Menadione derivatives and ferrous iron as cofactors of the nitrate reductase system of a coliform organism*

In a study of the nitrate reductase system of non-induced cells of the 1433 strain[1] of *Escherichia coli* it was found that the activity of suspensions of acetone powders depended upon the method of assay used. These preparations showed considerable activity when assayed anaerobically with leuco-methylene blue as H-donor (and electron-carrier), whereas less than 1 % of this activity was obtained in anaerobic assays (without methylene blue) by a modification of the method developed for the enzyme of *Neurospora crassa*[2], or by the recently described method of NICHOLAS AND NASON[3]. Because of this discrepancy, we have studied the cofactor requirements of the nitrate reductase system of crude acetone powders and of cells disrupted by sonic oscillation or grinding with alumina. Our results indicate that a menadione derivative, menadione reductase and possibly a cytochrome system are components of the nitrate reductase system, and ferrous iron is an activator.

Cells were grown aerobically at 30° for *ca.* 18 h in nutrient broth containing 0.5 % (w/v) glucose, harvested, washed twice and resuspended in distilled water. Suspensions were stored at 4° for 3 days before adding to 10 volumes of acetone at temperatures not exceeding —15° (cells precipitated shortly after harvesting gave powders of very low activity). The precipitate was further washed with cold acetone and stored at —15°. All of the activity of such preparations (suspended in a variety of buffers) was sedimented by centrifuging at 1400 *g* for 15 min, and we have therefore used crude acetone powders suspended in phosphate buffer (pH 7.2) for all experiments reported.

Addition of a concentrate (*in vacuo*) of the aqueous acetone extract to the virtually inactive acetone powder markedly stimulated the nitrate reductase activity as assayed with DPNH** or TPNH (as H-donor) in the presence of FMN or FAD. Of various substances tested as possible cofactors (including oxidized and reduced cytochrome *c* or hemin, tocopherol and ascorbic acid), only menadione (10^{-5}–10^{-4} *M*) caused appreciable stimulation of nitrate reductase activity. In the presence of menadione the activity was enhanced by ferrous iron (*M*/800), and still further stimulated by anaerobic pre-incubation of the enzyme preparation with the other components of the reaction mixture prior to addition of the nitrate substrate. Indeed, with freshly-prepared acetone powders the activity measured for enzyme pre-incubated in the presence of menadione and ferrous iron equalled that obtained with leuco-methylene blue as H-donor (Table I).

TABLE I

EFFECT OF PRE-INCUBATION WITH MENADIONE AND FERROUS IRON ON NITRATE REDUCTASE ACTIVITIES (expressed as μmoles nitrite/h/mg dry wt. of cells)

For assays with the methylene blue system each tube contained $5 \cdot 10^{-6}$ *M* leuco-methylene blue, $1.25 \cdot 10^{-3}$ *M* $FeSO_4$, $5 \cdot 10^{-3}$ *M* KNO_3, enzyme preparation and $2 \cdot 10^{-1}$ *M* KNa phosphate buffer (pH 7.2) to 2 ml. For assays with the menadione system each tube contained $2.5 \cdot 10^{-4}$ *M* DPNH, 10^{-6} *M* FMN, $5 \cdot 10^{-5}$ *M* ammonium molybdate, $5 \cdot 10^{-3}$ *M* $MgSO_4$, 10^{-5} *M* menadione, $1.25 \cdot 10^{-3}$ *M* $FeSO_4$, $5 \cdot 10^{-3}$ *M* KNO_3, enzyme and $2 \cdot 10^{-1}$ *M* phosphate buffer (pH 7.2) to 2 ml. In assays without pre-incubation enzyme was added from the stopper; in assays with pre-incubation nitrate was added from the stopper after incubation for 15 min.

All assays were performed in evacuated micro-Thunberg tubes with the equivalent of 1 mg dry wt. of cells and nitrite was estimated with the Griess-Ilosvay reagent. Duration of assay 15 min. Temperature 37°.

Preparation No.	*Methylene blue system*	*Menadione system without pre-incubation*	*Menadione system with pre-incubation*		
			Complete	*Omitting menadione*	*Omitting $Fe^{''}$*
1	0.8	0.5	0.8	—	—
2	0.6	0.1	0.6	—	—
3	1.0	0.3	1.1	0.0	0.3
4	1.2	—	1.2	0.1	0.2

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** The following abbreviations are used: di- and tri-phosphopyridine nucleotides, DPNH and TPNH; flavin mono- and di-nucleotides, FMN and FAD.

In addition to the obligate requirement for menadione and the activation by iron (Table I), we have found that flavin nucleotide was required for maximal activity of most of our preparations.

Various analogues of menadione have been tested (Table II) to obtain some indication of the specificity of the derivative presumed to be formed from the menadione during the pre-incubation. It is clear that the activity is markedly dependent upon the type of radicals substituted at carbon atoms 2 and 3. It seems possible that vitamin K_2, the form occurring naturally in bacteria, is the natural carrier of the nitrate reductase system.

TABLE II

RELATIVE ACTIVITIES OF MENADIONE ANALOGUES

Groups substituted in 1,4 naphthoquinone	*Relative activity*
nil	0
2-methyl- (Menadione)	100
2,3-dimethyl-	100
2-hydroxy-3(3-methyl-2-butenyl)- (Lapachol)	25
2-methyl-3-hydroxy-(2-hydroxy-3-methyl)- (Phthiocol)	12.5
2-methyl-3-phytyl- (Vitamin K_1)	0
2,6-dimethyl-	100
2-methoxy-	90
2-methyl-2,3-oxide-	0

Activities at equimolar concentrations were compared in the assay system given in the legend to Table I.

We are greatly indebted to Dr. W. W. UMBREIT for a gift of Vitamin K_1, and to Prof. L. F. FIESER for a gift of most of the other derivatives tested.

Dicumarol was a very potent inhibitor of the enzyme system (total inhibition at concentrations as low as 10^{-6} *M*) whether present during the pre-incubation (Table III) or when added with the nitrate substrate. Further, the inhibition was reduced by increasing the concentration of menadione, indicating that the inhibition was of the competitive type. Dicumarol only slightly inhibited the activity of intact cells (assayed as formerly[4]), but markedly inhibited the activity of toluenized cells*. Trimethyl benzoquinone inhibited the activities of acetone powders and of intact cells to approximately the same extent. Cysteine and glutathione also strongly inhibited the activities both of acetone powders and of intact cells. Dicumarol, cysteine and glutathione have all been shown to inhibit the menadione reductase enzyme of *E. coli*[5]. It therefore seems probable that the menadione derivative and reductase enzyme participate in the reduction of nitrate by intact cells.

TABLE III

INHIBITION BY DICUMAROL OF NITRATE REDUCTASE

Activity (expressed as μmoles nitrite/h/mg dry wt. of cells)

Acetone powder No.	*Menadione concentration*	*Dicumarol concentration*		
		0	$5 \cdot 10^{-7}$ *M*	10^{-6} *M*
1	$2 \cdot 10^{-5}$ *M*	0.6	0.1	0.0
	10^{-4} *M*	1.0	0.4	-
2	$2 \cdot 10^{-5}$ *M*	0.7	-	0.0
	10^{-4} *M*	1.5	-	0.2

Assays were performed with the equivalent of 0.8 mg dry wt. of cells in the menadione system with pre-incubation given in the legend to Table I.

With preparations of cells disrupted with alumina (and centrifuged at *ca.* 500 *g* for 10 min to remove the abrasive) the nitrate reductase activity assayed with TPNH as H-donor and in the presence of menadione was only slightly stimulated by ferrous iron, whereas with DPNH as H-donor there was a very marked stimulation by iron. A similar dependence of the effect of ferrous iron upon the nature of the H-donor has been observed for the reduction of cytochrome *c*[6]. It therefore seems possible that a cytochrome system is a component of the nitrate reductase system. Evidence from published studies of the effects of enzyme inhibitors (see[3,7] for references) is equivocal, but some of the confusion may perhaps be due to variations in the methods used for the assay of nitrate reductase activity.

The role of the ferrous iron has not yet been elucidated. In contrast to the effects obtained with alumina-ground cells, even with TPNH as H-donor, ferrous iron markedly stimulated the nitrate reductase activities of both acetone powders and cells disrupted by sonic oscillation. It

* Treatment with toluene of cells grown in complex media increased the apparent nitrate reductase activity; treatment of nitrate-induced cells or of cells grown in synthetic media or an acid hydrolysate of casein caused a marked loss of activity.

may also be recalled that ferrous iron (at the same optimal concentration of $M/800$) considerably reversed the "dilution effect" found with intact cells[4]. These findings suggest that perhaps a further electron-transporting system, in addition to the menadione and cytochrome systems, is a component of the nitrate reductase system of *E. coli* 1433.

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The role of nucleoside phosphorylase in erythrocyte preservation*

Erythrocytes stored in acid-citrate-dextrose (ACD) at 4° undergo a progressive loss of physiological viability, as measured by post-transfusion survival, and a simultaneous decline in the level of cellular phosphate esters, principally adenosine triphosphate and 2,3-diphosphoglycerate. Previous studies from this laboratory have demonstrated that after deterioration upon storage, cells may be rejuvenated by incubation with certain purine nucleosides[1,2]. The rejuvenation is indicated by a marked resynthesis of phosphate esters from orthophosphate (*cf.* Column I, Table I), and a concomitant restoration of the viability of the red cell**[3]. The ability of nucleosides to effect the resynthesis of phosphate esters is retained in hemolysates (*cf.* Column II) with the same order activity for the nucleosides: inosine > adenosine > guanosine > xanthosine. Deoxyadenosine and 2,6-diaminopurine riboside (kindly supplied by Dr. G. B. BROWN) were somewhat less effective than xanthosine in the intact cell system, whereas adenosine mono- and tri-sulfate (kindly supplied by Prof. Dr. A. HOCK) were inactive. Pyrimidine nucleosides (thymidine, uridine and cytidine) are inactive and do not inhibit the action of purine nucleosides. Purine bases and ribose, alone or in combination, are inactive. Trichloracetic acid filtrates of stored cells, which have been rejuvenated by the above nucleosides, contain quantities of the corresponding purine bases as revealed by paper chromatography in *n*-butanol/water (85/15). The above evidence suggests that red cell rejuvenation is initiated by the action of a purine nucleoside phosphorylase[5]:

$$\text{Purine-ribose} + P_i \longrightarrow \text{purine} + \text{ribose-1-P} \qquad (1)$$

which cleaves the nucleoside with the simultaneous conversion of orthophosphate (P_i) to ribose-1-phosphate. Subsequent metabolism of the phosphorylated pentose then provides energy for the resynthesis of phosphate esters.

TABLE I

RESYNTHESIS OF PHOSPHATE ESTERS BY NUCLEOSIDES

	*μM of organic P/100 ml red cells**	
	*Intact cells***	*Hemolysate****
Control§	400	229
Inosine	1305	917
Adenosine	1135	876
Guanosine	980	836
Xanthosine	894	687

* Phosphate measurements as described previously[4].

** Human blood, stored 25 days. 1300 μM nucleoside/100 ml cells; incubated for 45 min at 37°.

*** Hemolysate prepared from human blood stored 21 days. Values recalculated on the basis of 100 ml of cells. 1100 μM nucleoside/100 ml cells; incubated for 1 h at 37°.

§ Stored blood incubated in the absence of nucleoside.

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** Similarly, if nucleosides are added to fresh cells in ACE there is a prolongation of the effective period of storage, *i.e.* approximately 40–50 days in contrast to the value of 21 days which has been established for ACD alone[3].