Vitamin K-dependent nitrate reductase in *Escherichia coli*

A nitrate-reducing system has been studied by TANIGUCHI *et al.*[1] in *E. coli* grown in a peptone-broth, agar medium containing 0.5% KNO_3. This enzyme system catalyzes the reduction of nitrate to nitrite under anaerobic conditions in the presence of either methylene blue, formate or reduced diphosphopyridine nucleotide (DPNH) with participation of cytochrome *b* and flavin-adenine dinucleotide (FAD) functioning as electron carrier in the system. The DPNH-nitrate transport chain has at least some of the carriers in common with the DPNH-oxygen chain. The affinity for oxygen of the common part of the chain appears to be much greater than that for nitrate. On the other hand, NICHOLAS AND NASON[2] reported the finding of a reduced pyridine nucleotide-nitrate reductase, apparently of a molybdoflavoprotein nature, in *E. coli* strain B, grown in a synthetic medium.

We report here the finding, in cell-free extracts of *E. coli*, of a DPNH-dependent system which reduces nitrate to nitrite aerobically using vitamin K as electron carrier and apparently without participation of flavin in the process. The system is present in cells of *E. coli* grown either in a synthetic medium or in a complex broth medium containing 0.5% KNO_3, the specific activity being higher in the latter.

The cells were harvested by centrifugation and washed with 9% NaCl to remove nitrite. The cell-free extracts were prepared by grinding in a cold mortar for 10 min the frozen cells with twice their weight of alumina powder (Alcoa A-301) and for another 10 min with four times their weight of cold 0.1 *M* phosphate buffer, pH 7.5. After centrifugation in the cold for 15 min at 8000 × *g*, the supernatant and the sediment (the latter carefully separated from the alumina and suspended in phosphate buffer) were assayed for nitrate reductase activity.

In the experiments summarized in Table I, nitrate reductase activity was not detectable in the supernatant of the extract from *E. coli* grown in a synthetic medium, unless vitamin K_3 (as 2-methyl-1,4-naphthoquinone sodium bisulfite) was added. The system present in the supernatant and in the suspended sediment was not activated by addition of flavin-adenine dinucleotide, flavin mononucleotide or riboflavin. Atebrine can inhibit the system, but the fact that the inhibition could not be reversed by FAD but was completely reactivated by the addition of a similar amount of vitamin K_3 suggests that the inhibition cannot be attributed to flavin in the system.

TABLE I

EFFECT OF VITAMIN K

0.1 ml enzyme; 15 μmoles phosphate buffer, pH 7.5; 10 μmoles KNO_3; 0.4 μmole DPNH (enzymically reduced) and additions as indicated, in a final volume of 1 ml, were incubated for 10 min at 25°. Nitrites were determined by formation of the red azo compound[5]. Activity expressed as mμmoles nitrite produced in 10 min/mg protein[3]. A control assay was always included in which DPNH was omitted

Compound added	*Synthetic medium*		*Complex broth medium*	
	Supernatant	*Sediment*	*Supernatant*	*Sediment*
—	—	11	181	711
Boiled pig heart	—	11	192	707
0.1 μmole FAD	—	11	191	698
0.1 μmole Vitamin K_3	38	17	322	784
2 μmoles Atebrine		4	96	320
2 μmoles Atebrine + 0.4 μmole FAD	26	4	77	327
2 μmoles Atebrine + 0.1 μmole Vitamin K_3	27	15	360	775
2 μmoles Atebrine + 0.4 μmole Vitamin K_3	57			

Since *E. coli* has a menadione reductase, it appeared possible that the observed nitrate reduction could be produced chemically by the reduced vitamin K. This possibility can be excluded since purified menadione reductase[4] did not reduce nitrate in the presence of vitamin K_3. Besides, the menadione reductase is not inhibited by complexing agents and our system is markedly inhibited, as it is shown in Table II.

In view of these results, we suggest the presence of a system which is coupled to the menadione reductase and transports the electrons from reduced vitamin K to nitrate. This could perhaps explain the presence in *E. coli* of menadione reductase, whose physiological meaning is unknown.

The second part of the system seems to involve a metal and the participation of cytochrome *b* in the process is suggested by the high inhibition produced by 2-heptyl-4-hydroxyquinoline.

TABLE II

EFFECT OF INHIBITORS

Inhibitors were mixed with the enzyme before the addition of the other components of the assay as described in Table I and including 0.1 μmole vitamin K

Compound added	*Final concentration*	*% Inhibition*
KCN	10^{-3} *M*	100
α-α′ Dipyridyl	$6 \cdot 10^{-3}$ *M*	67
o-Phenanthroline	$5 \cdot 10^{-3}$ *M*	90
Na diethyldithiocarbamate	10^{-3} *M*	29
Salicylic acid	10^{-3} *M*	32
8-Hydroxyquinoline	10^{-3} *M*	29
Thiourea	10^{-3} *M*	41
2-Heptyl-4-hydroxyquinoline	$4 \cdot 10^{-4}$ *M*	95

Work is now in process to clarify this system and its physiological significance.

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Biosynthesis of hyaluronic acid by cell-free extracts of group-A streptococci*

Previous studies have demonstrated that both the glucosamine and glucuronic acid moieties of HA** synthesized by Group-A streptococci are derived from glucose[1]. The discovery of uridine nucleotides containing N-acetylated amino sugars[2] and glucuronic acid[3] and the established role of uridine nucleotides as glycosyl donors[4] suggested that these compounds are intermediates in the biosynthesis of mucopolysaccharides. CIFONELLI AND DORFMAN[5] have demonstrated the presence of UDPAG, UDPGA and other non-identified fractions containing uronic acid and N-acetylglucosamine in a strain of Group-A streptococcus grown under conditions optimal for the production of HA. The only direct evidence in support of the role of uridine nucleotides in acid mucopolysaccharide synthesis is given by the reports of GLASER AND BROWN[6,7] that extracts of Rous sarcoma incorporate into HA, ^{14}C from labeled UDPAG, AG-6-P+UTP, or UDPG. However, a large portion of the radioactivity was lost on reprecipitation or electrodialysis. No incorporation was obtained when labeled UDPGA was used.

It is the purpose of this communication to present evidence that a cell-free extract of a strain of Group-A streptococcus (A111, Type 18) incorporates glucoronic acid from UDPGA. This system has an absolute requirement for UDPAG and Mg^{++} and a relative requirement for AG-1-P and ATP.

Cells were grown as previously described[5], harvested by centrifugation at 30,000 × *g* for 10 min, and washed twice with 0.05 *M* phosphate buffer, pH 7.0. Following treatment in a

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** The following abbreviations are used in this paper: hyaluronic acid, HA; uridine diphospho-N-acetylglucosamine, UDPAG; uridine diphosphoglucoronic acid, UDPGA; uridine diphosphoglucose; uridine triphosphate, UTP; adenosine triphosphate, ATP; diphosphopyridine nucleotide, DPN; N-acetylglucosamine-1-phosphate, AG-1-P; N-acetylglucosamine-6-phosphate, AG-6-P.