SHORT COMMUNICATIONS

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Energy-linked reactions in digitonin particles from beef-heart mitochondria

The reduction of exogenous NAD^+ by succinate was first shown by Löw and co-workers[1,2] and various aspects of the reaction have been examined by several investigators[3–7]. That tetramethyl-*p*-phenylenediamine (TMPD) can also provide the reducing equivalents for external NAD^+ reduction was first demonstrated by Löw AND VALLIN[8,9].

Guanidine was first introduced as a respiratory poison in 1955 by HOLLUNGER[10]. PRESSMAN[11] showed that alkylguanidines were more effective than guanidine and that their effect was preferential for Site I phosphorylations. It was suggested by PRESSMAN[12,13] that phenylethylbiguanide acts selectively on Site II phosphorylations, and further studies by PRESSMAN[13] and HAAS[14] indicated that decamethylene diguanidine (Synthalin) also acted at a phosphorylation site other than the first.

The present study deals with the effect of these site-specific inhibitors on reversed electron transfer in digitonin and sonic fragments of beef-heart mitochondria.

Mitochondria and digitonin fragments were prepared by the method of HAAS AND ELLIOTT[15] and stored in a liquid nitrogen refrigerator. Sonic fragments were prepared from mitochondria treated in a Bronwill Biosonik for 90 sec, and then isolated in the same manner as the digitonin fragments[15]. Reduction of NAD^+ by succinate or TMPD *plus* ascorbate was followed at 340 nm in a Gilford 2000 multiple sample absorbance recorder in conjunction with a Beckman DU monochromator. The reaction mixture contained 250 mM sucrose, 50 mM Tris–HCl, 3 mM $MgCl_2$, 1 mM KCN, 0.67 mM NAD^+, 0.25 mM sodium arsenite, 10 mM nicotinamide, and either 30 mM succinate or 0.2 mM TMPD *plus* 30 mM sodium ascorbate, and approx. 0.5 mg protein in a final volume of 3.0 ml. The reaction was started by the addition of 2 mM ATP.

The rate of reduction of NAD^+ was found to vary somewhat among preparations, and was always higher with sonic particles than with digitonin particles. The range for the reaction with succinate was 14–25 nmoles/min per mg protein with digitonin particles, and 25–40 nmoles/min per mg protein with sonic particles. The rates for the TMPD and ascorbate reaction were 8–20 nmoles/min per mg protein, and 12–24 nmoles/min per mg protein for the digitonin and sonic particles, respectively.

Table I shows the effect of three site-specific inhibitors of oxidative phosphorylation on the reduction of NAD^+ by succinate in digitonin particles. It is apparent that at low concentrations, hexylguanidine inhibits the reaction while phenylethylbiguanide and Synthalin do not. At higher levels, the latter two poisons do indeed inhibit this reaction. This agrees with the observation of HOMMES[5] in sonic fragments. The results in Table I were compared to similar experiments on the reduction of NAD^+

Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine.

TABLE I

EFFECT OF "SITE-SPECIFIC" INHIBITORS ON THE REDUCTION OF NAD^+ BY SUCCINATE IN DIGITONIN PARTICLES

Reaction mixture as described in text.

Hexyl-guanidine (*μM*)	*Phenylethyl-biguanide* (*μM*)	*Synthalin* (*μM*)	*NAD^+ reduced* (*nmoles/min per mg protein*)	*Inhibition* (%)
0	0	0	18.3	0
28	0	0	8.7	53
56	0	0	7.5	59
0	80	0	18.5	0
0	170	0	18.3	0
0	340	0	11.7	36
0	680	0	8.3	55
0	0	11	18.4	0
0	0	22	18.0	2
0	0	33	15.0	18
0	0	50	10.3	44

TABLE II

NAD^+ REDUCTION BY TMPD AND ASCORBATE IN THE PRESENCE OF AN ALKYLGUANIDINE, A BIGUANIDE AND A DIGUANIDINE IN DIGITONIN FRAGMENTS OF BEEF-HEART MITOCHONDRIA

Reaction mixture as described in text.

Hexylguani-dine sulfate (*μM*)	*Phenylethyl-biguanide* (*μM*)	*Synthalin* (*μM*)	*NAD^+ reduced* (*nmoles/min per mg protein*)	*Inhibition* (%)
0	0	0	9.3	0
28	0	0	5.7	39
56	0	0	4.2	55
0	80	0	9.3	0
0	170	0	9.0	3
0	340	0	6.0	35
0	680	0	4.2	55
0	0	22	9.2	1
0	0	33	8.0	14
0	0	50	5.1	45

by TMPD and ascorbate, and are presented in Table II. These data appear to support the contention of HOMMES[5] that the reversal of electrons from succinate to NAD^+ involves more than one phosphorylation site, if indeed substances such as phenylethylbiguanide and Synthalin are specific for phosphorylation sites other than the first. However, since these reactions are driven by the addition of ATP, high energy intermediates are produced at all three phosphorylation sites[17–19] any of which can drive the reversal reactions. It was, therefore, deemed important to look at the effect of multiple additions of these guanidine derivatives. The data presented in Table III are for sonic particles. The results in digitonin fragments are similar but because of the higher rates, the sonic particles present a clearer picture. It is apparent that much of the energy for both reactions is provided by the first phosphorylation site. Once a large portion of this is removed by the addition of hexylguanidine, the resulting

TABLE III

EFFECT OF MULTIPLE ADDITIONS OF THREE GUANIDINE DERIVATIVES ON THE ENERGY-LINKED REDUCTION OF NAD^+ BY SUCCINATE OR TMPD IN SONIC FRAGMENTS OF BEEF-HEART MITOCHONDRIA

Reaction mixture as described in text.

Inhibitor(s)	*Succinate* ⟶ *NAD^+*		*TMPD* ⟶ *NAD^+*	
	nmoles NADH/min per mg protein	*Inhibition (%)*	*nmoles NADH/min per mg protein*	*Inhibition (%)*
None	27.5	0	14.0	0
Hexylguanidine sulfate (28 μM)	13.0	53	8.0	43
Phenylethylbiguanide (170 μM)	27.0	2	13.0	7
Hexylguanidine sulfate (28 μM) + phenylethylbiguanide (170 μM)	12.0	56	5.0	64
Synthalin (22 μM)	27.1	2	13.5	3
Hexylguanidine sulfate (28 μM) + Synthalin (22 μM)	13.1	52	7.0	50
Synthalin (22 μM) + phenylethylbiguanide (170 μM)	22.9	17	9.1	35
Hexylguanidine sulfate (28 μM) + phenylethylbiguanide (170 μM) + Synthalin (22 μM)	10.5	62	3.5	75

inhibition by the phenylethylbiguanide is much larger in the case of the TMPD to NAD^+ reaction. This is also true for the diguanidine (Synthalin) but to a lesser extent. Thus in the presence of 28 μM hexylguanidine, 170 μM phenylethylbiguanide produces a 64% inhibition of the TMPD to NAD^+ reversal. This is 21% greater than with hexylguanidine alone. Under the same conditions, there is little change in the succinate to NAD^+ reaction in the presence of hexylguanidine when 170 μM phenylethylbiguanide is introduced. The fact that the concentrations of the guanidine inhibitors which produce significant inhibitions in these reversal reactions are lower than those required for the forward reactions, is consistent with the findings of CHANCE AND HOLLUNGER[20] on the high sensitivity of the succinate-linked reduction reaction to known uncoupling agents.

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Interaction between NADH and succinate during simultaneous oxidation by non-phosphorylating submitochondrial particles from bovine heart

KREBS *et al.*[1] and KULKA *et al.*[2] reported a marked stimulation by succinate of the aerobic reduction of acetoacetate to β-hydroxybutyrate catalysed by homogenates prepared from several types of tissues. These workers suggested that succinate can "dominate" the respiratory chain, and that it is oxidised preferentially over NAD-linked substrates. KREBS AND EGGLESTON[3] have interpreted their results as showing that this effect of succinate is not due to energy-dependent reduction of NAD^+ (ref. 4). On the other hand, ERNSTER and co-workers (see *e.g.*, refs. 5 and 6) in studies of the succinate-linked reduction of acetoacetate by liver mitochondria, concluded that the major part of this reaction proceeds by an endergonic mechanism which proceeds by way of succinate dehydrogenase, the NADH dehydrogenase flavoprotein, NAD^+, and β-hydroxybutyrate dehydrogenase.

Our studies were initiated to gain additional information on the relative involvement of energy coupling and of substrate concentration on the interactions between the succinate- and NADH-oxidase systems. This report describes the results of experiments designed to show interaction between NADH and succinate in submitochondrial particles which carry out electron transport without the involvement of energy-linked functions. An NADH-generating system was used to maintain a relatively constant concentration of NADH throughout the reactions. This system was particularly useful in obtaining data on interactions between succinate and NADH at low concentrations of NADH (*i.e.*, at concentrations which are rate limiting for oxidation by the oxidase system), since these conditions may more closely mimic those 'seen' by the respiratory chain enzymes *in situ*.

Submitochondrial particles were isolated from heavy beef heart mitochondria[7] after sonic irradiation in 0.15 M KCl, washed once in 1 M KCl and twice in 0.5 M potassium phosphate buffer (pH 7.4), and finally suspended in the latter to a final