argue against this possibility. The absence of flavin groups from enzymes of this nature is not a novel observation. Also Thomson and Shapiro [17] noticed a lack of absorption bands in the visible region for the NADH:quinone oxidoreductase from *E. coli* membranes.

The enzymes described here resemble quinone reductases in properties, especially as regards the rather specific reduction of quinoid type substrates and their sensitivity to 2,4-dinitrophenol [13]. Dehydrogenases D-I and D-II may, therefore, also be classified as quinone reductases and may probably be identified with the ferricyanide-dependent oxidation of NADH noted by Lord and Beevers [12] to be brought about by the supernatant fraction of castor bean endosperm extracts. The enzymes are, therefore, not associated with organelles. This conclusion is also substantiated to some extent by the fact that Triton X-100 failed to stimulate enzyme activity at concentrations where Thomson and Shapiro [17] found activation of the membrane-bound quinone reductase of *E. coli*.

In spite of the widespread distribution of quinone reductases in the tissues of higher plants, the physiological role of these enzymes, in especially the soluble fractions of plant extracts, remains obscure. The occurrence of quinones in plants has led Martius [13] to postulate a possible relationship between NAD(P)H and phylloquinone or plastoquinone reduction, while Shichi and Hackett [6] suggested that the menadione reductase they have isolated from mung bean seedlings did not form part of the normal respiratory chain, but probably functioned in hydrogen transport in the cytoplasm itself. Wosilait *et al.* [9] suggested that the quinone reductase of pea seeds acted between quinone and the pyridine nucleotides as an intermediate in electron transfer between respiratory substrates and phenolase systems.

A plausible explanation was offered by Hendricks and Taylorson [21, 22] for the presence of quinone reductases in plant seeds. Evidence exists that seed germination is accompanied by an increase in glucose metabolism via the pentose phosphate pathway. Continuous functioning of this pathway depends on the concentration of $NADP^+$ and the reoxidation rate of NADPH as depicted in the following scheme:



Promotion of germination is thus thought to depend on coupling peroxidase action to NADPH oxidation which can regulate the pentose phosphate pathway of glucose metabolism. This process also involves the action of a pyridine nucleotide:quinone oxidoreductase which is present in dormant seeds. The enzymes isolated from castor bean seeds may possibly fulfil this function; a suggestion which finds some support in the observation that NADPH oxidation by D-I and D-II can be coupled to oxidation of hydroquinone by hydrogen peroxide and horseradish peroxidase (Fig. 2).

Apart from small differences in catalytic behaviour (D-I appears to be more active than D-II) and MW the enzymes show very similar characteristics as is evidenced by the physico-chemical properties reported here. They are, therefore, probably different molecular forms of the protein able to catalyse transfer of hydrogen from NAD(P)H to quinoid-type substrates. Studies are now in progress to characterize these interesting enzymes kinetically.

EXPERIMENTAL

The following products were supplied by Merck AG: DCPIP (Na salt), $NADPH_2$, $NADH_2$, 1-(2′-thenoyl)-3,3,3-trifluoroacetone, *p*-chloranil, *p*-quinone, Nile blue chloride and sodium pyruvate.

FMN and PES were products of Sigma while phenosafranine, BZV, INT, acetaldehyde and MW markers (range 14 700–71 500) for MW determination by electrophoresis, were bought from BDH Chemicals. FAD, cytochrome *c* and MW marker proteins (range 18 000–200 000) were obtained from Boehringer–Mannheim, and AcPyAD from P. L. Biochemicals. Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid) was purchased from K & K Laboratories and 2,4-dinitrophenol and hydroquinone from Hopkin & Williams. DEAE-Sepharose CL6B (Pharmacia) and Sephadex G-200 (Pharmacia) were prepared for CC as recommended by the manufacturers. Peroxidase from horseradish roots was obtained from Miles Laboratories. All other chemicals were of analytical reagent grade.

Castor bean seeds (an open pollinated variety known as UC-53) were supplied by Gunson's (South Africa) Ltd.

Enzyme assays. NAD(P)H dehydrogenase activity was determined by measuring spectrophotometrically the rate of reduction of possible electron acceptors in aerobic cuvettes (1 cm path length) containing 1 ml substrate soln in 0.05 M Tris–HCl, pH 7.5. One unit of enzyme activity is defined as that amount of enzyme causing a change in *A* at the specific wavelength of 0.001 *A* units/min. Dehydrogenase activity with various electron acceptors was monitored at the necessary wavelengths shown in Table 4, which also illustrates the extinction coefficients used for calculation of specific activities where applicable.

When pH–activity relationships were studied enzyme activity was followed by monitoring the reduction of DCPIP at 522 nm which is the isosbestic point of the dye [23]. At low pH the oxidation rate of DCPIP was corrected for the non-enzymic oxidation of this substrate. Glycolic acid oxidase and amine oxidase activities were determined as described in refs. [2] and [24], respectively, using glycolic acid and benzylamine as electron donors and DCPIP as electron acceptor. EtOH dehydrogenase and lactate dehydrogenase were assayed according to the method of ref. [25].

Coupling with peroxidase. The NAD(P)-dehydrogenase system was coupled with peroxidase which catalyses the oxidation of hydroquinone to quinone by H_2O_2. Reaction mixtures contained 56 μM H_2O_2, 0.056 mM hydroquinone and 38 μM NADPH in 1 ml 0.05 M Tris–HCl, pH 7.5. Dehydrogenase activity was monitored by measuring the decrease in *A* at 340 nm.

Table 4. Summary of the wavelengths at which NAD(P)H dehydrogenase activities with various electron acceptors were monitored and extinction coefficients used to calculate activities

Electron acceptor	Wavelength (nm)	Extinction coefficient (M^{-1}/cm)	Reference
DCIP	600	20 000	[39]
$K_3[Fe(CN)_6]$	420	1000	[39]
INT	485	12 000	[39]
BZV	540	13 050	[40]
AcPyAD	375	5200	[25]
Benzoquinone	340	6220*	[41]
Chloranil	340	6220*	[41]
FAD	450	11 300	[42]
FMN	450	12 200	[42]
Cytochrome c_{red-ox}	550	19 700	[39]
Phenosafranine	520	47 700	[43]
Nile blue	640	34 000	—†
Pyruvate	340	6220*	[41]
Acetaldehyde	340	6220*	[41]

*The wavelength at which decrease in absorption of NAD(P)H was measured was 340 nm.

†Personal observation.

Amino acid analysis. Protein samples were hydrolysed in redistilled constant boiling HCl (5.7 M) containing 0.2% (w/v) PhOH, in evacuated sealed tubes. Amino acid analyses were performed on the automated Beckman 120B amino acid analyser. Tryptophan was calculated spectrophotometrically according to the method of ref. [26]. Cysteine was determined as cysteic acid on samples oxidized with performic acid reagent substantially as described in ref. [27].

Protein concentration. Protein concn was evaluated by a modified Lowry procedure as described in ref. [28] and the data treated according to the method of ref. [29]. The estimate of concn was used in conjunction with *A* values at 280 nm to derive $A_{280\ nm}^{1\ mg/ml}$ values.

Electrophoresis and determination of molecular parameters. Disc electrophoresis on 7.5% polyacrylamide gels at pH 4.2 was performed according to the description of ref. [30], and SDS polyacrylamide gel electrophoresis was done essentially as described in ref. [31] as modified in ref. [32]. MWs of purified enzyme fractions were determined by SDS gel electrophoresis using MW markers in the range 14 300–71 500. Protein bands were detected on the developed gels by Coomassie blue stain according to the method of ref. [33]. Molecular size was also estimated by gel chromatography of the isolated enzymes and marker proteins as described in ref. [34]. A column of Sephadex G-200 (0.9 × 65 cm) was employed and elution effected with 0.1 M NH_4HCO_3 soln containing 0.3 M NaCl. The void vol V_0, and the total vol V_i, of the column were determined from the elution vols. of Dextran blue 2000 and mercaptoethanol, respectively. The column was calibrated with aldolase (158 000), bovine serum albumin (68 000), ovalbumin (45 000), chymotrypsinogen (25 000) and cytochrome *c* (12 500) as standard proteins, while results were plotted according to the suggestion in ref. [35], wherein the distribution coefficient, K_d is related to the Stokes radius, R_S, by:

$$R_S = a_0 + b_0\, erfc^{-1}(1 - K_d);$$

a_0 and b_0 being calibration constants for the gel and $erfc^{-1}$ is the inverse error function complement of K_d. Stokes radii were subsequently plotted as a function of MW in log-log fashion to obtain the MWs of the unknowns. The Stokes radii of the standards are those given in ref. [36]. Using the known Stokes radii for the isolated dehydrogenases, the diffusion coefficient, D, was calculated from:

$$D = kT/6\pi\eta R_S,$$

where k is the Boltzmann constant, and η is the viscosity of the solvent which was taken as 0.01 poise for dilute salt solns [36]. The frictional ratio f/f_0 was estimated by combining R_S with MW according to the equation [36]:

$$f/f_0 = R_S/(3\bar{v}M/4\pi N)^{1/3},$$

where $\bar{v}$ represents the partial specific vol. of the protein of MW M and N equals Avogadro's No. Partial specific vols. were determined from the amino acid composition of the enzymes as described in ref. [37].

Enzyme isolation. Castor bean seeds (400 g) were ground in a meat grinder and the pulp extracted with 1.5 l. 0.1 M NH_4HCO_3 soln. Extraction was continued for 1.5 hr in the cold. Longer extraction times were found to be unnecessary since enzyme activity did not increase beyond that time. The extract was strained through cheesecloth and centrifuged at 16 300 *g* for 35 min. The supernatant was subsequently filtered through glass wool and the turbid soln brought to 50% satn with solid $(NH_4)_2SO_4$. The suspension was stirred for 30 min and the ppt collected by centrifugation at 16 300 *g* for 35 min. Ppted protein was taken up in 400 ml H_2O and dialysed overnight in the cold against running H_2O. The dialysed sample was again centrifuged to remove insoluble material and lyophilised.

Lyophilised material (3 g) was suspended in 75 ml 0.025 M 2-aminoethanol–HCl, pH 9.5. After centrifugation the supernatant was adjusted to pH 9.5 with NH_4OH and applied to a DEAE-Sepharose column (2.6 × 35 cm) equilibrated and packed in 0.025 M 2-aminoethanol–HCl, pH 9.5. Desorption was obtained by means of a pH gradient established as described in ref. [38]. The gradient was produced by linear mixing of two buffers A and B having an equal pH; buffer A comprising 0.025 M 2-aminoethanol–HCl, pH 7.5, and buffer B consisting of 0.025 M Tris–HCl, pH 7.5. The gradient extended over 1.5 l. Material showing diaphorase activity was dialysed overnight in the cold against running H_2O followed by dialysis against 0.1 M NH_4HCO_3. The dialysed fractions were subsequently lyophilised.

The preparations from ion-exchange chromatography, fractions I and II, were finally fractionated by employing a step of gel chromatography. Active fraction I or II (500 mg) was taken up in 40 ml 0.1 M NH_4HCO_3, centrifuged at 12 100 *g* for 20 min and applied to a Sephadex G-200 column (5 × 270 cm) equilibrated in 0.1 M NH_4HCO_3 soln.

Chromatography was carried out at 10°. Peaks exhibiting diaphorase activity D-I and D-II were collected and lyophilised.

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