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FLUORIMETRIC ASSAY OF TOBACCO LEAF DEHYDROGENASES WITH RESAZURIN *

DONALD W. DE JONG and WILLIAM G. WOODLIEF

Tobacco Research Laboratory, Southern Region, Agricultural Research Service, U.S. Department of Agriculture, Oxford, N.C. 27565 (U.S.A.)

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Summary

A versatile fluorimetric assay based on the reduction of resazurin to resorufin demonstrated high specific activities for a number of important pyridine nucleotide-linked dehydrogenases in tobacco leaves. The Michaelis constant for the important photosynthetic enzyme, D-glyceraldehyde-3-phosphate:$NADP^+$ oxidoreductase (EC 1.2.1.13), determined by the fluorimetric method, was considerably lower than constants determined by conventional extraction and assay methods reported for the enzyme from other plants. The sensitivity of the fluorimetric method enabled the use of dilute enzyme preparations with resultant low background and high substrate specificity. Inclusion of the antioxidant diethyldithiocarbamate in the extraction medium preserved the enzymes during extraction. Primary amines inhibited competitively, and phenazine methosulfate non-competitively each of the eight dehydrogenases tested with the fluorimetric assay. The Mn^{2+} dependence of NADP-linked dehydrogenases specific for isocitrate and malate was confirmed. The method is rapid, requires a simple combination of ingredients and should be useful for surveying dehydrogenase activity in leaves.

Introduction

Recent work with plant dehydrogenases has focused on isoenzyme diversity in relation to genetic variability [1] and subcellular distribution [2]. The kinet-

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Abbreviations: PMS, phenazine methosulfate; Tricine, *N*-tris(hydroxymethyl)methylglycine; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; DCIP, 2,6-dichlorophenolindophenol.

ic properties of the abundant dehydrogenases * have been extensively studied, but the current assay methods for this important class of enzymes are not always reliable with crude enzymes from plant origin. Spectrophotometric measurement of changes in absorbance at 340 nm associated with reduction of the coenzymes NAD or NADP, and with the oxidation of their reduced counterparts, has been the standard technique used in recent years. Occasionally, redox dyes such as DCIP, with our without PMS, have been used to monitor oxidoreductase activity in plant extracts. Examples of the latter include succinate dehydrogenase [3], glycolate oxidase [4], and the NAD(P)-linked malate dehydrogenases [5]. Because of low sensitivity, high background rates and susceptibility to other interferences, such methods often are unsatisfactory for the assay of extracts of green plant tissues. Improvements in methodology should improve understanding of the respective roles of the dehydrogenases in plant growth and development.

Pyridine nucleotide-linked oxidoreductases of intermediary metabolism perform vital functions in all living organisms. The NADP-specific glucose-6-phosphate and 6-phosphogluconate dehydrogenases control operation of the pentose phosphate shunt; the glyceraldehyde-3-phosphate-NAD(P) dehydrogenases (EC 1.2.1.13) regulate the routing of triose phosphates into glycolytic and gluconeogenic pathways during respiration and photosynthesis; and oxidoreductases, acting on malate and isocitrate, furnish high energy compounds and organic acid intermediates through the sequence referred to collectively as the "Krebs' citric acid cycle". In animal systems [6], fluctuations in activities of certain dehydrogenases are sensitive indicators of changes in growth patterns and metabolic shifts. Although dehydrogenases are present in all organisms, the amounts of active enzymes vary in response to physiological needs and changing environments.

Studies are in progress to elucidate tobacco leaf metabolism with particular emphasis on biochemical differences between cultivars, changes during leaf maturation and diurnal fluctuations in dehydrogenase activity [7]. Work with tobacco leaf dehydrogenases has been hampered by the lack of convenient and accurate methods for their extraction and assay. Except for malate-NAD dehydrogenases, which is quite stable, most dehydrogenases in tobacco apparently are sensitive to conditions of extraction and assay. For prevention of denaturation by acid pH values and inactivation by quinones formed when polyphenoloxidases and endogenous polyphenols are mixed during leaf homogenization, plant enzymes must be protected during extraction by appropriate buffering and reducing conditions [8]. Frequently, the high concentrations of antioxidants needed for enzyme preservation interefere with oxidoreductase assays. These problems in enzyme extraction are common to most plant tissues, but are especially severe in mature tobacco leaves with their high organic acid and polyphenol contents.

We now describe a combination of techniques that surmounts the cited difficulties by use of relatively large volumes of buffer extraction in conjunction with an effective antioxidant; enzyme activity is assayed with a fluorimetric

* See Table I for systematic names and enzyme commission designations of dehydrogenases discussed in this paper.

technique based on reduction of resazurin to resorufin. The assay for purified dehydrogenases is a further modification of the method developed by Guilbault and Kramer [9,10] and adapted by Rhodes and Wooltorton [11], who used PMS with pyridine nucleotide-linked enzymes and resazurin to measure NAD(H) and NADP(H) in plant material. Our procedure appears to be suitable for assaying dehydrogenases in large numbers of samples and for monitoring enzyme enrichment during isolation and purification. For the analysis of extracts of green tissues, we have found it superior to published spectrophotometric methods.

Materials and Methods

Plant material. NC-95 and Pale Yellow hybrid (NC-95 T.I. 1372) plants *Nicotiana tabacum L.*) were grown in the greenhouse or in controlled environment chambers under conditions described previously [12]. For the comparative study of specific enzyme activities, fully expanded mature leaves, about 36 cm long, were taken from the third lowermost stalk position of 1-month-old plants. A 2 g sample of leaf blade/40 ml of buffer were thoroughly homogenized in a Virtis No. 60 blender for 1 min at 25 000 rev./min, filtered through Miracloth, and centrifuged at 1500 × *g* for 10 min to remove starch and chlorophyll aggregates. The preparation then was centrifuged at 32 000 × *g* for 30 min and the clear yellow supernatant was used for enzyme studies.

The extracting buffer consisted of 0.1 M potassium phosphate, pH 7.5, containing 1 mM EDTA * and 1 mM sodium diethyldithiocarbamate. The basic assay mixture contained 4.0 ml 0.1 M Tricine buffer, 0.1 ml of 0.02—0.2 M substrate, 0.05 ml of 5 mM coenzyme (NAD(P)), 0.1 ml of 100 μM resazurin, and 0.1 ml enzyme solution.

A Turner Model No. 111 Fluorimeter was adjusted for excitation at $\lambda_{540\,nm}$ and emission at $\lambda_{580\,nm}$ with primary filters No. 1—60 + 58 and secondary filter No. 23A. Sensitivity was set at position 1× and an interference filter of 20% transmission was placed on the secondary side. Calculation of the fluorescence yield of resorufin was based on total reduction of several concentrations of resazurin with excess $Na_2S_2O_4$ and measurement of the increase in fluorescence between 0 and 100 arbitrary units. A plot of fluorescence intensity versus concentration was linear over the 5 μM range. Fluorescence yield was found to be 1 units/23.8 nmol resorufin. Enzyme assays were run at 30° C for 5—15 min by continuously monitoring increase in fluorescence with the fluorimeter coupled to a Linear Instruments strip chart recorder. Activity was strictly proportional to the amount of enzyme. Diaphorase activity was assayed with a Gilford 240 spectrophotometer by the method described in the Worthington Enzyme Manual [13].

Protein was precipitated with cold 10% trichloroacetic acid and redissolved

* All agricultural chemicals recommended for use in this report have been registered by the U.S. Department of Agriculture. They should be applied in accordance with the directions on the manufacturer's label as registered under the Federal Insecticide, Fungicide, and Rodenticide act.

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in 0.1 M NaOH. Protein concentration was then determined with the Folin-Ciocalteau reagent [14].

For pH and kinetic studies, enzymes were extracted from leaves of cv. NC-95 plants that were grown in environmental chambers. Crude protein extracts were concentrated and partially purified by fractionation with $(NH_4)_2SO_4$ (between 30 and 60% saturation) followed by diafiltration through a P-30 membrane in an Amicon TCF-10 thin-channel apparatus. The dialysis chamber was pressured with nitrogen. Most of the dehydrogenase activity was present in this preparation. In all, eight NAD(P)-linked enzymes were studied. Further purification by ion-exchange chromatography or gel filtration, was not attempted.

With the exception of resazurin, obtained from Matheson, Coleman and Bell, all biochemicals were from Sigma Chemical Co. Other chemicals were reagent grade or better.

Results

Preliminary tests of the resazurin reduction method for the assay of glyceraldehyde-3-phosphate NADP dehydrogenase in tobacco leaf extracts established that Tris · HCl was a poor buffer for the reaction and that no electron carrier other than coenzyme was necessary. Because this information did not agree with the report by Guilbault and Kramer [10], we sought the optima for the buffer and conditions for assay. Several buffers at various pH values were tested with different substrates and coenzymes in the assay mixture. Contrary to published work [10], the pH range 8.0—9.0 was not always optimal. We, therefore, tested extraction and reaction conditions, including potential inhibitors and activators, for selected tobacco leaf dehydrogenases.

Extraction conditions

Due to the natural sensitivity of fluorimetry, leaf tissue could be extracted at unusually high dilution without falling below the level of enzyme detection. This factor was advantageous in achieving excellent protein recovery. Leaf material was homogenized in 20 volumes (w/v) of 0.1 M potassium phosphate buffer at pH 7.5 containing the appropriate protective adjuvants, i.e. diethyldithiocarbamate and EDTA. Protein content of the enzyme preparation was approx. 0.5—1.5 mg/ml, and varied according to the leaf source. The high protein values indicated that non-particulate protein was adequately dissolved and losses due to quinone complexing were minimal. Control of polyphenoloxidase by diethyldithiocarbamate (1 mM) was superior to that exerted by some more commonly used antioxidants, e.g. ascorbate, cysteine, β-mercaptoethanol, dithiothreitol and metabisulfite (unpublished results). The EDTA (1 mM) was included to improve protein extraction and enzyme recovery. Tricine at pH 8.0—9.0 was an excellent assay buffer for most of the dehydrogenases, but was inferior to phosphate as an extracting medium. When the ratio of tissue to buffer was doubled, the extract turned brown and some of the less stable enzymes were lost. The simple buffer-antioxidant prescribed above apparently protected the enzyme against inactivation without inhibiting the enzyme or causing non-substrate-specific reduction of resazurin to resorufin.

Temperature effects

Precise control of temperature during the enzyme assays was important because fluorimetric measurements are very temperature dependent. Enzyme activity normally increases with temperature (below the point of heat denaturation), but fluorescence efficiency increases with decreasing temperature. These two effects counterbalanced one another at 30°C which was subsequently adopted as the standard assay temperature.

pH effects

Guilbault and Kramer [9] stated that fluorescence yields were enhanced by alkaline conditions and suggested that pH 8.0—9.0 would probably be the most suitable. Our result showed a wider range. The optimum pH for each enzyme is shown in Table I. Tricine was the superior buffer in the pH 7.0—9.0 range for all but one of the dehydrogenases tested; viz. for isocitrate-NADP dehydrogenase, TES buffer at pH 6.5 was better.

Steady-state kinetics

When all components of the assay mixture were present in excess, enzyme activity was directly proportional to enzyme concentration, but not always linearly related to time during the entire course of the reaction. In leaf homogenates containing chlorophyll, enzyme activity invariably declined following a short linear phase but before levels of substrate, coenzyme, or dye became limiting factors. Furthermore, with enzymes exhibiting relatively low activity in crude extracts, a brief but distinct lag phase was commonly observed at the beginning of the assay (Fig. 1). The lag could not be attributed to inherent limitations of the assay system because it was never detected with malate-NAD dehydrogenase. Neither was the lag observed in concentrated and partially purified enzyme preparations.

The effect of substrate concentration was determined for selected dehydrogenases. They all showed classical Michaelis-Menten kinetics. The Michaelis constant for NAD-linked glyceraldehyde-3-phosphate dehydrogenase was within the range published for other plants, i.e. 255 μM [15], but the K_m for NADP-

TABLE I
MICHAELIS CONSTANTS AND pH OPTIMA FOR TOBACCO LEAF DEHYDROGENASES ASSAYED FLUORIMETRICALLY WITH RESAZURIN

Enzyme	pH optimum	K_m (substrate)
D-Glyceraldehyde-3-phosphate : NAD^+ oxidoreductase (EC 1.2.1.12)	9.0	255 μM
D-Glyceraldehyde-3-phosphate : $NADP^+$ oxidoreductase (EC 1.2.1.13)	9.0	56 μM
L-Malate : NAD^+ oxidoreductase (EC 1.1.1.37)	8.5	1170 μM
D-Glucose-6-phosphate : $NADP^+$ 1-oxidoreductase (EC 1.1.1.49)	8.5	227 μM
6-Phospho-D-gluconate : $NADP^+$ 2-oxidoreductase (EC 1.1.1.44)	8.5	105 μM
L-Glutamate : NAD^+ oxidoreductase (EC 1.4.1.2)	7.5	18 μM
L-Malate : $NADP^+$ oxidoreductase (EC 1.1.1.40)	7.0	3300 μM
L_S-isocitrate : $NADP^+$ oxidoreductase (EC 1.1.1.42)	6.5	4 μM

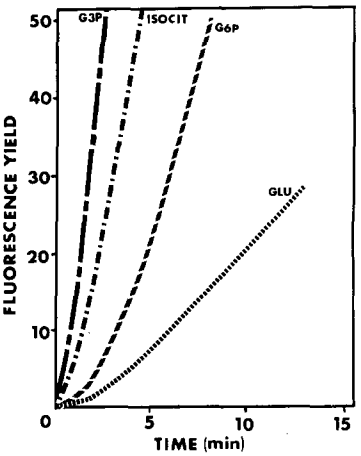

Fig. 1. Typical time course of fluorimetric assay for selected tobacco leaf dehydrogenases in crude extracts. Note initial lag phase for enzymes with low activity. Substrates and coenzymes were: G3P, glyceraldehyde 3-phosphate + NADP; ISOCIT, isocitrate + NADP; G6P, glucose 6-phosphate + NADP; GLU, glutamate + NAD. Data transcribed directly from recorder chart without alteration.

linked activity was much lower, i.e. 56 μM, than published values. Whether the affinity of the latter coenzyme for substrate would be increased for the tobacco enzyme under other conditions has not been tested. The substrate K_m values for the eight enzymes investigated are given in Table I.

The K_m values were not determined for the coenzymes NAD and NADP because the line V/K_m, on a double reciprocal plot [16] of data from our assay system, passed through the origin. Dixon [17] attributed such enzyme behavior to liberation of one of the products at some intermediate stage in a series of reactions. Our results suggest that after the coenzyme was reduced, it was reoxidized subsequent to its dissociation from the enzyme-substrate complex.

Enzyme modifiers

A number of inhibitors and cofactors were examined for their ability to modify the resazurin assay system. The poor results with Tris · HCl were found to involve competitive inhibition. Although the Tris molecule contains a reactive primary amine group, Tris has been customarily used as a buffer for glyceraldehyde-3-phosphate-NAD(P) dehydrogenases in plant extracts [18,19]. We proceeded to test other low molecular weight amines for inhibition on the assumption that the amino group in Tris was inhibitory. Surprisingly, addition of β-alanine did not affect the reaction rate. However, inhibition with ethanolamine was similar to the effect of Tris at low concentrations (i.e. 50% inhibition at 0.5 mM) and twice as inhibitory at concentrations higher than 1.0 mM. Ethylenediamine was the most potent inhibitor of glyceraldehyde-3-phosphate-NAD(P) dehydrogenases (i.e. 50% inhibition at 0.05 mM) but diethylamine had no effect whatsoever.

A different type of inhibition was discovered when PMS was added to the reaction mixture to facilitate electron transport from NAD(P)H to resazurin. Guilbault [20] indicated that either diaphorase or PMS was required to complete this reaction sequence, but we found that low concentrations of PMS

were very inhibitory while diaphorase had no stimulating effect. When PMS was added to the assay mixture together with Tris, the inhibitions were additive, indicating that different mechanisms were operative in the two instances. With PMS held constant at 3.75 μM and data plotted by the double reciprocal method, inhibition by PMS of dehydrogenases, under standard conditions, was noncompetitive. All the dehydrogenases exhibited similar behavior with PMS.

To examine diaphorase participation in the resazurin assay, we determined the DCIP-NAD(P)H oxidoreductase (i.e. diaphorase) activity in leaf samples before and after ammonium sulfate fractionation. In a crude supernatant, activity was 2.4 units/0.1 ml with NADH and 4.6 units/0.1 ml with NADPH (1 unit = ΔA/min of 1.000). When the extract was boiled, both the DCIP-reducing activity and the dehydrogenase activity assayed fluorimetrically were destroyed. Overnight dialysis of crude enzyme halved the diaphorase activity. Distribution of diaphorase varied widely among the five ammonium sulfate fractions. Activity was barely detectable, in the 0—30% fraction, highest in the 40—50% fraction (e.g. 16.8 units/0.1 ml with NADPH), substantial in the 30—40% fraction (e.g. 5.1 units/0.1 ml with NADPH), and low in the 50—60% and 60% soluble fractions. Diaphorase activity was persistent in tobacco extracts and was not affected by standard inhibitors, such as *N*-ethylmaleimide [13], iodoacetate, and *p*-chloromercuibenzoic acid. However, mersalyl acid inhibited diaphorase more than it did malate-NAD dehydrogenase suggesting that, if diaphorase was indeed required for the resazurin reaction to proceed, extremely low concentrations were adequate. Furthermore in the 0—30% fraction, where diaphorase was hardly detectable, the malate-NAD dehydrogenase isozyme [5] expressed full activity in the resazurin system, with or without added diaphorase. We were not, however, able to absolutely rule out participation of endogenous diaphorase in the fluorimetric dehydrogenase assay with resazurin.

We noted that in some preparations, activity of malate-NADP dehydrogenase often was either unusually low or absent, and was not stimulated by dithionite or lipoate [21]. Furthermore, in our ammonium sulfate-fractionated and diafiltered preparations, Mn^{2+} was essential for malate-NADP dehydrogenase activity. This strict dependence is not generally observed in spectrophotometric assays of partially purified plant enzymes [5], but in bacterial extracts, malate-NADP dehydrogenase (malic enzyme) is Mn^{2+} dependent [22]. An activation curve for malate-NADP dehydrogenase, obtained by titrating with Mn^{2+}, showed that the optimum concentration of the metal cofactor was 0.5 mM, above which enzyme activity was inhibited. Isocitrate-NADP dehydrogenase was not strictly Mn^{2+} dependent; however, its activity was stimulated 10-fold by 0.5 mM Mn^{2+} in partially purified preparations.

Application to enzyme survey

After establishing optimal parameters for the eight dehydrogenases, we surveyed enzyme levels in leaf extracts from two tobaccos, NC-95 and its Pale Yellow hybrid (T.I. 1372 x NC-95). Specific activities were highest for malate-NADP and -NAD dehydrogenases, followed closely by glyceraldehyde-3-phosphate-NADP and -NAD dehydrogenases (Table II). All activities were substantially higher than published values. In general, activity was higher in Py than in NC-95 samples. We did not relate the data to plant type.

TABLE II

COMPARISON OF FLUORIMETRICALLY DETERMINED DEHYDROGENASE ACTIVITIES IN TOBACCO LEAF EXTRACTS

Activity expressed in units of μmol/min per mg protein. Samples were unfractionated leaf extracts.

Enzyme system			Units specific activity	
Substrate	Coenzyme	Metal cofactor	Plant variety	
			NC-95	Pale yellow
Malate	NADP	Mn^{2+}	42.88	43.83
Malate	NAD	—	33.54	41.86
Glyceraldehyde 3-phosphate	NADP	—	33.54	39.32
Glyceraldehyde 3-phosphate	NAD	—	27.25	34.25
Isocitrate	NADP	Mn^{2+}	27.87	31.15
6-Phosphogluconate	NADP	—	27.25	32.98
Glucose 6-phosphate	NADP	—	17.82	16.49
Glutamate	NAD	Zn^{2+}	8.58	7.79

Discussion

High dilution of leaf sample in extraction buffer was one major improvement in our extraction-assay protocol. In most homogenization schemes, the ratios of plant tissues to buffers range from 1 : 1 to 1 : 4. Under these conditions, protein solubilization is probably not complete although protein levels are invariably higher in concentrated than in dilute extracts. However, comparisons of enzyme activity among plants, leaves or treatments will be of questionable value if protein extraction is not exhaustive. Dilution of endogenous phenols and their respective oxidases by high buffer volumes and inhibition of polyphenoloxidase with diethyldithiocarbamate, apparently had an additive effect on preservation of leaf proteins. Satisfactory control of polyphenoloxidase probably contributed to the proportionately high levels of active protein in our diluted tobacco leaf extracts; a second extraction of the leaf residue after filtration yielded little additional protein and negligible enzyme activity. Additionally, diethyldithiocarbamate used as an antioxidant and polyphenoloxidase inhibitor, appeared to protect the enzymes without interfering with the fluorimetric assay. Resazurin can be readily reduced by some of the more commonly used antioxidants, including cysteine, dithiothreitol and dithionite.

Guilbault and Kramer [9] indicated that the resazurin method for dehydrogenase was extremely sensitive because of the high fluorescent yields of resorufin. For our analyses, we used the lowest senstivity setting for the Turner instrument and a secondary 20% neutral density filter to keep the assay within a measurable range. Instrumental sensitivity was adequate for measuring even lower enzyme activities. Furthermore, because of the intrinsic selectivity of fluorescence, background color was of little significance. Increase in enzyme sample size caused little loss of resolution, but we found evidence that such an increase raised some endogenous substrates to interfering levels. For example, the concentration of malate in crude tobacco leaf extracts apparently gave

spurious background activity with NAD or with NADP plus Mn^{2+}. Negligible background was found with NADP alone and the NADP plus Mn^{2+} background was optimal at pH 7.0. Dialysis of the extract for 3 h removed the interference providing further confirmation that malate was the responsible contaminant. Interestingly, extracts from leaves of pokeberry (*Phytolacca americana L.*) showed no endogenous activity with NAD although enzyme activity was within the same general range as in tobacco (data not shown).

The higher than usual levels of specific dehydrogenase activity we observed in leaf extracts, could be physiologically significant. Malate-NAD dehydrogenase activity in green plants is usually reported to be much higher than any other pyridine-linked dehydrogenases [4,23,24]. In tobacco leaf material, we examined, the disparity between malate-NAD dehydrogenase and the other dehydrogenases was less than previous reports have indicated. The relative activities of these essential enzymes might not differ too greatly in green plants growing under normal physiological conditions. Tolbert et al. [4], who surveyed enzymes in subcellular fractions from several plants, found specific malate-NAD dehydrogenase activity of 1.108 units in tobacco. For that enzyme, we reported 3.12 units in bud tissue where its concentration in tobacco is highest [7], and Benedict [23] demonstrated activity as high as 8.3 units in spinach. In contrast, the specific activities reported for glyceraldehyde-3-phosphate-NADP dehydrogenase, a critical photosynthetic enzyme in plants, generally have not exceeded 0.25 unit [23,24]. The predominance of malate-NAD dehydrogenase frequently seen might be a result of its greater stability in contrast to other dehydrogenase enzymes that are more easily inactivated during extraction and that as a consequence are more apt to be underestimated. The excellent preservation of glyceraldehyde-3-phosphate-NADP dehydrogenase in our preparations was shown, not only by the high specific activity, but also by the lack of requirement for adjuvants, such as cysteine, NaF or sodium arsenate in the assay medium [18].

The inhibitory action of primary amines on glyceraldehyde-3-phosphate dehydrogenases has not been reported. Guilbault and Kramer [9,10] assayed commercially available enzymes in 0.1 M Tris buffer, pH 8.0—9.0. Tris has been extensively used for glyceraldehyde-3-phosphate dehydrogenase analyses in plant extracts, so apparently Tris does not inhibit enzyme activity in spectrophotometric assays. Evidence that ethylenediamine was more inhibitory to that enzyme than monoamines, such as ethanolamine, might provide a clue as to the mechanism of perturbation of the enzyme receptor site but we have not explored the system in detail. The competitive nature of the inhibition suggested that formation of the enzyme-substrate complex was reversibly blocked by the amine functional group; a response usually observed with structural analogues of the substrate. Inhibition by Tris and ethanolamine was common to all the dehydrogenases examined, whereas, inhibition with ethylenediamine was unique for the glyceraldehyde-3-phosphate-NAD(P) dehydrogenases. Inhibition by monoamines apparently was a function of either the assay system or the dehydrogenase reaction mechanism. The responses to the two monoamine inhibitors differed, however. For example, 1 mM Tris inhibited malate-NAD dehydrogenase by 67% and glucose-6-phosphate-NADP dehydrogenase by only 33%; conversely, 1 mM ethanolamine inhibited malate-NAD dehydrogenase by 25%,

but glucose-6-phosphate-NADP dehydrogenase by 75%.

The inhibitory effect of PMS on the resazurin assay is difficult to explain. It might be that PMS, instead of serving as an electron transfer agent, interferes with the reaction by diverting electrons from the terminal acceptor, resazurin. By intercepting electrons between the NAD(P)H $\xrightarrow{2e^-}$ resazurin step in the reaction, the auxilary redox dye could be retarding the rate of resorufin formation. Also, PMS is somewhat unstable and can photooxidize in solution [11]. A less likely explanation for the inability of PMS to act as an NAD(P)H-resazurin coupler in our system could be a mechanism whereby PMS catalyzes a rate-limiting step between endogenous diaphorase and resazurin. Although non-competitive inhibition by PMS in dehydrogenase assays apparently has not been reported previously, the inhibition was genuine and should be investigated whenever this enzyme assay is used.

We also found that isocitrate-NADP dehydrogenase was inhibited by simultaneous addition of 1 mM glyoxalate and 1 mM oxaloacetate confirming an earlier report of similar concerted inhibition of this enzyme in protozoan extracts [25]. The enzyme block was not immediate, but required preincubation of enzyme with the reagents for 30 min to obtain 100% inhibition. Feedback inhibition of this step in organic acid metabolism probably occurs in plants as well as in microorganisms and the results provided further substantiation for the validity of the resazurin assay method.

Specific enzyme activities differed between the two tobaccos examined, and evidence (data not shown) suggests that activities change selectively during plant growth and development. The Py hybrid is more vigorous than the NC-95 parent and has better photosynthetic capabilities [12], even though Py leaves contain less chlorophyll and senesce more abruptly than leaves of the normal parental line. For crop plants, such as tobacco, that are selectively bred for certain traits, correlation of those traits with biochemical properties such as patterns of enzyme activities would be valuable. The different responses of tobacco cultivars to chemical treatments or environmental stresses could result from enzyme lesions, involving induction and repression at the gene level, or from positive/negative feedback control by metabolites. A convenient assay method for dehydrogenases could help to resolve these possibilities.

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