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UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY A STABLE FREE RADICAL AND ITS DIAMAGNETIC HOMOLOG

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SUMMARY

The stable free radical 1,1′-diphenyl-2-picrylhydrazyl and its hydrazine homolog are very potent uncouplers of oxidative phosphorylation in whole mitochondria. These molecules exhibit many of the same effects on respiratory kinetics and ATPase as does 2,4-dinitrophenol, but at low concentrations near 10^{-7} M, and appear to act at a mechanistic locus common to other "true" uncouplers of oxidative phosphorylation.

Uncouplers of oxidative phosphorylation have a wide variety of molecular structures. Some of the more potent have been described by Heytler and co-worker[1,2], and by Williamson and Metcalf[3]. Despite the growing list of molecules found capable of uncoupling oxidative phosphorylation, their mode of action remains obscure.

A recent proposal on the mechanism of oxidative phosphorylation has centered around the possibility of free radical participation[4–6]. With such a hypothetical mechanism in mind, the experiments reported here relate to the uncoupling of oxidative phosphorylation by a stable free radical, as well as its non-radical homolog.

These data show that the radical 1,1′-diphenyl-2-picrylhydrazyl (DPPH), as well as the diamagnetic hydrazine homolog, behave almost identically as very potent respiratory stimulators and ATPase enhancers in the manner of classical uncouplers of oxidative phosphorylation, in whole rat liver mitochondria.

Rat liver mitochondria were prepared according to the method of Kielley and Kielley[7] in 0.25 M sucrose, and resuspended such that an average of 3.4 mg protein were employed per O_2 uptake incubation. The mitochondrial protein was determined by a modified biuret procedure[8] using bovine serum albumin and ovalbumin as standards.

O_2 uptake was measured polarographically with a YSI Model 53 oxygen monitor in a final volume of 3.0 ml at a controlled temperature of 25 °C. Respiratory control and P:O ratios were calculated graphically by the method of Chance and Williams[9].

Conditions for mitochondrial incubations are tabulated under the appropriate figure.

All reagents for these studies were the purest grades commercially obtainable.

Abbreviation: DPPH, 1,1′-diphenyl-2-picrylhydrazyl.

All biochemical reagents were purchased from Sigma Chemical Co. or Calbiochem. Quartz-redistilled deionized water was used throughout the work.

Fig. 1 shows typical oxygen electrode tracings obtained with DPPH, as well as with the non-radical hydrazine homolog (not shown in Fig. 1). At final concentrations of these agents slightly above 0.11 μM the kinetics of oxygen consumption were observed to be inhibited progressively with time after an initial rapid stimulation, in the absence of ADP. Such an inhibition of mitochondrial respiration has previously been observed with other well characterized uncouplers.

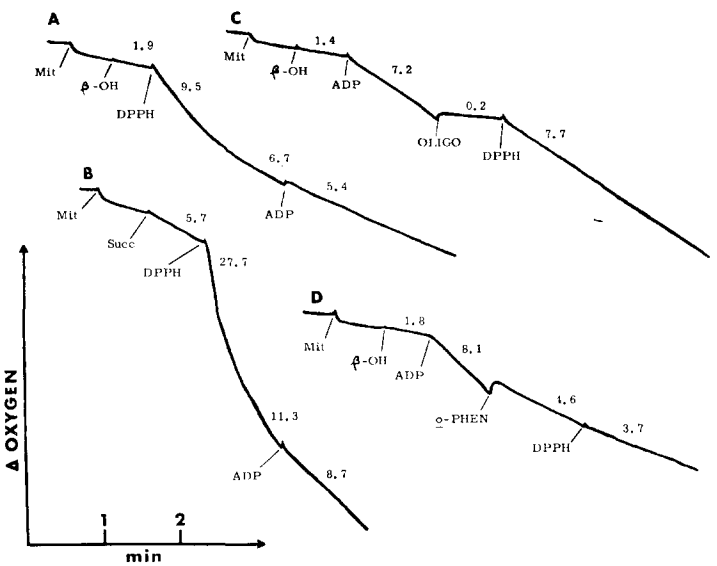

Fig. 1. Oxygen electrode tracings: effects on respiration of DPPH (as radical or hydrazine). Conditions: 3.0 ml; 25 °C; medium consisted of 250 mM sucrose, 10 mM P_i (as buffer, pH 7.39 at 23 °C) and 5 mM $MgCl_2$. Substrates were either succinate (2.5 mM) or β-hydroxybutyrate (5.0 mM). Trace A, 0.44 μM DPPH or hydrazine and 250 μM ADP; Trace B, 0.44 μM DPPH or hydrazine and 250 μM ADP; Trace C, 250 μM ADP, 0.5 μg/ml oligomycin and 0.11 μM DPPH or hydrazine; Trace D, 250 μM ADP, 1.6 mM *o*-phenanthroline and 0.11 μM DPPH or hydrazine. Incubations averaged 3.4 mg mitochondrial protein, and were magnetically stirred. The probe sleeve was reconstructed of Kel-F, since the uncouplers adhered to lucite even after repeated washing between incubations. Numbers beside tracings indicate rates of O_2 uptake in μatoms oxygen/min per mg protein per incubation.

Fig. 1 also demonstrates that the subsequent addition of ADP to DPPH- or hydrazine-stimulated respiration instantly resulted in a partial inhibition of the respiratory rate. The extent of this respiratory rate inhibition was observed to depend upon the uncoupler concentration added to the incubation in the presence of constant amounts of ADP and substrate. Several groups[10–12] have noted the ADP inhibition of uncoupler-stimulated respiration in mitochondria. Van Dam and Slater[11] in fact proposed that the inhibition might be attributable to the non-availability of energy, otherwise necessary for substrate transport across the mitochondrial outer membrane to the respiratory chain locus.

However, there still appears to be some ambiguity as to the reason for the lack

of correspondence between the uncoupler concentration promoting maximal respiration, and that causing maximal inhibition of P_i esterification in the coupled formation of ATP. It often appears that larger concentrations of uncoupler are required to inhibit phosphorylation than are necessary to promote maximal linear O_2 uptake (see, for example, Table I of ref. 3 for 2,4-dinitrophenol *vs* other uncouplers). Thus, maximal uncoupling is often associated with respiratory kinetics showing progressive inhibition of O_2 uptake.

Despite the inability to fully explain these interesting kinetic phenomena, it is clear from the data reported here that by choosing the appropriate empirical concentration-balancing conditions, one may ascertain a range of uncoupler concentrations, probably within a factor of two, which may be viewed as "optimal" for the uncoupler in question.

Such an approach was taken in the respiratory studies reported here, and the

TABLE I

RATE OF OXYGEN CONSUMPTION AS A FUNCTION OF UNCOUPLER CONCENTRATION

Incubations were in 3.0-ml volumes at 25 °C, pH 7.4, and contained 250 mM sucrose, 10 mM phosphate (as buffer), 5 mM $MgCl_2$, 2.5 mM succinate or 5.0 mM β-hydroxybutyrate, and 3.4 mg protein per incubation, in addition to the indicated uncoupling agents, added as ethanolic solutions. Incubations were magnetically stirred, and O_2 uptake determined with a Clark-type electrode, as shown in Fig. 1.

Substrate	*Additions*	*Concn (μM)*	*μatoms oxygen consumed/min per mg protein per incubation**		*Ratio: Initial rate / Final rate*
			Initial rate	*Final rate (+ADP)*	
Succinate	ADP (control)	250	20.0	—	—
β-Hydroxybutyrate	ADP (control)	250	8.1	—	—
Succinate	DPPH	0.67	23.7	7.3	3.2
	Hydrazine	0.67	23.4	8.0	2.9
	DPPH	0.44	31.5	11.0	2.9
	Hydrazine	0.44	28.2	11.3	2.5
	DPPH	0.22	23.6	23.6	1.0
	Hydrazine	0.22	22.0	22.0	1.0
	DPPH	0.11	19.6	19.6	1.0
	Hydrazine	0.11	17.6	17.6	1.0
β-Hydroxybutyrate	DPPH	0.67	10.3	7.0	1.5
	Hydrazine	0.67	9.6	7.0	1.4
	DPPH	0.44	9.3	7.0	1.3
	Hydrazine	0.44	8.9	5.7	1.5
	DPPH	0.22	9.6	7.0	1.4
	Hydrazine	0.22	9.9	8.4	1.2
	DPPH	0.11	7.2	7.2	1.0
	Hydrazine	0.11	7.7	7.7	1.0

* Mean values; 6 incubations each.

data are given in Table I. Initial rates and final rates refer to the resulting uncoupler-stimulated respiration, and the resulting uncoupler *plus* 250 μM ADP-induced respiratory rate, respectively.

In the vicinity of 0.3 μM DPPH or hydrazine, the O_2 uptake rates clearly became progressively inhibited with time, even without the later treatment with P_i acceptor. It can be observed from the table that for the NAD-linked substrate, DPPH or hydrazine concentrations approaching 0.1 μM offered such an "optimum" level for the uncoupling response on the respiratory rate, inasmuch as subsequent ADP treatment resulted in no inhibition (given by the ratio of 1.0 in the table's last column). For succinate oxidation, on the other hand, the uncoupler concentration "optimum" appeared to be somewhat higher.

The DPPH or hydrazine concentration displaying minimal perturbation of a linear respiratory rate in the presence of P_i, with ADP added afterward, was taken as that optimal concentration necessary for the superficial evaluation of uncoupling potency for these experiments.

This method of evaluating uncoupler potency achieves empirical validity from results obtained by the author, Coleman, P. S. (unpublished observations) on $^{32}P_i$ incorporation employing the classical uncoupler 2,4-dinitrophenol. In reference to these unpublished $^{32}P_i$ incorporation experiments, a concentration of 25 μM 2,4-dinitrophenol yielded a stimulated respiratory rate approximately equal to that given by the presence of ADP alone in control incubations. At this concentration of 2,4-dinitrophenol the subsequent addition of ADP to uncoupler-triggered rapid respiration was observed to yield a ratio of initial to final O_2 consumption rates very close to 1.0 (0.97 ± 0.05). Yet, this same uncoupler concentration inhibited the phosphorylation reactions by only 82%. On the other hand, for 2,4-dinitrophenol concentrations greater than 30 μM, the addition of ADP to the uncoupled mitochondria (after linear respiration was established) displayed inhibited O_2 uptake kinetics, in agreement with Nijs[12] and Wenner[13].

These findings suggest that both DPPH and the non-radical hydrazine homolog behave as do other uncouplers of oxidative posphorylation, and possess a respiratory stimulating potency more than 250 times that of the classical 2,4-dinitrophenol.

Further investigation with these uncouplers showed that oligomycin-inhibited respiration could be overcome by DPPH (as the free radical or the hydrazine), while *o*-phenanthroline-inhibited respiration with NAD-linked substrates could not. Such results are consistent with the proposed chemical intermediate hypothesis[14,15] of coupling electron transfer to phosphate esterification.

Fig. 2 concerns the effect of *o*-phenanthroline with respect to uncoupler-mediated respiration. Although required in relatively high doses, 1.6 mM *o*-phenanthroline severely inhibited NAD-linked β-hydroxybutyrate oxidation in the presence of uncoupling quantities of either 2,4-dinitrophenol or DPPH. Kawakita and Ogura[16] place the site of action of the phenanthroline at an electron transfer chain locus on the oxygen side of a non-heme iron moiety affiliated with NADH:cytochrome *c* reductase (EC 1.6.99.3). Oligomycin is thought to block the oxidative phosphorylation sequence at a site between that proposed for uncoupler activity and that for terminal phosphorylation of ADP[17].

Fig. 3 demonstrates the large stimulatory effect of DPPH or the hydrazine on mitochondrial ATPase, which aids in characterizing these molecules as true uncou-

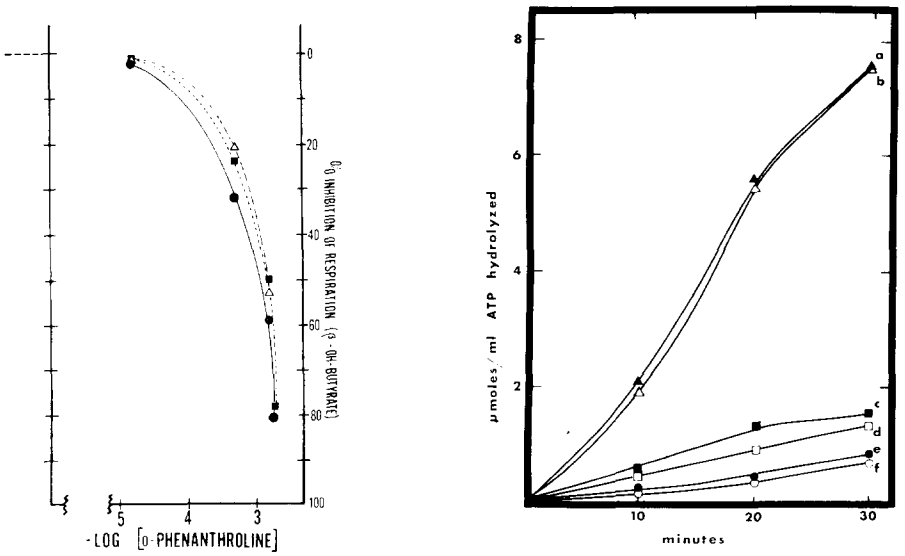

Fig. 2. Effect of *o*-phenanthroline on β-hydroxybutyrate respiration in the presence of uncoupling agents. Incubations were performed as described for Fig. 1 and Table I. ●—●, 250 μM ADP + *o*-phenanthroline; ■---■, 0.11 μM DPPH + *o*-phenanthroline; △---△, 25 μM 2,4-dinitrophenol + *o*-phenanthroline.

Fig. 3. Effect of DPPH or non-radical hydrazine on mitochondrial ATPase. Incubations were performed in a shaking bath at 23 °C in a total volume of 5.0 ml, which contained 10 mM ATP, 5 mM $MgCl_2$, 100 mM sucrose and 20 mM Tris buffer, (pH 7.23). Each incubation contained an average of 1.74 mg protein/ml. 1-ml aliquots were removed at 10-min intervals, and the reaction immediately quenched with 1.0 ml cold trichloroacetic acid (10%, w/v). P_i release was assayed according to Fiske–SubbaRow[19], and corrected for non-enzymic hydrolysis of ATP. DPPH, the hydrazine homolog, oligomycin or *o*-phenanthroline were added as ethanolic solutions, never exceeding 5% of the total volume in ethanol. An independent experiment could not distinguish between control incubations run over the same time course in either the presence or absence of 5% ethanol. ▲—▲, 1 μM DPPH or hydrazine; △—△, 1 μM DPPH or hydrazine + 2 mM *o*-phenanthroline; ■—■, 2 mM *o*-phenanthroline; □—□, 0.5 μg/ml oligomycin + 1 μM DPPH or hydrazine; ●—●, control (no additions); ○—○, 0.5 μg/ml oligomycin.

plers. As expected from the respiratory data, *o*-phenanthroline did not inhibit the DPPH-stimulated ATPase activity, whereas the presence of oligomycin did display inhibition.

These data point to a site of uncoupling activity of DPPH and the hydrazine between those oxidative phosphorylation loci affected by *o*-phenanthroline and oligomycin, in agreement with general proposals on the manner in which other true uncouplers behave[15].

There is an obvious topological similarity between the phenylhydrazones of carbonyl cyanide and the non-radical diphenylpicryl hydrazine employed in these studies. An intriguing question arising from the data reported here concerns the role, if any, of free radical participation in the coupling–uncoupling mechanism. The hypothesis put forth by Wang and co-workers[4–6] based on model experiments, implicates imidazolyl and possibly flavinyl species, endogenous to the mitochondrial composition, as active participants in the coupling scheme. The conceivable existence

of phenylhydrazone derivatives as free radical moieties, in addition to the DPPH homologs discussed here, provides a preliminary conceptual framework upon which to examine, in more detail, the mechanism of uncoupling. Indeed, many, if not all, of the known uncouplers of oxidative phosphorylation display structures capable of resonance stabilization of an unpaired electron[18], and even a tenuous relationship between a free radical coupling–uncoupling phenomenology warrants closer examination.

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