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Improvements of the Nitrite Color Development in Assays of Nitrate Reductase by Phenazine Methosulfate and Zinc Acetate[1]

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ABSTRACT

Nitrate reductase activity is most commonly assayed by measurement of product formation. Excess NADH and factor(s) present in the enzyme extract that interfere with the diazotization and azo color complex of nitrite cause a depression of apparent nitrate reductase activity. Two postassay treatments were found that markedly enhanced the extent of nitrite color formation and apparent nitrate reductase activity. The procedure involves stopping the reaction with zinc acetate (50 μmoles per ml of reaction mix), followed by removal of the precipitate by centrifugation. Presumably the zinc acetate removes extract factor(s) that interfere with color development, because it does not remove the NADH. Phenazine methosulfate (15 nmoles per ml of reaction mix) is added to aliquots of the supernatant and allowed to stand for 20 min at 30 C to oxidize the residual NADH before color development.

The most sensitive method for assaying nitrate reductase in plant extracts is by colorimetric measurement of the product, nitrite (12). Nitrite is diazotized with sulfanilamide and then reacted with N-(1-naphthyl)ethylenediaminedihydrochloride to produce the azo dye which is measured spectrophotometrically at 540 nm. However, excess NADH from the reaction medium interferes with the full development of color (9), although NAD does not.

Several methods for the removal of excess NADH before color development have been suggested. Although barium acetate- or zinc acetate-ethanol treatments enhance the recovery of NO_2^- produced in the NR[3] assay (9, 10, 11), they are not effective in removing NADH from the medium (14). R. H. Hageman (unpublished) has shown that a marked enhancement of color development can be obtained by stopping the reaction with zinc acetate, followed by centrifugal clarification, before color development. Presumably the zinc acetate precipitates one or more components of the crude enzyme extract which interfere with color development. The effectiveness of this treatment varies with species, genotype, and plant age. The removal of NADH with charcoal, as proposed by Stulen (14), is laborious and not always reliable, as discussed later. While dialysis removes excess NADH (6), it requires specialized equipment. Enzymatic oxidation is also satisfactory (5), except for time and expense. Phenazine methosulfate rapidly oxidizes NADH (2, 7, 8), but it has not been used to oxidize excess NADH in the NR assay.

The objectives of this study were to test the effectiveness of PMS in the oxidation of excess NADH in the NR assay medium before color development and to determine if PMS could be used in conjunction with zinc acetate to improve NO_2^- recovery in the NR assay.

MATERIALS AND METHODS

Corn (*Zea mays* L.) and soybeans (*Glycine max* L. Merr.) were used as the source of NR for these studies. Leaves from corn seedlings, variety "Oh43 × B14", were harvested after 4 to 5 hr of illumination, and NR was extracted and assayed by methods similar to those described by Hageman and Hucklesby (3). The exceptions were that the fresh leaf tissue was ground in a TenBroeck homogenizer in 10 volumes of extraction medium. Standard (reference) assays, containing 50 μmoles of potassium phosphate (pH 7.5), 20 μmoles of KNO_3, 0.8 μmole of NADH, and 0.2 ml of the crude extract in a final volume of 4 ml, were incubated 15 min at 30 C. Blank assays were identical except NADH was omitted. The reactions were terminated by boiling for 2 min, and NO_2^- color was developed by adding an equal volume of a 1:1 mixture of the color reagents (3).

Fresh soybean leaf discs were frozen with liquid N_2 in a precooled mortar. After evaporation of the liquid N_2, the tissue was rapidly ground to a powder (approximately 15 sec), while still in a frozen state. The extraction medium (twenty volumes of 25 mM potassium phosphate (pH 7.8), 1 mM cysteine, 5 mM KNO_3, 5 mM EDTA, and 25 μM FAD) was added and allowed to freeze in the mortar. The tissue was suspended with additional grinding as the mixture thawed, using the shearing action of the ice crystals to aid in grinding. Upon complete liquefaction, the solution was transferred and centrifuged at 2 to 3 C (15 min at 20,000*g*). The supernatant was used as the enzyme source. The standard (reference) assays contained 30 μmoles

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[3] Abbreviations: NR: nitrate reductase; PMS: phenazine methosulfate; FAD: flavin adenine dinucleotide.

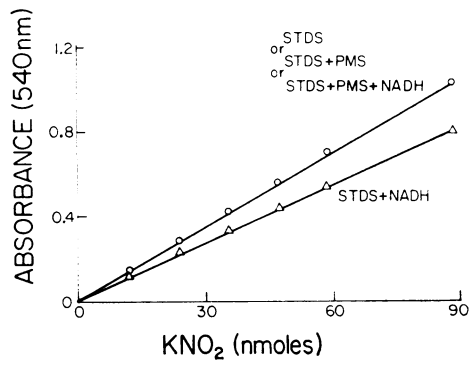

FIG. 1. Effect of NADH and PMS on color development of nitrite standards (STDS). The amounts of PMS and NADH added were 30 and 400 nmoles, respectively. Assays were incubated for 20 min. Color reagents were added 20 min after addition of PMS. Two ml of color reagents were added to all tubes to bring final volume to 4 ml.

of potassium phosphate (pH 6.5), 20 μmoles of KNO_3, 0.5 μmole of NADH, 1.2 nmoles of FAD, and 0.1 ml of crude extract in a final volume of 2 ml. Reactions (15 min, 35 C) were terminated by adding in sequence 1 ml of each of the color reagents (3). The *in vivo* assay with which the *in vitro* assays were compared was conducted as previously described (4), with the following modifications. The assay volume was 10 ml and contained 0.1 M potassium phosphate buffer (pH 7.5), 50 mM KNO_3, and 1% (v/v) *n*-propyl alcohol.

The following postcolor development procedure modifications were tested with the corn and soybean nitrate reductase assays. Reactions were terminated either by boiling (2 to 3 min) and cooling to 30 C (PMS is ineffective in hot reaction mixes), by zinc acetate addition (50 μmoles/ml of reaction mix), or by boiling followed by zinc acetate addition. Reaction terminations involving zinc acetate addition (or boiling, with the soybean assays) were centrifuged (1,000*g* for 10 min) to remove the resulting precipitate. Nitrite color development was determined directly, following the respective termination treatments, or after PMS addition (15 nmoles/ml of reaction mix) to oxidize the excess NADH. Phenazine methosulfate was allowed to react 20 min before color development. In all instances appropriate corrections were made for differences in volume of reaction mixtures used in color development.

RESULTS AND DISCUSSION

Elimination of NADH Interference by PMS (Nitrite Standards). Phenazine methosulfate (15 nmoles/ml of reaction mix) does not interfere with the NO_2^- color development and permits complete recovery of NO_2^- in the presence of 400 nmoles of NADH (Fig. 1). Higher concentrations of PMS interfere with color development. In the absence of PMS, the detrimental effect of the constant amount of NADH on NO_2^- color development was a constant proportion of the NO_2^- concentration.

Elimination of NADH Interference by PMS (Nitrate Reductase Assay). The addition of PMS to the assay medium after incubation eliminated the NADH interference with nitrite color development over a wide range of NADH concentrations (Fig. 2). Phenazine methosulfate had no effect at NADH concentrations up to 400 nmoles/4 ml of reaction mix, presumably because this much NADH was oxidized during the incubation period.

Effects of PMS and Zinc Acetate on Apparent Nitrate Reductase Activity and Recovery of Added Nitrite—Extracts from Corn. Phenazine methosulfate and zinc acetate were tested, separately and in combination, for ability to eliminate NADH interference of NO_2^- recovery from standards and nitrate reductase assays (Table I). The standard 15 min enzyme assay, a zero time enzyme blank, and a reagent blank were tested with and without added NO_2^-. Highest apparent nitrate reductase activity of the corn extract, without added NO_2^-, was obtained when both PMS and zinc acetate treatments were used (Table I). Phenazine methosulfate was more effective than zinc acetate, when each was used separately. As previously observed, zinc acetate enhances apparent nitrate reductase activity, although it removes only a small portion of the residual NADH from the assay medium (14 and unpublished data). With these extracts from young corn seedlings, PMS was also more effective than zinc acetate in enhancing the recovery of the added nitrite. Regarding the recovery of added NO_2^-, the NADH inhibition of color development was about 30% when no enzyme, PMS, or zinc acetate was added. When the extract was present, color development was inhibited about 10% in minus NADH assays, indicating a negative effect of the extract on color development. Neither addition of zinc acetate nor PMS, whether added separately or in combination, seemed to abolish this "extract factor" effect completely. In other experiments with extracts from older corn tissue, zinc acetate treatments were more effective than PMS in overcoming the adverse extract effect, but not the NADH interference.

Phenazine methosulfate was completely effective in counteracting the NADH inhibition on the nitrite assay, based upon recovery of added NO_2^-. Zinc acetate, as expected (14), did not completely overcome the inhibition. The presence of zinc acetate, however, did not interfere with the ability of PMS to oxidize NADH, and analysis of variance revealed that the effects of PMS and zinc acetate treatments on color development were independent.

Effects of PMS and Zinc Acetate on Apparent Nitrate Reductase Activity—Extracts from Soybeans. The ability of zinc acetate, zinc acetate + PMS, boiling, or boiling + PMS treatments to oxidize or remove NADH and extract factor(s) that interfere with NO_2^- color development was also determined with extracts from soybean leaves. Highest apparent nitrate reductase activity was obtained when PMS treatment followed either zinc acetate or boiling (Table II). While both boiling and zinc acetate, in conjunction with PMS, seemed to be equally effective, the standard error for the zinc acetate + PMS treatment was consistently smaller. Zinc acetate without PMS gave greater recoveries of NO_2^- than boiling without PMS.

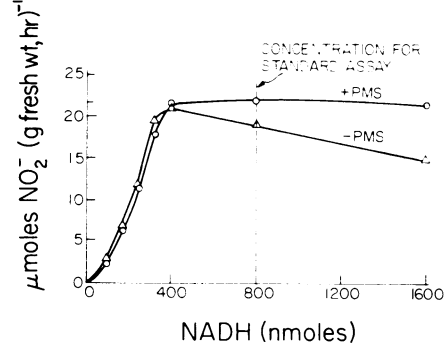

FIG. 2. Effect of PMS on apparent nitrate reductase activity of corn assayed with variable NADH concentrations. Assay procedure described in "Materials and Methods," except for variable NADH concentrations. All reactions were stopped by boiling. Reaction volumes were 4 ml to allow subaliquoting.

Table I. *Effectiveness of PMS in Eliminating NADH Interference in Assays of Nitrate Reductase Activity of Corn Tissue and in the Recovery of Nitrite Added to the Reaction Mixture, With and Without Removal of Protein by Zinc Acetate*

Zinc Acetate	PMS	Time of Reaction	Amount Extract[1]	Enzyme Activity[2] (without NO_2^-)	Recovery of Added NO_2^- (35.25 nmoles/assay)			
					−NADH		+NADH	
		min	*ml*	*nmoles/tube*	*nmoles*	%	*nmoles*	%
		...	0	...	35.1 ± 0.5[3]	100	24.7 ± 0.5[3]	70
−	−	0	0.2	...	31.9 ± 0.7	90	23.4 ± 0.5	66
		15	0.2	39.4	31.9 ± 0.6	90	27.6 ± 0.6	78
		...	0	...	36.9 ± 0.7	105	35.9 ± 0.5	102
−	+	0	0.2	...	32.4 ± 1.5	92	31.1 ± 1.2	88
		15	0.2	51.1	32.4 ± 0.8	92	34.9 ± 1.2	99
		...	0	...	35.1 ± 0.7	100	29.0 ± 1.5	82
+	−	0	0.2	...	32.7 ± 0.9	93	27.6 ± 0.7	78
		15	0.2	45.4	32.7 ± 0.7	93	27.4 ± 1.0	78
		...	0	...	34.6 ± 0.6	98	35.8 ± 0.5	102
+	+	0	0.2	...	32.8 ± 1.5	93	33.3 ± 2.0	94
		15	0.2	54.3	33.0 ± 0.7	94	32.4 ± 0.7	92
LSD 0.05				3.3				

[1] Enzyme extract prepared from corn leaves contained approximately 1 mg protein/ml.
[2] Details of experiment are given in the text.
[3] Mean ± SE of the mean.

Table II. *Removal of NADH and Other Factor(s) that Interfere with NO_2^- Color Development in the in vitro Nitrate Reductase Assay from Soybeans*

Assay[1]	Experiment I		Experiment II			
	Growth chamber	Greenhouse	Growth chamber		Greenhouse	
	2nd[2]	3rd	1st	3rd	3rd	5th
	μmole NO_2^- (*g fresh wt, hr*)$^{-1}$ ± SE *of mean*					
In vitro precolor treatment						
None (Standard assay)	25.2 ± 2.5	18.6 ± 0.5	3.0 ± 0.4	21.9 ± 1.4	23.3 ± 1.5	21.4 ± 0.5
Zn acetate	38.2 ± 1.3	30.5 ± 1.6	6.4 ± 0.4	34.9 ± 1.5	36.1 ± 1.0	42.6 ± 0.9
Boiling	32.6 ± 4.2	27.1 ± 0.3	3.8 ± 0.6	28.1 ± 3.5	25.4 ± 1.3	31.0 ± 3.9
Zn acetate + PMS	48.3 ± 1.6	43.7 ± 0.4	10.8 ± 0.3	44.4 ± 0.4	43.7 ± 0.5	47.2 ± 0.3
Boiling + PMS	47.7 ± 1.7	42.7 ± 1.8	8.1 ± 1.1	42.8 ± 0.9	46.2 ± 0.6	47.4 ± 1.5
In vivo	27.9 ± 3.6	20.9 ± 0.5	11.7 ± 1.8	29.5 ± 0.8	20.0 ± 1.2	25.9 ± 2.7

[1] Assay mixtures were comparable to those described in "Materials and Methods," except that a final volume of 4 ml was used. The larger volume permitted subaliquoting to test the various color development treatments.
[2] Trifoliolate positions numbered from bottom to top of plant. The uppermost fully expanded leaf was sampled in all experiments, and a lower leaf was included in Exp. II for comparison of leaf age.

In addition to the residual NADH, the soybean extract seems to contain more of the factor(s) that interfere with color development than the corn extract (Tables I and II). The 30 to 70% increase in apparent activity from zinc acetate or boiling treatments (without PMS) over normal control (Table II) is greater than can be accounted for by removal of NADH; especially in view of the additional enhancement of color development from subsequent addition of PMS.

Except for the oldest tissue assayed (first trifoliolate, experiment II, Table II), the *in vitro* activity, with zinc acetate and PMS treatments, was 1.5- to 2.2-fold that of the *in vivo* activity. While the extraction and postassay techniques are a marked improvement over those previously published (13), additional improvements are still needed to provide a valid *in vitro* estimate of nitrate reductase activity in mature soybean leaves. The reasons for this statement are: that tissues of species that have no or low levels of inhibitors and interfering factors consistently show higher (2- to 5-fold) *in vitro* than *in vivo* activity (1); and that higher *in vitro* than *in vivo* activity is expected, because saturating levels of NADH are present in the *in vitro* assays, which may not be the case in the *in vivo* assay.

Elimination of NADH Interference with Nitrite Recovery by Charcoal. The effectiveness of activated charcoal in removing NADH from nitrite standards before color development was tested as described by Stulen (14). Charcoal (Norit "A", Fisher Scientific Co., Fairlawn, N. J.) was effective in removing NADH from the assay media, although the use of charcoal caused other problems. When charcoal was added to nitrite standards without added NADH, recoveries were lower than theoretical. Evidence was obtained that some of the NO_2^- was bound to the charcoal and could be removed by washing. When charcoal was added to nitrite standards with NADH, recoveries were higher than theoretical. The cause for this was not determined. The percentage of recovery was variable and was affected by source of charcoal and amount of charcoal added.

In addition, the charcoal treatment is a relatively laborious procedure compared to PMS addition.

General Discussion. The postassay treatments improved the measured *in vitro* nitrate reductase activity of both corn and soybeans, the latter showing greater improvement. The procedure used with corn extracts, boiling to stop the reaction followed by zinc acetate and PMS additions, is effective. These procedures can be further simplified, as demonstrated with the soybean work, where boiling was eliminated, and zinc acetate was used to stop the reaction. Subsequent study has shown that with corn extracts the PMS can be added immediately after zinc acetate addition, before centrifugation, with equivalent results (data not shown).

The marked effect of zinc acetate with soybean extracts is attributed to removal of extract factor(s) that interfere with NO_2^- color development. Although zinc acetate was not markedly beneficial with the Oh43 × B14 corn seedlings used in this study, subsequent work has indicated that zinc acetate is more beneficial with other corn genotypes and with older tissue (unpublished, R. H. Hageman). Therefore, both zinc acetate and PMS postassay treatments are recommended when comparisons are made among genotypes with diverse background or with markedly different levels of nitrate reductase activity. It is suggested that the use of either the *in vitro* or *in vivo* assay as an estimate of the *in situ* contribution of reduced nitrogen for a species or genotype be adopted only after evaluation against actual accumulation of reduced nitrogen (1).

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