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# ELECTRON DONORS AND INHIBITORS OF NITRATE REDUCTASE FROM *CYANIDIUM CALDARIUM*

CARMELO RIGANO and GIOVANNI ALIOTTA

*Istituto di Botanica dell'Universita di Napoli, Via Foria 223, 80139 Napoli (Italy)*

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## Summary

Studies on nitrate reductase (NAD(P)H:nitrate oxidoreductase EC 1.6.6.2) of *Cyanidium caldarium* revealed that the enzyme is inhibited by excess of electron donor, NADPH, reduced benzylviologen and FMN. Also dithionite, used to reduce benzylviologen and FMN, inactivates nitrate reductase; however, FMN at an optimal concentration and nitrate, added before the dithionite, protect the enzyme against this inactivation.

Cyanide, cyanate and carbamyl phosphate inhibit the enzyme competitively with respect to nitrate, and $K_i$ values are reported. Organic mercurials, 0.1 mM, act preferentially on NADPH activity, whereas $Ag^+$ and $Hg^{2+}$ at the same concentration inactivate 80—90% of the benzylviologen and FMN activities. ADP is very poor inhibitor. Urea 4 M in 2 h destroys 90% of NADPH activity and only 30% of the benzylviologen and FMN activities. The apparent $K_m$ values for NADPH, benzylviologen, FMN and nitrate have been determined.

## Introduction

Studies on the assimilatory nitrate reductase (NAD(P)H:nitrate oxidoreductase EC 1.6.6.2) from different organisms have shown that this enzyme is a molybdoflavoprotein [1,2] with two different enzymatic activities associated: and NAD(P)H dehydrogenase and the nitrate reductase proper activity [3,4]. The presence of NAD(P)H dehydrogenase activity is supported by the fact that purified preparations of nitrate reductase reduce compounds such as ferricyanide or cytochrome *c* utilising pyridine nucleotides as electron donors.

Nitrate reductase utilises for nitrate reduction the reduced forms of pyridine nucleotides, flavins and viologens. However, whereas the NAD(P)H dehydrogenase activity can be selectively destroyed by mercurials and mild heating suppressing all NAD(P)H activity, the same treatments do not affect the nitrate

reductase activity itself which continues to be functional with benzylviologen and FMN [3,4].

For many nitrate reductases from different organisms an excess of electron donor has been shown to be inhibitory [5—8], and, in some organisms this inhibition has been regarded as a regulatory mechanism [5—7]. The nitrate reductase of *Cyanidium* is also inhibited by an excess of reducing equivalents, and this is reported in the present paper. In addition, inhibition by some other inhibitors is considered and discussed.

## Material and Methods

*Cyanidium* was cultivated autotrophically in acidic media at 42°C using nitrate as the sole nitrogen source as previously described [9]. The crude extract, obtained by passing the cells resuspended in buffer 3 mM phosphate (pH 7.5), through a French press and centrifugation at 27 000 × *g*, was centrifuged at high speed (150 000 × *g*) for 2 h and the supernatant, which contains the nitrate reductase activity, treated with $(NH_4)_2SO_4$ to 40% satn. The precipitate, dissolved in 3 mM sodium-phosphate (pH 7.5), plus 1 mM EDTA, and desalted on Sephadex G-25, was used as the enzyme source.

As previously reported [9], nitrate reductase occurs in cell-free extracts of *Cyanidium* grown on nitrate, partially as a latent enzyme. However, the latency of the enzyme is lost upon $(NH_4)_2SO_4$ precipitation and so the preparation of nitrate reductase used here contains enzyme only in the fully active form.

*Enzyme assay*

Nitrate reductase was assayed in systems of 2 ml, containing: extract; 60 mM phosphate buffer (pH 7.5)/benzylviologen or FMN/nitrate/3 mM dithionite. In some instances NADPH was used as electron donor. The concentration of nitrate, benzylviologen, FMN and NADPH varies, and will be indicated in the text. The reaction was usually started, with a few indicated exceptions, with the addition of dithionite, or NADPH, as the last component. After 10 min incubation at 30°C the reaction was stopped by addition of 1 ml of barium acetate 0.15 M and 1 ml of ethanol at 0°C. After vigorous shaking and centrifugation at low speed, 1 ml of the reagent mixture for nitrite was added and the absorbance measured at 540 nm after 10 min. The 1 ml of nitrite reagent mixture was composed of 0.67 ml of sulphanilamide (2% in 3 M HCl) plus 0.33 ml of *N*-(1-naphthyl)ethylenediamine · 2 HCl (0.08% in water).

## Results

*Electron donors*

In the present study we have tested NADPH, benzylviologen and FMN as electron donors for *Cyanidium* nitrate reductase.

It was reported by Hewitt and Nicholas [10] that reduced pyridine nucleotides interfere with color development in the colorimetric estimation of nitrite. To overcome this source of error in the nitrate reductase assay, several methods have been proposed, one of which consists of the precipitation of excess NAD(P)H by barium or zinc acetate plus 5 vol. of ethanol [10]. We have

TABLE I

SPECTROPHOTOMETRIC ASSAY OF NADPH PRECIPITATION BY BARIUM ACETATE AND ETHANOL IN PRESENCE OF PHOSPHATES

The mixture contained in a final volume of 4 ml: 120 μmol Tris·HCl or phosphates buffer (pH 7.5); 1 μmol NADPH; 0.15 mmol barium acetate; 1 ml ethanol. Immediately, the mixture was shaken, centrifugated at low speed and the absorbance estimated at 340 nm.

| Composition of the mixture | Absorbance at 340 nm |
|---|---|
| Tris, NADPH | 1.160 |
| Tris, NADPH, barium acetate | 1.160 |
| Tris, NADPH, barium acetate, ethanol | 1.240 |
| Phosphates, NADPH | 1.150 |
| Phosphates, NADPH, barium acetate | 0.670 |
| Phosphates, NADPH, barium acetate, ethanol | 0.003 |

modified this procedure using only 1 ml of ethanol/4 ml, which is sufficient for immediate precipitation of NADPH in presence of phosphates (Table I). This modified procedure however, does not work with NADH.

Double reciprocal plot shown in Fig. 1 indicates that at high concentration of NADPH inhibition by excess of substrate occurs. The apparent $K_m$ value calculated in the non-inhibitory range of NADPH is of $3.7 \cdot 10^{-5}$ M.

In Figs 2 and 3 the double reciprocal plot of nitrate reductase activity vs benzylviologen and FMN concentration reveals that these two electron donors above 37 and 500 μM, respectively, are strong inhibitors of nitrate reductase.

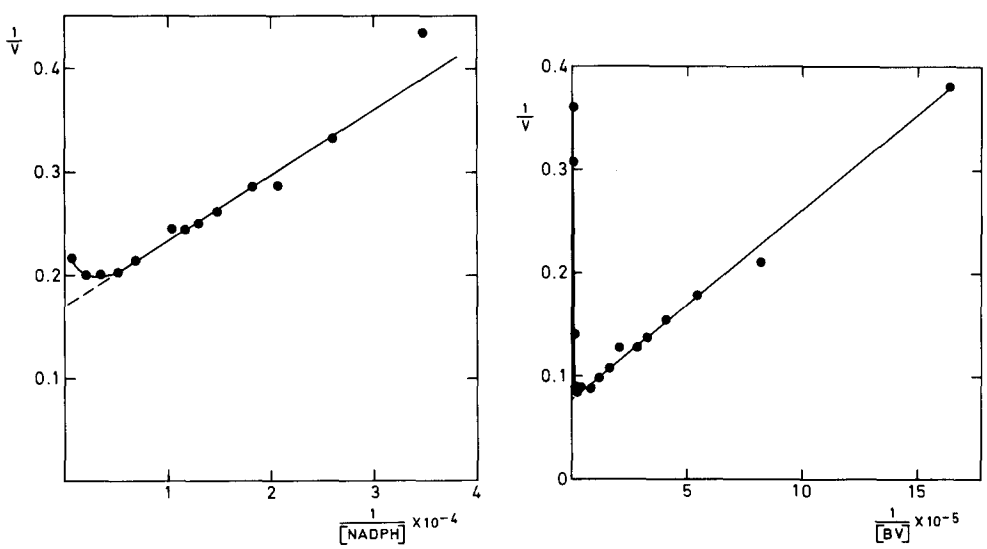


**Fig. 1. Double reciprocal plot of *Cyanidium* nitrate reductase activity vs NADPH concentration at fixed nitrate concentration (25 mM). The activity was determined as reported under Material and Methods. Activity: nmol nitrite produced/min/system.**

**Fig. 2. Double reciprocal plot of *Cyanidium* nitrate reductase activity vs benzylviologen concentration at fixed nitrate 25 mM. Activity as in Fig. 1.**

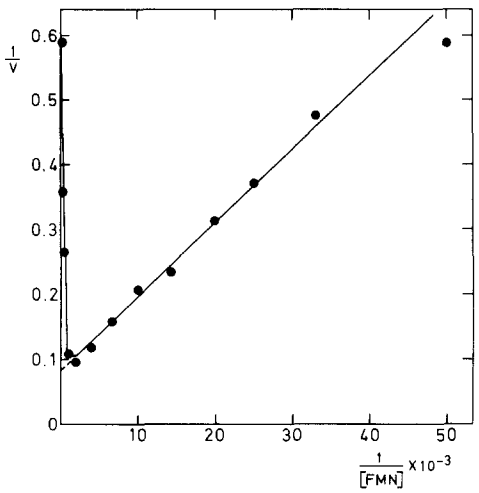


Fig. 3. Double reciprocal plot of *Cyanidium* nitrate reductase activity vs FMN concentration at fixed nitrate 25 mM. Activity as in Fig. 1.

The apparent $K_m$ values calculated in the non inhibitory range are: $2.35 \cdot 10^{-6}$ M for benzylviologen and $1.3 \cdot 10^{-4}$ M for FMN.

*Activity as a function of nitrate concentration*

When reciprocal of nitrate reductase activity is plotted against the reciprocal of nitrate concentration, at a fixed 0.5 mM FMN or 0.2 mM NADPH, straight lines are obtained. The concentration of electron donors chosen supports the maximum of activity of enzyme without inhibitory effect by excess reducing power. The apparent $K_m$ value for nitrate is: $4 \cdot 10^{-4}$ M when FMN is the electron donor and $8.4 \cdot 10^{-5}$ M when NADPH is the reductant.

*Effect of the composition of preincubation mixture on the enzyme activity*

Usually in the nitrate reductase assay the reaction was started by addition of dithionite (or NADPH) as the last component. In a series of experiments we have varied the order of addition of assay mixture components as follow: (1) buffer, enzyme, dithionite, and, after 6 min of preincubation, the reaction started with the addition of nitrate plus benzylviologen or FMN; (2) buffer, enzyme, benzylviologen or FMN, dithionite and, after 6 min, nitrate; (3) buffer, enzyme, nitrate, dithionite and, after 6 min, benzylviologen or FMN. The activity was assayed as a function of the time. Benzylviologen was 0.122 mM and FMN 1 mM. Nitrate concentration varied with the experiments, and concentrations of 25, 6 and 1.5 mM were used.

With benzylviologen as can be seen in Fig. 4, when dithionite is added as the last component, the enzyme activity results linear only during a period of 10—12 min with 25 mM nitrate and of 6—8 min with 6 mM nitrate, after which it tends to decrease. Preincubation with dithionite alone or with dithionite plus benzylviologen inactivates the enzyme which exhibits an activity of 70% with 25 mM nitrate and of 30% with 6 mM nitrate with respect to the control (dithionite added as the last component). By contrast, if the enzyme is prein-

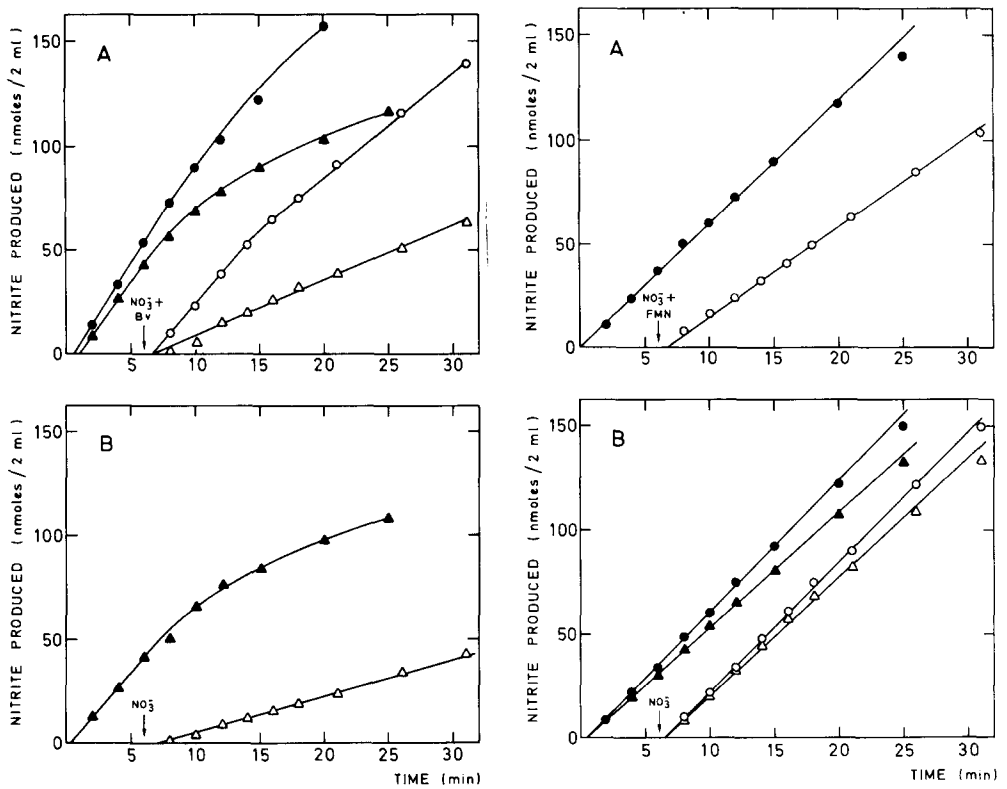


Fig. 4. Effect of preincubation mixture on *Cyanidium* nitrate reductase activity when benzylviologen is the electron donor. The assay mixture was of 22 ml and the final concentration of the components was: nitrate, 25 mM (full and open circles) or 6 mM (full and open triangles); enzyme 0.16 ml; benzylviologen 0.122 mM; dithionite 3 mM; phosphate buffer (pH 7.5), 0.06 M. The activity was followed with the time. Full circles and triangles in A and B: activity starting with the addition of dithionite as the last component. (A) (open symbols). Enzyme preincubated with buffer and dithionite in 20 ml, after which the activity started with the addition of benzylviologen plus nitrate at the time indicated by the arrow. (B) (open symbols). Enzyme preincubated with all components in 21 ml except nitrate which was added as the last component at the time indicated by the arrow.

Fig. 5. Effect of preincubation mixture on *Cyanidium* nitrate reductase activity when FMN is the electron donor. The composition of assay mixture was as indicated in Fig. 4 except that FMN, 1 mM, was used instead of benzylviologen, and that nitrate was 6 mM (full and open circles) or 1.5 mM (full and open triangles). Full symbols in A and B. Activity starting with the addition of dithionite as the last component. (A) (open symbols), enzyme preincubated with all components in 20 ml except FMN and nitrate which were added at the time indicated by the arrow. (B) (open symbols), enzyme preincubated with all components in 21 ml except nitrate added at the time indicated by the arrow.

cubated with dithionite but in presence of nitrate, the reaction starting with the addition of benzylviologen, it exhibits 100% of activity (not shown).

With FMN, the activity is linear with the time also at very low concentration of nitrate (1.5 mM). When the enzyme is preincubated with dithionite alone and then assayed with nitrate 6 mM, it exhibits only 70% of activity (Fig. 5A). By contrast, when the enzyme is preincubated with dithionite plus FMN, it exhibits 100% of activity (Fig. 5B).

These results clearly show that the enzyme is inactivated by contact with dithionite and that nitrate and FMN protect against this inactivation; a large

excess of nitrate and FMN partially reverses it; benzylviologen does not protect against this inhibition and does not reverse it.

Oxidation with air reverses 60—90% the inactivation by dithionite (not shown). In this respect we must point out that the reoxidation is a very delicate procedure which, if no executed properly (for instance by gently shaking or bubbling with air) brings about denaturation of enzyme. We have found that the best procedure is to reoxidize dithionite by dilution.

*Inhibitors of nitrate reductase*

The action of some inhibitors have been tested with nitrate reductase of *Cyanidium*. Carbamyl phosphate, cyanate and cyanide inhibit competitively with respect to nitrate, and similar results were obtained whether the electron donor was NADPH or FMN. The $K_i$ values calculated were: 0.26 $\mu$M cyanide (Fig. 6); 2.8 $\mu$M cyanate, 71 $\mu$M carbamyl phosphate.

The effect of mercurials, heavy metals and urea is shown in Tables II and III. 50 $\mu$M *p*-Chloromercuribenzoate inhibit 100% the NADPH activity and only 10% the benzylviologen and FMN activities. By contrast, $Ag^+$ and $Hg^{2+}$ at the same concentration inhibit 100% the NADPH activity and 60—80% the benzylviologen and FMN activities. Urea 4 M in 2 h inactivates 90% the NADPH activity and only 30% the benzylviologen and FMN activities. These latter are 90% destroyed by urea 6 M in 2 h.

ADP, which is a strong inhibitor of nitrate reductase from *Chlorella fusca*, tomato and spinach, is almost without effect on the enzyme from *Cyanidium* (not shown). Indeed, this nucleotide is without effect also if preincubated with the enzyme in presence of a reductant.

## Discussion

Inhibition by reduced benzylviologen and FMN was described by Pichino-

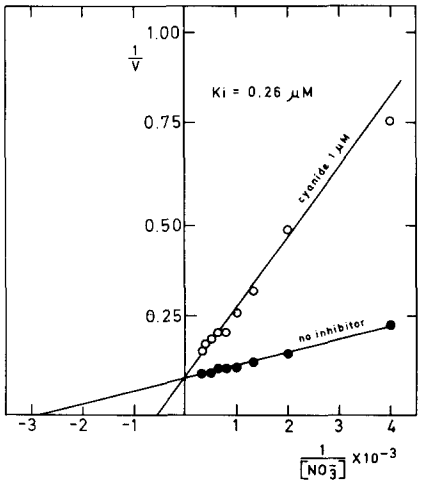


**Fig. 6. Competitive inhibition of *Cyanidium* nitrate reductase by KCN 1 $\mu$M, with respect to nitrate. FMN, 0.5 mM, was the electron donor. For experimental procedure see Material and Methods. Activity: nmol nitrite produced/min per system.**

TABLE II

EFFECT OF SULPHYDRYL GROUP REAGENTS ON NITRATE REDUCTASE ACTIVITY

The enzyme activity was assayed in the presence of inhibitor in systems of 2 ml of the following composition: enzyme, 0.1 and 0.035 ml for NADPH and benzylviologen or FMN activities respectively; 25 mM nitrate; 0.06 mM benzylviologen, or 0.5 mM FMN; 0.25 mM NADPH. The other components of the assay systems, and the procedure, were as reported under Materials and Methods.

| Sulphydryl group reagent | Concn ($\mu$M) | % of activity | | |
|---|---|---|---|---|
| | | NADPH | FMN | Benzyl-viologen |
| Control | | 100 | 100 | 100 |
| Mersalyl | 10 | 62 | 90 | 100 |
| | 50 | 16 | 80 | 82 |
| | 100 | 0 | 93 | 68 |
| *p*-Chloromercuribenzoate | 10 | 69 | 100 | 100 |
| | 50 | 0 | 100 | 86 |
| | 100 | 0 | 77 | 70 |
| $Hg(Cl)_2$ | 10 | 53 | 46 | 48 |
| | 50 | 0 | 21 | 17 |
| | 100 | 0 | 13 | 5 |
| $AgNO_3$ | 10 | 65 | 81 | 80 |
| | 50 | 0 | 27 | 39 |
| | 100 | 0 | 13 | 22 |

ty and Méténier [8] with respect to a nitrate reductase from *Hansenula anomala*; inhibition by the same reductants was found by Hewitt in spinach (personal communication) and inhibition by the combined action of dithionite and nitrate by Guerrero et al. [11] in *Azotobacter*. Finally inhibition by dithionite/methylviologen was described by Rivas et al. for nitrate reductase of *Torulopsis* [12]. In all cases reoxidation reverses the inhibition as it occurs in *Cyanidium*.

Inhibition by reducing agents is not an exceptional characteristic of nitrate reductase: inhibition by NADPH is reported for a NADPH-cytochrome *f*

TABLE III

EFFECT OF PREINCUBATION WITH UREA ON NITRATE REDUCTASE ACTIVITY

The enzyme was preincubated with the reported concentrations of urea at 0°C. At intervals aliquots of 0.2 and 0.1 ml were taken and assayed with NADPH and benzylviologen or FMN respectively. Assay procedure as reported in Table II. The values of activity reported are those found after 2 h of incubation with urea.

| Urea | % of activity | | |
|---|---|---|---|
| | NADPH | FMN | Benzylviologen |
| Control | 100 | 100 | 100 |
| 3 M Urea | 18 | 73 | 80 |
| 4 M Urea | 9 | 70 | 68 |
| 5 M Urea | — | 70 | 53 |
| 6 M Urea | — | — | 7 |

reductase of spinach chloroplast [13]. This enzyme is also inactivated by dithionite, and reoxidation with air brings it once more fully active. $NADP^+$, which can bind to the enzyme at a different binding site with respect to NADPH, protects the enzyme against the inhibition by the reductant by poising the enzyme in a redox state favorable to the preservation of the native structure.

With respect to *Hansenula* nitrate reductase, Pichinoty and Méténier have proposed that in presence of reducing conditions labile disulphide bonds are opened and the subsequent change in conformation of enzyme leads to a loss of activity. The same hypothesis could explain the inhibitory effect of excess benzylviologen and FMN on the nitrate reductase of *Cyanidium* and, furthermore, the inactivation by dithionite. In fact, it is well known that the disulphide bonds are subjected to cleavage by mild reducing agents [14].

In *Cyanidium* the inhibition of nitrate reductase by dithionite is prevented by nitrate or FMN; besides, like in *Hansenula*, an high concentration of nitrate prevents inhibition by low levels of reduced benzylviologen, whereas it is ineffective against the inhibition by excess benzylviologen or FMN. Perhaps nitrate and FMN protect through a mechanism similar to that proposed for the protection by NADP of NADPH-cytochrome *f* reductase against the inactivation by NADPH above reported.

A relevant difference found in the present study with respect to nitrate reductase from other organisms, is that the enzyme from *Chlorella fusca* [7], tomato [15] and spinach [16] is inhibited by ADP, whereas the enzyme from *Cyanidium* is very little inhibited by this nucleotide. This fact is very significant since in the former organisms the inhibition by ADP assumes the significance of a regulatory mechanism. In *Chlorella*, moreover, the inhibition by ADP depends on the simultaneous presence of a reductant, is prevented by nitrate, and represents a part of the mechanism which is at the basis of enzyme inactivation promoted by the presence of ammonia in vivo. Also in *Cyanidium* in vivo the presence of ammonia brings about inactivation of nitrate reductase, but, in the light of the present study we must exclude that in this alga the factors which control the inactivation of enzyme are ADP plus a reductant, as in the green algae. Indeed, also in *Chlorella vulgaris* no inhibitory effect by ADP was found by Solomonson [17]. Moreover, that the mechanism which is at the basis of ammonia-dependent inactivation of nitrate reductase in intact cells, should be different in *Cyanidium* with respect to green algae was suggested also by experiments reported in previous papers [18—20].

Cyanide is a powerful inhibitor of nitrate reductase. There are reports that the degree of sensitivity to this inhibitor [17,21,22] and the type of inhibition [22], depend on the oxidation state of the enzyme. In the oxidized state the enzyme is less sensitive, and the inhibition is of competitive type with respect to nitrate, whereas, when the enzyme is reduced, the inhibition is non competitive [22]. In *Chlorella fusca*, nitrate prevents the inhibition by cyanide, but the inhibition results of non-competitive type [21]. By contrast, with *Cyanidium* nitrate reductase the degree of inhibition seems to be independent from the redox conditions, and the type of inhibition is always strictly competitive with respect to nitrate.

Cyanate and carbamyl phosphate inhibit the nitrate reductase of *Cyani-*

*dium* competitively with respect to nitrate, and this fits in with general reports [21,23].

The results with urea agree with those obtained with mercurials or heating, and confirm that the active site for NAD(P)H and benzylviologen or FMN are distinct. Beside, they corroborate that the part of nitrate reductase which bears the NAD(P)H site is more labile and is selectively destroyed by denaturing agents of proteins.

Finally, concerning the action of sulphydryl group reagents, it is significant that organic mercurials act preferentially on NAD(P)H activity, whereas $Ag^+$ and $Hg^{2+}$ act appreciably also on the benzylviologen, FMN, activity. It is evident that, besides sulphydryl groups which are especially associated with pyridine nucleotide dependent activity, there exist other sulphydryl groups, not approached by mersalyl or *p*-chloromercuribenzoate, but approached by $Ag^+$ and $Hg^{2+}$ which, very presumably, are involved in the maintenance of the structure of the more stable part of nitrate reductase.

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