shock, when given a vasoexcitatory synthetic polypeptide, PLV-2, showed increased survival rates. The mechanism(s) involved in the beneficial action of this drug cannot yet be precisely identified but may be related to a limited vasoconstrictor action as evidenced by a plateau-like pattern of the blood pressure response to its administration.

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Phosphorylation in a Partially Restored Bacterial System. (28904)

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Studies on the electron transport system of *Bacillus stearothermophilus* indicated that the light sensitive naphthoquinone component in the succinate oxidase complex did not participate in the direct line of electron flux but apparently functioned in a collateral manner(4). Such data are in conformity with the sequence of components in the succinoxidase chain as formulated for mammalian systems (6). However, the mechanism of the quinone mediated oxidation of succinate and NAD linked substrates in the microbial system appears to differ in various species(7,8). Recent studies on the electron transport sequence in the thermophile indicated a commensal relationship between soluble and particulate oxidative components was possible.

Methods and materials. The microorganism, electron transport particles (ETP) and soluble fraction were prepared as described previously(4). Soluble NADH—cytochrome *c* reductase was obtained from a supernatant (100,000 × *g*; 90 min) in the manner of Segel *et al*(11). The reduced nicotinamide adenine dinucleotide (NADH) and beef heart cytochrome *c* were obtained from Sigma Chemical Co., St. Louis; antimycin A, Kyowa Fermentations, LTD., Tokyo, Japan; 2,3-dimercapto propanol (BAL), gramicidin and ubiquinone 30, Mann Research Laboratories, N. Y., and amytal sodium, Eli Lilly & Co., Indianapolis.

Results and discussion. A cytochrome-bearing, electron transport particle (ETP) and a non-cytochrome, soluble fraction, isolated from *B. stearothermophilus*(4), both catalyzed the oxidation of reduced nicotinamide adenine dinucleotide (NADH). The oxidative rate in the soluble fraction was aided considerably by addition of cytochrome *c* and to a lesser extent by a naphthoquinone (NQ_b) isolated from the thermophile (Table I). No oxidation of added NADPH was observed.

The oxidation of NADH in the ETP was aided slightly by addition of cytochrome *c* or NQ_b. Examination of the catalysis in a mixture of the 2 systems indicated the rates were additive. These results suggested that the soluble NADH oxidase is limited by the availability of acceptor whereas the ETP maintains its initial degree of respiratory control and is limited by some other factor.

Flavin analyses, in the manner of Burch *et al*(2) disclosed a ratio of 3 to 1 between

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TABLE I. Oxidation of NADH by a Particulate and a Soluble Fraction Isolated from *Bacillus stearothermophilus*.

Fraction	Activity (μmoles NADH oxidized/min/mg protein)		
	Acceptors added		
	None	Cytochrome *c**	NQ_b †
Particulate (P)	.16	.20	.19
Soluble (S)	.23	1.13	.69
P + S	.41	1.24	.77

* Beef heart cytochrome *c*; Sigma.

† Naturally occurring naphthoquinone isolated from *B. stearothermophilus*.

Reaction cells contained: 33 mM Tris-Cl (pH 8.0). 15 mM KCl, 3 mM ADP, 6 mM Pi, ETP (2.0 mg protein) or soluble fraction (4.3 mg protein), 0.026 mM cytochrome *c*, 34 μg NQ_b, 1 mM NADH, and H_2O to 3.0 ml volume. Control cuvette lacked the enzyme. Temperature 60°C.

the soluble and particulate fractions, the latter containing approximately 1.31 mμmoles FAD per mg protein. Although the soluble fraction contained little quinone and no cytochrome *c*, either of these acceptors or a dye (2,6-dichlorophenol indolphenol) was essential for maximal NADH oxidation.

The soluble NADH-quinone reductase was incapable of phosphate esterification whereas P/O ratios of 1.75 were consistently observed in the ETP with NAD^+, sodium malate and malic dehydrogenase present in substrate amounts. Phosphorylation (P/O) of the mixture did not exceed this latter figure.

It appears that the particles retained enough organization for acquisition of at least half of the chemical bond energy available via the respiratory brigade.

Several respiratory inhibitors were tested for effects on the ETP and soluble fraction. Oxidation of NADH by the ETP was inhibited by 2,3-dimercapto propanol (BAL), azide and cyanide as determined by oxygen consumption and the change in absorbance at 340 mμ. Oxidation of NADH via the soluble system was inhibited by p-chloromercuribenzoate (CMB) and amytal. Antimycin A, gramicidin, 2-amino-1, 4-naphthoquinone and ubiquinone 30 (Q_6) had no effect on either the ETP or soluble fraction at concentrations ranging from 0.01 to 100 times those reported as inhibiting mammalian systems.

The mode of inhibition of malate and succinate oxidation by BAL appeared to be similar to that of antimycin A(9,10). A sigmoid curve is obtained when the inhibitor concentration is plotted against velocity (Fig. 1). Rate of endogenous and exogenous cytochrome *c* reduction by the ETP was similarly affected by BAL.

It was of interest to investigate the oxidative and phosphorylative behavior of the mixed system (ETP plus soluble fraction) in the presence of BAL, since the latter had no inhibitory effect on the soluble fraction, while phosphorylation depended upon the oxidation mediated by the ETP. The soluble enzymes catalyzed reduction of particle bound cytochrome *c* in a BAL blocked preparation (Fig. 2). The extent of participation of bound cytochrome *c* as electron acceptor for the NADH donor system was indicated when the rate reduction of cytochrome *c* was found to be very similar to the rate oxidation of NADH in a separately tested system. Since comparatively little is known about the BAL sensitive component in the respiratory chain of *B. stearothermophilus*, nothing other than speculation would make it analogous to the so-called "Slater Factor"(12) in chemical character or function. However, as a working hypothesis I assumed it to function beyond the flavin site and parallel to or beyond the cytochrome *b* locus. The question arose as to whether the particulate system retained any phosphorylative capability in the presence of BAL. If not, would addition of an active soluble fraction restore activity to the BAL inhibited system? Phosphorylation was determined as previously described(4) on a mixture under conditions of BAL inhibition. The P/O in the mixture was approximately 0.85, a value which, although considerably lower than the full potential of the ETP, could represent ATP formation at the remaining operative segment of the respiratory chain. Phosphorylation was inhibited by 2, 4-dinitrophenol but not by dicumarol. This would suggest that a non-phosphorylating diaphorase mechanism is coupled to the remaining functional hemeprotein segment at the cytochrome *c* locus.

The partial restoration of the BAL sensitive particulate system via addition of a reductase which contained no detectable qui-

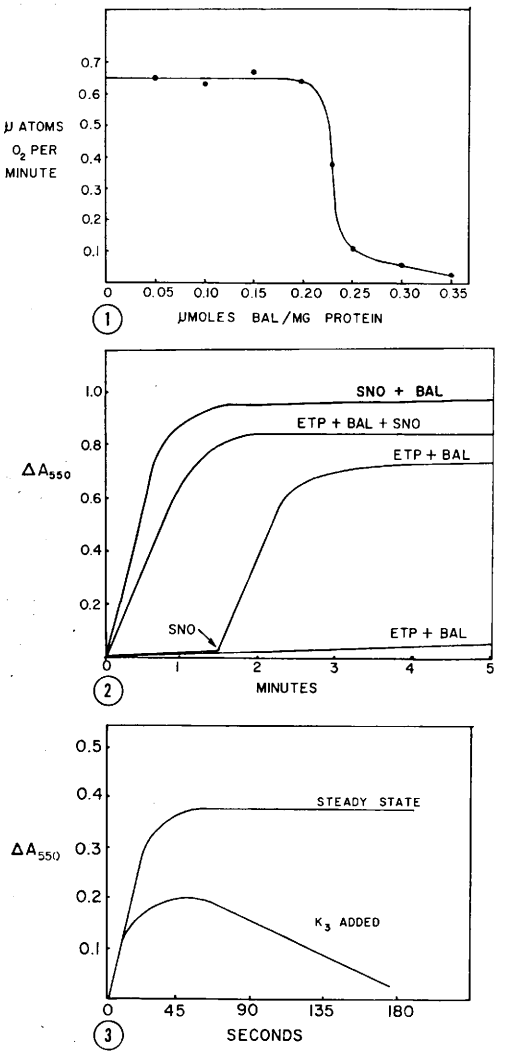

FIG. 1. Inhibition of O_2 uptake in the ETP by BAL (2,3-dimercaptopropanol). Reaction vessels contained: 33 mM Tris-Cl (pH 8.0), 3 mM KCl, 6 mM Pi, 3 mM ADP, 0.1 mM NAD^+, 12 mM Na-malate or Na-succinate, 0.01 mM EDTA, ETP (2.0 mg protein), inhibitor in concentration given and H_2O to a 3.0 ml volume. Gas phase: air, Tem. 50 C. Control contained inactivated enzyme and no substrate.

FIG. 2. Reduction of endogenous (bound cytochrome *c* in a BAL blocked system by the soluble NADH Cyto. *c* reductase. Reaction cells contained: 33 mM Tris-Cl (pH 8.0), 2.0 mM Na-Azide, 20 mM Pi, 10 mM ADP, 0.5 mM BAL, ETP (45.0 mg protein), soluble fraction (14 mg protein), 4 mM NADH and H_2O to 3.0 ml volume. Reference cuvette lacked substrate. Temp. 60 C.

FIG. 3. Effect of menadione (K_3) on reduction of cytochrome *c* by the soluble fraction. Reaction cell contained: 33 mM Tris-Cl (pH 8.0), 0.16 mM cytochrome *c*, 2.0 mM menadione, soluble fraction (0.2 mg protein), 0.3 mM NADH and H_2O to volume of 3.0 ml. Temp. 60 C. Control cuvette contained heat inactivated enzyme and no substrate.

none prompted examination of the effects of menadione (K_3) when added to the mixture as well as its separate composite fractions. The reduction of beef heart cytochrome *c* by the soluble fraction was arrested at an early stage by menadione (Fig. 3). When the concentration of cytochrome *c* was less than 0.1 the concentration of K_3 in the test system the shift in equilibrium occurred in favor of reoxidation of the cytochrome. The initial rates of reduction of cytochrome *c* by NADH in presence of and absence of K_3 were equal. This would support the concept that the quinone acts as a reservoir for electrons and can also contribute to reoxidation of cytochrome *c* when its normal oxidation is inhibited. Although the concentration of natural naphthoquinone in the thermophile is normally greater than 10 times that of cytochrome *c*, its reoxidation of bound ferrocytochrome *c* has not been observed by this author.

A dicumarol sensitive diaphorase has been isolated from rat liver(5). It catalyzed NADH and NADPH oxidation with a variety of acceptors and was postulated to link cytoplasmic NADH oxidation to the mitochondrial respiratory chain between the amytal sensitive site and cytochrome *c*(3). The latter investigators also reported esterification of phosphate in a Vit. K_3 mediated respiration in an amytal blocked mitochondrial system. They concluded that the quinone acts as an artificial link between a DT diaphorase and the main respiratory sequence although amytal inhibition seemed to be a necessary prerequisite.

A solubilized NADH-quinone reductase from a particulate fraction from *E. coli* was inhibited by dicumarol (3×10^{-3}M) but not by CMB (1.5×10^{-3}M)(7). Likewise a NADH-cytochrome *c* reductase of the parent fraction was inhibited by irradiation with light at 360 mμ. Addition of Vit. K_2 restored activity while benzoquinone (Q_{10}) did not.

The photolability of bound quinones permits their irreversible inactivation(1) under

certain conditions without impairment of neighboring catalysts. Restoration of overall oxidative ability of the ETP then depends upon the accessibility of exogenous quinone to the natural donor. Menadione, although easily reduced by soluble reductases, does not seem to complete with bound quinones in non-irradiated systems except under anaerobic conditions(8). Regardless of this diverse specificity *in vitro* with bacterial systems the functional acceptor of quinone mediated electrons appears to be cytochrome *b*(6,7). The resistance of bacterial systems to antimycin A, as reported here, would suggest another character and function for this hemeprotein.

Attempts to couple an active soluble NADH-quinone reductase to a light (360 mμ) inactivated ETP preparation from *B. stearothermophilus* were unsuccessful. It would seem that NADH specificity, dicumarol sensitivity and cytochrome *c* reactivity serve to distinguish the quinone reductase of the thermophile from the DT diaphorase of rat liver and the NADH-quinone reductase of *E. coli.*

Although an inescapable disparity in steric relationships accompanies Vit. K_1, K_2, and K_3 participation in the quinone reductase segment of a branch pathway, the growing evidence that quinones transfer electrons to endogenous acceptors offers a sizable prospect for future study of the interplay between soluble and particulate oxidative systems of the cell.

Summary. A cytochrome bearing ETP and a soluble, non-heme fraction, isolated from *B. stearothermophilus,* both catalyzed the oxidation of NADH. Although the oxidative rate in the soluble fraction was aided considerably by addition of cytochrome *c* and to a lesser extent by a naphthoquinone isolated from the bacillus, the rate in the ETP was only slightly increased. Flavin analysis disclosed a ratio of 3 to 1 between the soluble and particulate fractions. The former was incapable of phosphorylation whereas the ETP exhibited a P/O of 1.75. Oxidation of NADH was inhibited in both systems by 2,3-dimercaptopropanol (BAL) and in the soluble system by p-chloromercuribenzoate and amytal. Phosphorylation in an ETP suspension, inactivated by BAL or light (360 mμ) was partially restored by addition of soluble fraction. The P/O in such a mixture was 0.85, a figure which apparently represented ATP formation in the remaining functional segment of the cytochrome sequence. This phosphorylation was inhibited by 2,4-dinitrophenol but not by dicumarol.

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