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In previous work from our laboratory [1-4]), it was shown that flavin nucleotides (either FMN or FAD) could mediate, in the light, the transfer of electrons from spinach grana to nitrate in the presence of nitrate reductase purified from spinach leaves. In the dark, flavin nucleotides, reduced either by sodium hydrosulfite [5]) or by hydrogen gas-*Clostridium pasteurianum* hydrogenase [6]) or by $NADPH_2$-spinach $NADPH_2$ diaphorase [2]), could also donate electrons to the nitrate-nitrate reductase system.

The principal aim of this communication is to summarize the work of our laboratory on the enzymatic reduction of nitrate with $NADH_2$, placing special emphasis on recent results.

BEEVERS et al. [7]) showed that nitrate reductase extracts from different plant species had a specific or preferential requirement for $NADH_2$ versus $NADPH_2$ as electron donor. Since we found [8]) that chloroplast extract from spinach could reduce nitrate with $NADH_2$, we decided to compare the reduction of nitrate with $NADH_2$ and $NADPH_2$ using a higly purified nitrate reductase preparation from spinach [9]).

TABLE 1. — *Comparative effect of $NADH_2$ and $NADPH_2$ on the reduction of nitrate by nitrate reductase* (PANEQUE and LOSADA [9])).

Electron donor	Addition	NO_2^- formed (µmoles)
$NADH_2$	None	0.63
$NADPH_2$	FMN, NADP reductase	0.55
$NADPH_2$	FMN	0.04
$NADPH_2$	NADP reductase	0.02

Table 1 shows that the reduction of nitrate with $NADH_2$ as the electron donor required only nitrate reductase. By contrast, when the electron donor was $NADPH_2$, the system required, besides nitrate reductase, the addition of FMN and NADP reduc-

tase [10-12]). The nitrate reductase preparation also contained $NADH_2$-specific diaphorase activity, being able to catalyze the oxidation of $NADH_2$, but not of $NADPH_2$, by cytochrome c, menadione, ferricyanide or indophenol dyes.

In the transfer of electrons from $NADH_2$ to nitrate, catalyzed by the highly purified nitrate reductase, both $NADH_2$ diaphorase and nitrate reductase activities participate. As can be seen in Table 2, the reduction of nitrate with $NADH_2$ was inhibited when the enzyme was heated at 45° for 5 minutes or when the reaction was carried out in the presence of p-chloromercuribenzoate, sodium azide or potassium cyanide.

TABLE 2. — *Effect of inhibitors and heat treatment on the reduction of nitrate with $NADH_2$, catalyzed by a highly purified spinach nitrate reductase preparation.*

System	Inhibition (per cent)
Complete, enzyme heated at 45° for 5 min	95
Plus pCMB, 0.1 mM	95
Plus NaN_3, 1.0 mM	100
Plus KCN, 1.0 mM	100

The reactions were carried out aerobically and followed spectrophotometrically by measuring at 340 mμ the enzymatic oxidaton of $NADH_2$ by nitrate (cf. [2]).

In order to locate the site of action of inhibitors we separately assayed $NADH_2$ diaphorase activity by measuring ferricyanide reduction [9]), and nitrate reductase itself by measuring nitrite appearance using FMN chemically reduced by sodium hydrosulfite as electron donor [5]).

TABLE 3. — *Effect of inhibitors and of heat treatment on the terminal nitrate reductase activity exhibited by a highly purified spinach nitrate reductase preparation.*

System	Inhibition (per cent)
Complete, enzyme heated at 45° for 5 min	12
Plus pCMB, 0.1 mM	18
Plus NaN_3, 1.0 mM	100
Plus KCN, 1.0 mM	100

Nitrate reductase activity was determined with FMN chemically reduced by sodium hydrosulfite as the electron donor by colorimetrically measuring the formation of nitrite [5]). For avoiding interference of azide on the colorimetric estimation of nitrite, the effect of this inhibitor was assayed as previously described by measuring ammonia formation after the addition of spinach nitrite reductase and ferredoxin [21]).

Table 3 demonstrates that, under the specified conditions, the reduction of nitrate was not affected either when the enzyme was heated or when the assay was carried out in the presence of p-chloromercuribenzoate, whereas potassium cyanide and sodium azide completely inhibited the reaction.

Fig. 1 shows, on the other hand, that $NADH_2$-specific diaphorase was not operative either when the enzyme preparation was heated or when p-chloromercuribenzoate was present. KCN and NaN_3 did not affect the $NADH_2$ reduction of ferricyanide. The results presented in Table 3 and Fig. 1 explain, therefore, why any of the inhibitors or the heat treatment will block the transfer of electrons from $NADH_2$ to nitrate.

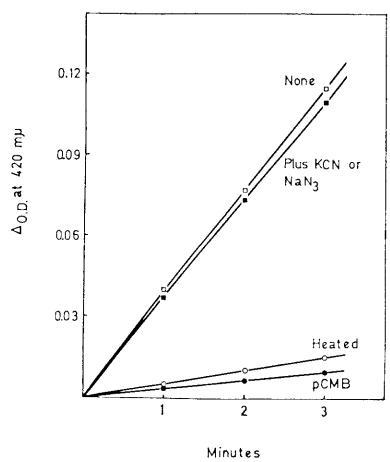

FIG. 1. — Effect of heat treatment and of inhibitors on the $NADH_2$ diaphorase activity exhibited by a highly purified spinach nitrate reductase preparation.
The reaction mixture included, in a final volume of 3 ml, 150 μmoles Tris buffer pH 8.0, 1 μmole potassium ferricyanide, 30 μg spinach nitrate reductase, and 0.3 μmoles $NADH_2$. Where indicated, either 30 μg heated (45° for 5 min) nitrate reductase or 0.3 μmoles pCMB or 3 μmoles KCN or 3 μmoles NaN_3 was added.

The differential behavior against inhibitors suggest that $NADH_2$ diaphorase and nitrate reductase itself are distinct proteins or, at least, two moieties of the same protein. Thus far it has not been possible to separate them physically.

Although SHIN and ARNON [13]) concluded that under physiological conditions only NADP but no NAD will be reduced in chloroplasts, HEBER and SANTARIUS [14]) reported that upon illumination of intact leaves, chloroplasts exhibit a rapid increase in $NADPH_2$ and $NADH_2$. The latter work prompted us to investigate if the photosynthetic reduction of NAD could be coupled with the reduction of nitrate.

It can be seen in Table 4 that the reduction of nitrate by illuminated chloroplast fragments depended on the presence of nitrate reductase and of catalytic amounts of NAD, and was stimulated by the addition of ferredoxin [15]). When NADP substituted

for NAD, no reduction of nitrate was observed. If the ferredoxin present in the chloroplast preparation was totally removed by washing, its addition was an absolute requirement for the reaction. Since under our experimental conditions both NAD and NADP could be reduced in the light in a ferredoxin-dependent reaction, it is followed that the $NADH_2$ thus formed can then act as the direct electron donor for the reduction of nitrate to nitrite catalyzed by nitrate reductase through the mechansim above described.

TABLE 4. — *Photoreduction of nitrate by chloroplasts depending on ferredoxin and NAD* (LOSADA and PANEQUE [15]).

System	NO_2^- formed (μmoles)
1. Complete	0.54
2. Minus ferredoxin	0.33
3. Minus NAD	0.02
4. Complete, but NADP instead of NAD	0.04

Finally, we shall mention that chloroplast extract contains a new $NADH_2$-specific diaphorase which can reduce either FMN or FAD, and that this system can be coupled to the one involved in the reduction of nitrate to nitrite, using nitrate reductase lacking activity of the previously described $NADH_2$ diaphorase. Table 5 shows the reduction of nitrate by nitrate reductase with FMN reduced by the $NADH_2$-$NADH_2$ diaphorase system.

TABLE 5. — *Reduction of nitrate with $NADH_2$ through the combined action of $NADH_2$-diaphorase, FMN and nitrate reductase* (PANEQUE, APARICIO, CATALINA and LOSADA [16]).

System	NO_2^- formed (mμmoles)
Complete	225
Minus $NADH_2$ diaphorase	18
Minus FMN	3
Minus nitrate reductase	6

The new $NADH_2$-specific diaphorase was purified from spinach chloroplast extract through a procedure which included adsorption and elution on alumina C_Y and on DEAE-cellulose bed [16]). The activity was measured spectrophotometrically by the oxida-

tion of $NADH_2$ with flavin nucleotides and it was inhibited by 0.1 mM p-chloromercuribenzoate or by heating the enzyme preparation at 45° for 5 minutes. Table 6 indicates the different electron

TABLE 6. — *Electron acceptor for the $NADH_2$-specific diaphorases involved in nitrate reduction.*

Electron acceptors	Diaphorase I	Diaphorase II
Ferricyanide	+	+
Menadione	+	—
Cytochrome c	+	—
Ferredoxin	—	—
Flavin nucleotides	—	+

acceptors for this new diaphorase (which we call diaphorase II) and for the previously described, also NADH-specific, present in the nitrate reductase preparation (diaphorase I).

Although assimilatory nitrate reductase from leaves [17-19]) have been considered as molybdoflavoproteins, we have now found that highly purified preparations of spinach nitrate reductase prepared as previously described [9]) and treated in addition with protamine sulfate to remove nucleic acids, have not shown any indication of absorption peaks for flavin nucleotides. Fig. 2 shows the absorption spectrum of spinach nitrate reductase in 0.1 M sodium pyrophosphate pH 7.0. It seems therefore that this enzyme, in contrast with nitrate reductase from other origins, is not a flavoprotein.

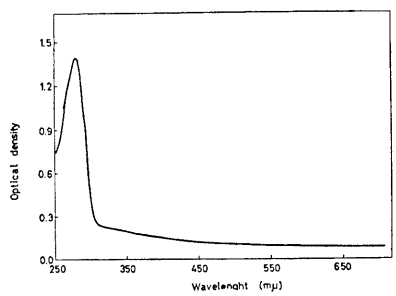

FIG. 2. — Absorption spectrum of a highly purified (1200-fold) nitrate reductase preparation from spinach leaves in 0.1 M sodium pyrophosphate buffer, pH 7.0. Light path, 1 cm.

Our actual evidence suggests also that spinach nitrate reductase may not be a molybdoflavoprotein: 1) Molibdate reduced by sodium hydrosulfite could not act as electron donor for the reduction of nitrate catalyzed by spinach nitrate reductase. 2) The most purified preparations analyzed by spectrographic and colorimetric [20]) methods did not contain detectable amounts of molybdenum, even using 10 mg of protein.

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SUMMARY — Nitrate reductase purified from spinach leaves as previously described in this laboratory can reduce nitrate to nitrite using either $NADH_2$ or $FMNH_2$ as electron donor.

In the transfer of electrons from $NADH_2$ to nitrate participates a $NADH_2$-specific diaphorase present in the nitrate reductase preparation. However, this $NADH_2$-specific diaphorase, which can not use FMN as electron acceptor, is not involved in the reduction of nitrate when $FMNH_2$ is the electron donor. Chloroplasts contain also a separate $NADH_2$-specific diaphorase that can reduce FMN with $NADH_2$ and therefore work in conjuction with nitrate reductase itself in the reduction of nitrate.

The best preparations of spinach nitrate reductase representing about 1000 fold increase in specific activity neither showed evidence of any absorption bands corresponding to flavin nucleotides nor contained detectable amounts of molybdenum. This enzyme seems therefore not to be, in contrast with nitrate reductases from other origins, a molybdoflavoprotein.

RÉSUMÉ — Comme préalablement on a décrit dans ce laboratoire la nitrate reductase de feûilles d'épinard purifiée peut réduire le nitrate à nitrite en usant comme donneur d'électrons le $NADH_2$ ou le $FMNH_2$.

Dans le transfer d'électrons du $NADH_2$ au nitrate particípe une diaphorase spécifique du $NADH_2$ présente à la préparation de la nitrate reductase. Cependant cette diaphorase spécifique du $NADH_2$, laquelle ne peut pas user du FMN comme accepteur d'électrons, n'est pas enveloppée dans la reduction du nitrate lorsque le donneur d'électrons est le $FMNH_2$.

Les cloroplastes contiennent aussi une diaphorase spécifique du $NADH_2$ diferente qui peut reduire le FMN et pourtant elle peut travailler conjointement avec la nitrate reductase propement dite dans la reduction du nitrate.

Les meilleures préparations de la nitrate reductase d'épinard, en representant una augmentation d'activité spécifique de 1000 fois, ne monstraient pas évidence d'aucune bande d'absorption correspondante aux flavinnucleotides ni elles contenaient aucune quantité detectable de molibdene. Pourtant cette enzyme parait ne pas être, en contraste avec les nitrate reductases d'autres origins, une molibdoflavoproteine.

ZUSAMMENFASSUNG — Aus früheren Veröffentlichungen dieses Laboratoriums geht hervor, dass die gereinigte Nitratreduktase von Spinatblättern das Nitrat zu Nitrit reduzieren kann, wenn als Elektronendonator entweder $NADH_2$ oder $FMNH_2$ verwendet wird.

Bei der Überfrägung der Elektronen vom $NADH_2$ zum Nitrat nimmt eine spezifische Diaphorase des $NADH_2$ teil, die in der Präparation der Nitratreduktase gegenwärtig ist. Diese spezifische Diaphorase des $NADH_2$, die das FMN nicht als Elektronenacceptor gebrauchen kann, hat jedoch keinen Anteil an der Reduktion des Nitrats, wenn der Elektronendonator $FMNH_2$ ist.

Die Chloroplaste enthalten ebenfalls eine besondere spezifische Diaphorase des $NADH_2$, die FMN mit $NADH_2$ reduzieren kann, und daher ein gemeinsamer Einsatz mit der eigentlichen Nitratreduktase zur Reduktion des Nitrats möglich ist.

Die besten Präparationen der Spinat-Nitrat-Reduktase zeigten bei einer 1000-fachen Zunahme an zpezifischer Aktivität keine Evidenz weder irgendeines den Flavinnukleotiden entsprechenden Absorptionssreifens noch die eines feststellbaren Molybdängehaltes. Dieses Enzym scheint also folgerichtig, im Gegensatz zu den Nitratreduktasen anderen Ursprungs, kein Molybdoflavoprotein zu sein.

RESUMEN — Como previamente se describió en este laboratorio la nitrato reductasa de hojas de espinaca purificada puede reducir el nitrato a nitrito usando como donador de electrones $NADH_2$ or $FMNH_2$.

En la transferencia de electrones de $NADH_2$ a nitrato participa una diaforasa específica del $NADH_2$ presente en la preparación de nitrato reductasa. Sin embargo, esta diaforasa específica de $NADH_2$, la cual no puede usar FMN como aceptor de electrones, no esta envuelta en la reducción del nitrato cuando el donador de electrones es $FMNH_2$.

Los cloroplastos contienen también una diaforasa específica de $NADH_2$ distinta que puede reducir FMN y, por tanto, puede trabajar conjuntamente con la nitrato reductasa propiamente dicha para la reducción del nitrato.

Las mejores preparaciones de nitrato reductasa de espinaca representando un aumento de actividad específica de 1000 veces no mostraban evidencia de ninguna banda de absorción correspondiente a flavinnucleótidos ni contenía ninguna cantidad detectable de molibdeno. Este enzima parece por consiguiente no ser, en contraste con la nitrato reductasas de otros orígenes, una molibdoflavoproteína.

RIASSUNTO — La nitrato-reduttasi di foglie di spinacio purificata può ridurre il nitrato a nitrito usando come dontatore di elettroni $NADH_2$ o $FMNH_2$.

Al passaggio di elettroni da $NADH_2$ a nitrato partecipa una diaforasi specifica del $NADH_2$ presente nella preparazione della nitrato-reduttasi.

Tuttavia, questa diaforasi, che non può adoperare FMN come accettore di elettroni, non agisce nella riduzione del nitrato quando il donatore di elettroni è $FMNH_2$.

Anche i cloroplasti hanno una diaforasi specifica di $NADH_2$ che può ridurre FMN con $NADH_2$ e, pertanto, agire insieme alla nitrato-reduttasi propriamente detta per la riduzione del nitrato.

Le migliori preparazioni di nitrato-reduttasi di spinacio, pur presentando un'attività specifica 1000 volte superiore, non registravano nessuna banda di assorbimento corrispondente a flavin-nucleotidi né contenevano una quantità apprezzabile di molibdeno. Da ciò si deduce che tale enzima, in contrasto con la nitrato-reduttasi di altra origine, non sia una molibdoflavoproteina.

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