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Comparative Studies on Nitrate Reductase in *Agrostemma githago* Induced by Nitrate and Benzyladenine[1,2]

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ABSTRACT

NADH-nitrate reductase activity in excised embryos of *Agrostemma githago* develops in response to nitrate as well as benzyladenine. Induction of nitrate reductase by benzyladenine was much more susceptible to inhibition by a mixture of amino acid analogues and by cordycepin than induction by nitrate. In contrast, only induction of nitrate-nitrate reductase was decreased by chloramphenicol.

NADH-cytochrome *c* reductase and reduced flavin mononucleotide-nitrate reductase activities were found to be associated with NADH-nitrate reductase and were induced by both nitrate and benzyladenine. When a partially purified enzyme sample was centrifuged in a linear 5 to 20% sucrose density gradient, a minor and a major band of NADH-cytochrome *c* reductase activity were observed. NADH-nitrate reductase cosedimented with the major band.

The characteristics of nitrate-nitrate reductase and benzyladenine-nitrate reductase were compared by four methods but no differences could be detected: (*a*) Both enzymes sedimented with the same velocity during sucrose density gradient centrifugation. (*b*) Their distribution among fractions obtained by differential precipitation with $(NH_4)_2SO_4$ was identical. (*c*) The elution profile of nitrate-nitrate reductase and benzyladenine-nitrate reductase after chromatography on diethylaminoethyl Sephadex A-25 columns showed no significant difference. (*d*) On polyacrylamide gel, the electrophoretic migration of the two enzymes was also identical.

NR[3] in excised embryos of *Agrostemma githago* can be induced by incubating them in media containing either nitrate or a cytokinin (1–3). The physiological significance of the hormonal induction of NR, as opposed to substrate induction, is not known at this point. Accumulating evidence indicates, however, that cytokinins are acting in a manner different from nitrate in inducing NR. BA loses its effectiveness as an inducer of NR after 64 hr of seed germination, whereas the effect of nitrate is not time-limited (4). The level of NR decreases upon removal of nitrate or BA; NR can be induced a second time by BA but not by nitrate (2). The development of NR activity by nitrate and cytokinin exhibits differential sensitivity toward a variety of metabolic inhibitors (2–4). Last, the effects of nitrate and BA at optimal concentrations are additive with regard to induction of NR (2). Experiments described below were designed to characterize further hormone and substrate-induced NR.

MATERIALS AND METHODS

Plant Material. Excised embryos of *Agrostemma githago* L. were prepared according to Kende *et al.* (2).

Induction of Enzyme Activities. NR and associated enzyme activities were induced as described by Kende *et al.* (2). NR activities induced by nitrate and BA are designated as nitrate-NR and BA-NR, respectively. Metabolic inhibitors included in the induction medium were filter-sterilized and were present during the entire period of induction.

Enzyme Preparations. Crude homogenates were prepared as described by Kende and Shen (3) and were used directly as an enzyme source or as a starting material for partial purification of the respective enzymes. For fractionation with $(NH_4)_2SO_4$, the desired amount of a saturated $(NH_4)_2SO_4$ solution (pH 7.5) was added slowly and under continuous stirring to the enzyme preparation. The mixture was left standing in an ice bath for 30 min, after which time the precipitate was collected by centrifugation at 15,000*g* for 20 min at 2 C.

Enzyme Assays. Catalase was assayed as described by Luck (7), except that 0.25 ml of H_2O_2 (30% w/v) was diluted with 100 ml of 67 mM phosphate buffer (pH 7.0), instead of 0.16 ml of H_2O_2.

$FMNH_2$-NR was assayed according to Wray and Filner (14). The step of flushing the reaction mixture with N_2 gas was omitted as it was found to be unnecessary.

NADH-NR activity was measured according to Sanderson and Cocking (11), as adapted by Wray and Filner (14).

NADH-Cyt *c* reductase was assayed by following spectrophotometrically the rate of reduction of Cyt *c*. The reaction mixture contained 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.5), 0.1 ml of 10 mM KCN, 0.05 ml of 2% (w/v) Cyt *c* (horse heart, Sigma), 0.05 ml of 8 mM NADH and the enzyme preparation to give a final volume of 1.0 ml. The reaction was started by adding NADH; controls were conducted without NADH. The change in extinction at 550 nm was followed with a Gilford spectrophotometer equipped with a linear chart recorder. The activity was expressed as μmole Cyt *c* reduced/hr and calculated using 21 mM^{-1} cm^{-1} as ϵ_{550} (reduced-oxidized) for Cyt *c* (9).

Sucrose Density Gradient Centrifugation. The procedure of Martin and Ames (8) as modified by Wray and Filner (14) was

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[2] Dedicated to the memory of Ernest Sondheimer.

[3] Abbreviations: NR: nitrate reductase; BA: benzyladenine; NiR: nitrite reductase; $FMNH_2$: reduced flavin mononucleotide; FAD: flavin adenine dinucleotide; DTT: dithiothreitol.

followed. A crude cell-free extract was prepared from 30 embryos incubated for 7 hr with either 50 mM KNO_3 or 3 μM BA, and purified partially by $(NH_4)_2SO_4$ precipitation. The precipitate obtained at 25 to 50% saturation was redissolved in 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 10 μM FAD, 1 mM EDTA, and 1 mM L-cysteine. Of this, 0.1 ml was layered on top of a 4.8-ml 5 to 20% (w/v) sucrose gradient. The sucrose solutions were prepared in 0.1 M potassium phosphate buffer (pH 7.5) with 10 μM FAD and 1 mM EDTA. The centrifugation was carried out at 45,000 rpm for 11 hr at 2 C, using a SW 65 rotor and a Beckman L-2 ultracentrifuge. After centrifugation, the centrifuge tubes were punctured and 21 fractions of 20 drops were collected. The sucrose concentration was determined with a Bausch and Lomb Abbe-32 refractometer.

Stepwise Fractionation by Differential Precipitation with $(NH_4)_2SO_4$. A protein fraction precipitated between 20 and 45% saturation of $(NH_4)_2SO_4$ was prepared from a crude extract of 50 embryos incubated for 6 hr in either 50 mM KNO_3 or 3 μM BA. The precipitate was redissolved in 3 ml of the original homogenizing medium, and 2 ml of saturated $(NH_4)_2SO_4$ solution adjusted to pH 7.5 were slowly added to it. The mixture was left standing in an ice bath for 30 min and was subsequently centrifuged at 15,000*g* for 15 min at 2 C. The resultant supernatant solution contained a protein fraction which precipitated at 45% saturation of $(NH_4)_2SO_4$ but not at 40% saturation. An aliquot from this supernatant was assayed for NADH-NR activity without further purification. The remaining pellet, which was insoluble in 40% saturated $(NH_4)_2SO_4$, was sequentially extracted with $(NH_4)_2SO_4$ solution of 35, 30, 25, and 20% saturation. The NR activity of each extracted fraction (40–35%, 35–30%, etc.) was assayed directly. The protein concentration in each fraction was determined by the method of Lowry *et al.* (6) using BSA as a standard.

Fractionation on DEAE Sephadex A-25 Columns. A protein fraction precipitated with $(NH_4)_2SO_4$ between 25 and 50% saturation was prepared from a crude extract of 50 embryos incubated for 6 hr with either 50 mM KNO_3 or 3 μM BA. The precipitate was redissolved in 2 ml of a buffer solution consisting of 25 mM potassium phosphate, pH 7.5, 1 mM EDTA, and 1 mM L-cysteine (buffer A). It was desalted by passage through a small Sephadex G-25 column (1.2 × 8 cm). The eluate (6 ml) containing the protein was clarified immediately before chromatography by centrifugation at 27,000*g* for 20 min. Equal portions (2 ml each) and activities of nitrate-NR and BA-NR were mixed and loaded onto a column of DEAE Sephadex A-25 (0.9 × 18.0 cm) equilibrated with buffer A, followed by elution with a linear KCl gradient; the mixing chamber contained 100 ml of buffer A, and the reservoir 100 ml of 0.3 M KCl in buffer A. Finally, the column was eluted with 100 ml of 1 M KCl in buffer A. The eluate was collected in 3.2-ml fractions at a flow rate of 40 ml/hr. KCl concentrations in the eluate were determined with a Bausch and Lomb Abbe-32 refractometer.

Polyacrylamide Gel Electrophoresis. The combined $(NH_4)_2SO_4$ extracts containing the bulk of the NR (30–25% and 25–20%, Table II) were pooled and the protein was reprecipitated with $(NH_4)_2SO_4$ solution at 30% saturation. This precipitate was redissolved in 0.2 ml buffer consisting of 0.1 M potassium phosphate, pH 7.5, 50 mM glycine, 1 mM EDTA, 1 mM DTT, and 1 μM FAD. This buffer was used during electrophoresis.

Gels (0.7 × 9.0 cm) were made from 4% (w/v) acrylamide, 0.135% (w/v) methylene bisacrylamide, 0.06% (v/v) N,N,N′-tetramethylethylene diamine, 0.14% (w/v) ammonium persulfate, 1 mM DTT, 1 mM EDTA, and 1 μM FAD in 0.1 M potassium phosphate buffer, pH 7.5. The gels were pre-run with the above buffer for 2 hr, whereupon 20 μl of the enzyme sample were applied to the gel. Electrophoresis was performed at 8 mA/gel for 11 hr at 4 C. At this time, the marker dye, bromophenol blue, had reached the end of the gel. The gels were cut into 4-mm sections and NADH-NR was located by assaying each slice in a mixture of 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.5), 0.1 ml of 0.1 M KNO_3, and 0.1 ml of 1 mM NADH. The incubation was carried out at 25 C for 1 hr. The production of nitrite was determined as in the standard assay.

Each experiment was repeated at least three times with similar results.

Table I. *Effect of Metabolic Inhibitors on Nitrate-NR and BA-NR*

Four embryos were incubated per 25-ml Erlenmeyer flask containing 1 ml medium at 30 C. BA was given at a concentration of 3 μM and nitrate at 50 mM. Incubation time was 6 hr for the amino acid analogue experiment, 5 hr for the chloramphenicol experiment, and 4 hr for the cordycepin experiment. The mixture of amino acid analogues consisted of DL-7-azatryptophan, L-canavanine, L-ethionine, DL-2-fluorophenylalanine, and thioproline, each at 0.1 mM. A mixture of the corresponding amino acids had no significant effect. The concentrations of chloramphenicol and cordycepin were 2.5 mg/ml and 0.1 mM, respectively.

Treatment	NADH-NR Activity		Inhibition
	Control	+Amino acid analogues	
	nmole NO_2^- formed/hr·embryo		%
H_2O	61	33	
BA	104 (43)[1]	30 (−3)	100
KNO_3	166 (105)	80 (47)	55
	Control	+ Chloramphenicol	
H_2O	62	54	
BA	82 (20)	76 (22)	0
KNO_3	122 (60)	89 (35)	42
	Control	+ Cordycepin	
H_2O	51	54	
BA	137 (86)	60 (6)	93
KNO_3	153 (102)	104 (50)	51

[1] Numbers in parentheses indicate NR activities minus background in H_2O control. Per cent inhibition is based on these values.

RESULTS

Effect of Amino Acid Analogues, Chloramphenicol, and Cordycepin on the Development of Nitrate-NR and BA-NR. Several metabolic inhibitors showed differential effects on the induction of nitrate-NR and BA-NR (3). A series of additional inhibitors, namely, chloramphenicol, cordycepin, and a mixture of amino acid analogues, were tested for their ability to inhibit induction of NR by BA and nitrate. The results are summarized in Table I. A mixture of amino acid analogues inhibited the induction of NR by BA completely but the induction of nitrate-NR by only 55%. A mixture of the corresponding amino acids had no significant effect on the induction of

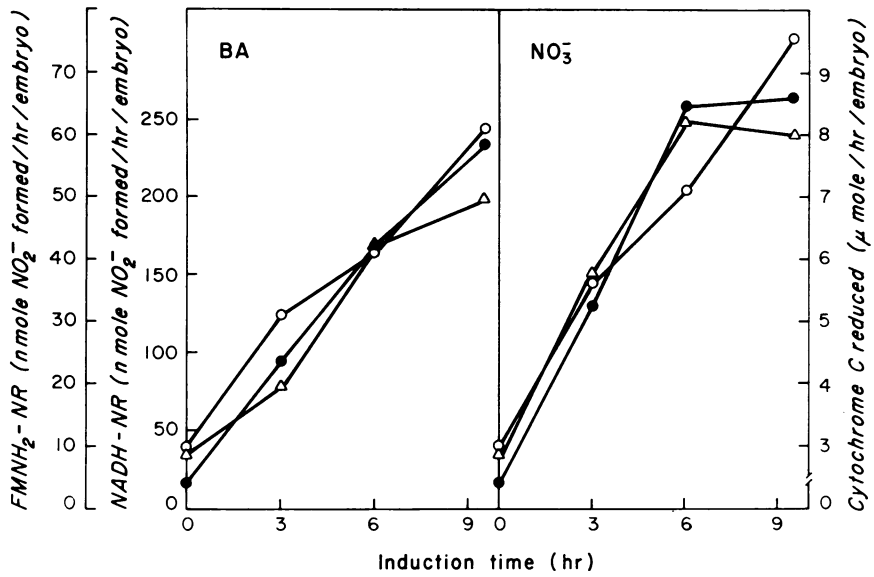

FIG. 1. Time-course study on the development of NADH-Cyt *c* reductase (○) and $FMNH_2$-NR (●) activities associated with NADH-NR (△) activity. Four embryos were incubated per flask for the times indicated. At the end of the induction, the embryos from two identically treated flasks were extracted in a total of 2.5 ml of extraction medium. NADH-Cyt *c* reductase activity was assayed immediately after extraction as the enzyme activity was relatively unstable.

either NR activity. Chloramphenicol (2.5 mg/ml) inhibited the induction of nitrate-NR only; at the concentration used to maintain sterility of the incubation media (20 μg/ml), chloramphenicol did not interfere with the development of either enzyme activity. Cordycepin inhibited induction of BA-NR to a greater extent than that of nitrate-NR.

Development of NADH-Cyt *c* Reductase and $FMNH_2$-NR Activities. The original finding of Nicholas and Nason (10) that NR in *Neurospora* also exhibited NADH-Cyt *c* reductase (diaphorase) activity has been confirmed with NR from higher plants, such as spinach (5), corn (12), and barley (14). In these three cases it has also been shown that $FMNH_2$, in addition to NADH, can serve as electron donor for the reduction of nitrate.

In embryos of *A. githago* NADH-Cyt *c* reductase activity was also found to be associated with a NR which could utilize NADH and $FMNH_2$ as cofactors. BA and nitrate enhanced the activity of both $FMNH_2$-NR and NADH-Cyt *c* reductase (Fig. 1). The activities of the two enzymes increased parallel with that of NADH-NR, for 6 hr in the case of nitrate-NR and at least for 9 hr in that of BA-NR.

Sucrose Density Gradient Centrifugation. Wray and Filner (14) found three bands of NADH-Cyt *c* reductase activity when an enzyme preparation from barley shoots was centrifuged in a sucrose density gradient. Of the three, one was associated with NR, and its activity increased in response to nitrate. In *A. githago,* sucrose gradient centrifugation of a partially purified enzyme preparation yielded two bands of NADH-cyt *c* reductase activity; a minor one, which sedimented to the bottom of the tube, and a major one, which was lighter than the added catalase marker (Fig. 2). NADH-NR activity was associated with the major band. There was no difference in the sedimentation velocity of the enzyme preparations obtained from nitrate and BA-induced embryos.

Distribution of NR Activity in Fractions Obtained by Differential Precipitation with $(NH_4)_2SO_4$. All NR activity from embryos of *A. githago* precipitated between 20 and 45% saturation of $(NH_4)_2SO_4$. This pellet was sequentially extracted

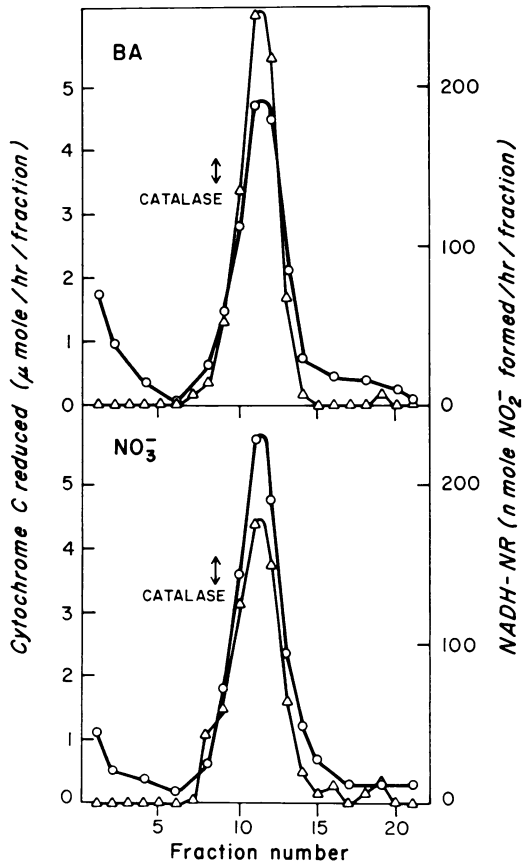

FIG. 2. Distribution of NADH-Cyt *c* reductase activity (○) and NADH-NR activity (△) after centrifugation on a sucrose density gradient. A partially purified enzyme sample was layered on top of a linear 5 to 20% (w/v) sucrose gradient. The centrifugation was performed for 11 hr at 2 C and at 45,000 rpm using a SW 65 rotor in a Beckman L-2 ultracentrifuge.

Table II. *Distribution of NADH-NR Activity in Fractions Obtained by Differential Precipitation with $(NH_4)_2SO_4$*

Fifty embryos each were induced with 50 mM nitrate and 3 μM BA for 6 hr and extracted. The precipitate obtained at 20 to 45% saturation of $(NH_4)_2SO_4$ was redissolved in buffer, an aliquot was assayed for NR, and precipitation was repeated at 40% saturation of $(NH_4)_2SO_4$. The resulting supernatant was designated as the 45 to 40% extract. The pellet was further sequentially extracted with 35, 30, 25, and 20% saturated solutions of $(NH_4)_2SO_4$. The respective supernatant solutions were assayed for NR and their protein content was determined.

Sample	Fraction	Specific Activity	Total Activity	Recovery
		μmole NO_2^- formed/hr·mg protein	μmole NO_2^- formed/hr·fraction	%
BA-NR	20–45% precipitate	0.287	8.082	100
	45–40% extract	0.026	0.071	0.9
	40–35% extract	0.062	0.108	1.4
	35–30% extract	0.171	0.293	3.6
	30–25% extract	1.036	3.647	45.2
	25–20% extract	0.519	1.830	22.6
Nitrate-NR	20–45% precipitate	0.274	8.045	100
	45–40% extract	0.026	0.071	0.9
	40–35% extract	0.062	0.103	1.3
	35–30% extract	0.213	0.394	4.9
	30–25% extract	1.099	4.658	57.9
	25–20% extract	0.455	1.434	17.8

with decreasing concentrations of $(NH_4)_2SO_4$ solutions. The bulk of the NR activity was found in the 30 to 25% $(NH_4)_2SO_4$ extract (Table II). There was no significant difference in the distribution pattern of NR activity between nitrate-NR and BA-NR.

DEAE Sephadex A-25 Ion-Exchange Chromatography. An attempt was made to separate nitrate-NR activity from BA-NR activity by ion-exchange chromatography on DEAE Sephadex A-25 columns. When nitrate-NR and BA-NR were chromatographed separately, they were eluted by KCl concentrations varying from 0.09 to 0.15 M KCl. When equal amounts of BA-NR and nitrate-NR were mixed and chromatographed together, we always obtained only one peak of activity (Fig. 3).

Polyacrylamide Gel Electrophoresis. Preliminary electrophoresis experiments indicated that nitrate-NR and BA-NR had the same mobility on starch gels (Shen, unpublished results). Electrophoresis of NR on polyacrylamide gels confirmed these findings (Fig. 4). It has not been determined whether or not the NR activity observed at the top of the gel was due to a different NR from the one that migrated. However, it appeared that part of the protein sample had precipitated at the top of the gel and longer periods of electrophoresis did not cause migration of NR from this precipitate.

DISCUSSION

The metabolic inhibitors used in this study add to an accumulating number of compounds which have differential effects on the development of nitrate-NR and BA-NR. A mixture of amino acid analogues inhibited induction of BA-NR much more than induction of nitrate-NR, behaving in this respect like two inhibitors of protein synthesis, cycloheximide and puromycin (3). Since amino acid analogues, at least initially, cause the formation of nonfunctional enzyme molecules rather than inhibiting protein synthesis, the present results may indicate that nitrate-NR and BA-NR have either a different amino acid composition, or that nitrate-NR and BA-NR are derived from separate amino acid pools. The inhibitory effect of chloramphenicol, which is limited to nitrate-NR only, seems to support the latter assumption. However, chloramphenicol at such a high concentration can cause damage to the normal functions of plastids; thus, the inhibition of nitrate-NR induction by this antimetabolite does not necessarily reflect a direct effect (13). The results with cordycepin, an inhibitor of RNA synthesis, appear to contradict the earlier finding (3) that actinomycin D had no effect on the induction of either NR activity. However, our knowledge on the precise mechanism of action of these inhibitors in higher plants is limited, and it is

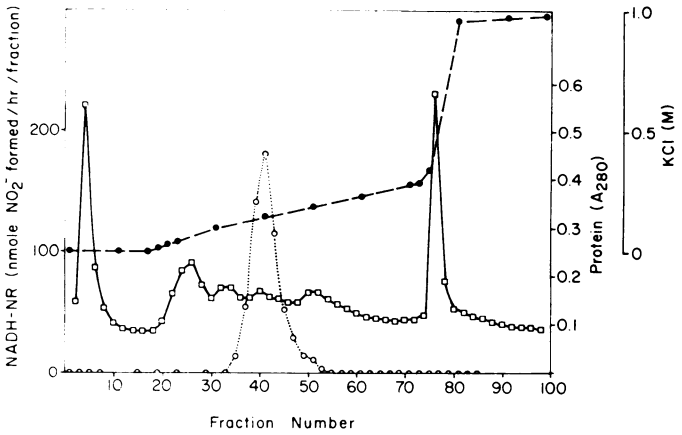

FIG. 3. Fractionation of a mixed sample of nitrate-NR and BA-NR on a DEAE Sephadex A-25 column. The BA-NR had an activity of 1.43 μmole of NO_2^- formed hr^{-1} ml^{-1}, the nitrate-NR an activity of 1.37 μmole NO_2^- formed hr^{-1} ml^{-1}. □: Absorbance at 280 nm; ○: NADH-NR activity; ●: KCl concentration.

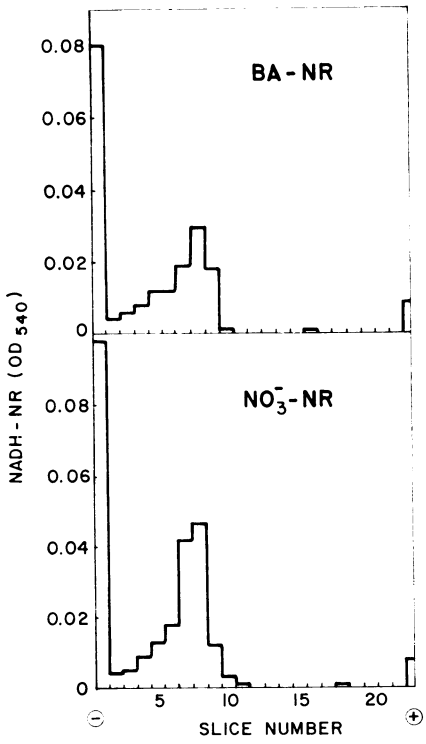

FIG. 4. Polyacrylamide gel electrophoresis of nitrate-NR and BA-NR. Electrophoresis was performed for 11 hr at 8 mamp per gel. The concentration of acrylamide was 4%. Immediately after electrophoresis, a gel was sliced into 4 mm-sections and each slice was assayed for NADH-NR activity.

therefore difficult to draw meaningful conclusions from inhibitor studies alone. It is significant that the induction of nitrate-NR and BA-NR is differentially affected by a number of metabolic inhibitors. This observation was unexpected as BA and nitrate both appear to induce *de novo* synthesis of at least part of the NR. This was concluded from density labeling experiments showing that nitrate-NR and BA-NR incorporated the same amount of deuterium during the induction period (3).

The results illustrated in Figures 1 and 2 tend to rule out the possibility that BA may affect only a part of the NR complex, showing that the activities of both of its components, NADH-Cyt *c* reductase and $FMNH_2$-NR, were enhanced by BA.

Attempts to find differences in physico-chemical properties of nitrate-NR and BA-NR have been unsuccessful. The results show that the two NR preparations behaved very similarly when subjected to differential precipitation with $(NH_4)_2SO_4$ (Table II), to DEAE Sephadex A-25 ion-exchange column chromatography (Fig. 3), and to polyacrylamide gel electrophoresis (Fig. 4). Thus, the question of whether BA-NR and nitrate-NR are different remains still unanswered.

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