Short Communications and Preliminary Notes

Oxidative phosphorylation with ferricyanide as terminal electron accepter

Previous attempts to localize the steps of oxidative phosphorylation in mitochondria have shown phosphorylation to occur during the transfer of electrons to either added cytochrome c[1,2] or ferricyanide[3]. Rates obtainable in the presence of cytochrome c can be greatly accelerated by hypotonic pretreatment of the mitochondria[2,4] or addition of DPN[5] and inhibited by antimycin A. Values as high as 1.6 have been reported for the $\Delta P/\Delta 2\varepsilon$ ratio of the DL-β-hydroxybutyrate to cytochrome c electron transfer[2] and 0.8 for the corresponding transfer to ferricyanide, the latter being insensitive to antimycin A[3].

We have been able to confirm the findings reported for ferricyanide under conditions similar to those used by the previous workers. When the ferricyanide system was adapted for spectrophotometric measurements however, as shown in Table I, $\Delta P/\Delta 2\varepsilon$ ratios approaching 2 resulted and the system was now found sensitive to antimycin A inhibition. Exactly where the critical difference between these two systems lies has not been ascertained but the ferricyanide concentration and versene have been eliminated as the source of the discrepancy. The substrate oxidation can be uncoupled from phosphorylation by 2,4-dinitrophenol and the latter does not occur in the absence of either substrate or ferricyanide. Fumarate, L-glutamate and L-malate, although oxidized at widely differing rates, gave similar $\Delta P/\Delta 2\varepsilon$ ratios while as expected the ratio with succinate was lower by about 1. Pyruvate or α-ketoglutarate could not be tested in this system since α-keto acids remove the cyanide, required for inhibition of the cytochrome oxidase, from the reaction mixture. This effect, also recently observed by SLATER[6], is apparently not due to simple cyanohydrin formation since the ability of cyanide to form a compound to which the α-keto acid assay[7] does not respond is dependent on prior incubation with the mitochondria.

TABLE I

PROPERTIES OF FERRICYANIDE ACCEPTOR SYSTEMS

Substrate	$\Delta\mu$ *eq.* $PO_4^{=}$/*g liver*	$\frac{\Delta\mu \text{ eq} \cdot \varepsilon}{2}$/*g liver*	$\Delta P/\Delta 2\varepsilon$
DL-β-Hydroxybutyrate	15.4	7.9	1.95
less ferricyanide	0.0	—	
less substrate	0.0	0.4	
antimycin A added ($2\ \gamma$/g liver)	0.0	0.3	
2,4-dinitrophenol added (0.03 μM/ml)	0.0	8.2	
DPN added (0.2 μM/ml)	16.6	9.4	1.76
L-Glutamate	17.3	9.0	1.92
Fumarate	2.5	1.45	1.7
L-Malate	1.9	1.06	1.8
Succinate	15.3	20.4	0.75

Rat liver mitochondria, prepared essentially according to the method of SCHNEIDER[9], with 0.25 M sucrose containing 0.001 M versene, and representing 40 mg liver/cuvette, were incubated in a final volume of 3.2 ml containing/ml the following: 37.5 μM DL-β-hydroxybutyrate or 19 μM of other substrate; 0.95 μM ferricyanide; 0.37 μM KCN; 31 μM KF; 0.9 μM versene; 1.25 μM AMP; 0.62 μM ATP; 156 μM TRIS; 46 μM $MgCl_2$; 0.18 mM sucrose; 7.3 μM phosphate labeled with ^{32}P; 15 μM glucose; 0.63 mg crystalline serum albumin; and excess yeast hexokinase; final pH, 7.4. Cells were incubated at 22° and samples withdrawn for phosphate analysis at 5.75 and 15.75 minutes. Values expressed in the above table apply to the ten minute interm. The reduction of ferricyanide, as followed by decrease of 420 mμ absorption, (light path 1 cm), proceeded linearly. The reaction was started by adding the enzyme, suspended in 2 ml with all other components, to cuvettes containing the phosphate, substrate, hexokinase and ferricyanide.

In Table II the ferricyanide and cytochrome *c* acceptor systems are compared with respect to the effects of a two minute hypotonic treatment of the mitochondria (0.025 *M* sucrose at 0°) and added DPN. The somewhat low $AP/\Delta 2\varepsilon$ ratios are possibly due in part to the lower phosphate concentrations found desirable to increase the sensitivity of the analytical method[8] used for measuring the relatively small phosphorylations occurring in the cytochrome *c* systems. When the mitochondria were not pretreated, low rates of cytochrome *c* reduction resulted accompanied by such large endogenous incorporations of ^{32}P into the organic phase, even in the absence of substrate, that meaningful $AP/\Delta 2\varepsilon$ ratios could not be estimated. It seems significant that this incorporation is greatly suppressed by antimycin A. Whether or not mitochondria are hypotonically pretreated, added DPN greatly accelerates the reduction of cytochrome *c*, but this is not accompanied by increased phosphorylation. The effects of DPN on the ferricyanide systems are comparatively slight. From the ferricyanide data of Table II it appears that the hypotonicity treatment, so necessary for demonstrating substrate-dependent phosphorylation in the cytochrome *c* systems, is capable of inducing some loss in the potential ability of mitochondria to oxidize substrate and lowering the phosphorylation efficiency.

TABLE II

COMPARISON OF FERRICYANIDE AND CYTOCHROME *c* ACCEPTOR SYSTEMS

Acceptor	*β-Hydroxybutyrate*	*DPN*	*Nonpretreated mitochondria*			*Pretreated mitochondria*		
			Aμ eq. P	*Aμ eq. ε 2*	*AP/Δ2ε*	*Aμ eq. P*	*Aμ eq. ε 2*	*AP/Δ2ε*
Cytochrome *c*			1.33	0.09		0.87	0.53	1.64
			1.18	0.34		2.86	2.60	1.10
	+	+	1.44	1.87		0.8	6.19	0.13
Ferricyanide			7.5	5.8	1.30	4.3	3.9	1.10
	+		7.8	6.4	1.22	4.4	4.2	1.05

Experimental conditions were the same as in Table I except that the phosphate was lowered to 1.8 μM/ml. When ferricyanide was replaced by cytochrome *c* (0.067 μM/ml) enzyme concentrations were adjusted when required to give suitable reaction rates; samples for phosphate analysis were then withdrawn at 1.5 and 13.5 minutes, values in the above table having been corrected to the equivalent rates/10 minute interval. The reduction of cytochrome *c* was followed as increase in 550 mμ absorbtion with a light path of 0.3 cm (total volume, 1.10 ml).

It is concluded that ferricyanide is capable of accepting electrons from mitochondria at a site very close to that at which cytochrome *c* does; this site could indeed be bound cytochrome *c* itself in both cases. Because it encounters less difficulty in reaching its point of action than does added cytochrome *c*, ferricyanide offers certain advantages in studying oxidative phosphorylation.

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