A NEW CLASS OF UNCOUPLING AGENTS - CARBONYL CYANIDE PHENYLHYDRAZONES

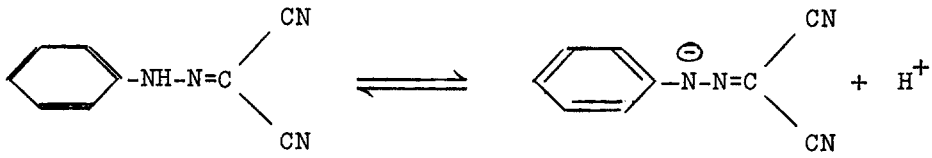
P. G. Heytler and W. W. Prichard

Central Research Department *
E. I. du Pont de Nemours and Company, Inc.
Wilmington, Delaware

Received March 21, 1962

We have observed that many ring-substituted derivatives of carbonyl cyanide phenylhydrazone (CCP) strongly inhibit transport processes in plant tissues and microorganisms, depress growth and stimulate respiration (1). Investigation of these effects led to the finding, reported here, that these compounds are extremely effective uncouplers of oxidative phosphorylation in mitochondrial systems. In addition, CCP derivatives inhibit cyclic photophosphorylation in spinach chloroplasts, a system which is relatively resistant to most uncouplers.

At least one of these materials, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (p-CF_3O-CCP) exhibits uncoupling effects at concentrations down to 10^{-8} molar. To our knowledge, this is the most potent synthetic uncoupling agent yet reported.



Carbonyl cyanide phenylhydrazone (CCP)

* Contribution No. 785

Experimental Methods

Carbonyl cyanide phenylhydrazones were synthesized by diazotizing the corresponding aniline and coupling the resulting diazonium salt with malononitrile in mildly basic aqueous solution. The yellow CCP derivatives precipitated on acidification and were recrystallized from benzene or chloroform. The stable aqueous solutions of the sodium or "tris" salts were used in the assays. Mitochondria were prepared from mouse liver by homogenizing in 5 volumes 0.25 M sucrose + .001 M EDTA and centrifuging at 1,000 x g for 12 minutes and 10,000 x g for 20 minutes. The mitochondria sedimented in the latter step were washed once with 0.25 M sucrose.

Beef heart mitochondria were prepared by Green's technique (2), with the addition of .001 M EDTA to the homogenizing medium.

Oxidative phosphorylation runs were made at 25 or 26° C. for 15 minutes. The 3 ml system contained, in μmols/ml: 50 succinate or 17 β-OH-butyrate; 17 P_i(pH 7.4); 0.3 ADP; 67 glucose; 67 KCl; 3 $MgCl_2$; 0.1 cyt. c; 7 NaF; 17 KM units/ml hexokinase; 0.4-1 mg/ml mitochondrial N. The reaction was stopped by rapid chilling and 25,000 x g centrifugation. Succinate oxidation was measured manometrically in a Warburg apparatus. Oxidation of β-hydroxybutyrate was determined by colorimetric assay of acetoacetate (4). Glucose-6-phosphate formed was assayed spectrophotometrically by the reduction of TPN in the presence of glucose-6-phosphate dehydrogenase (Sigma, type III).

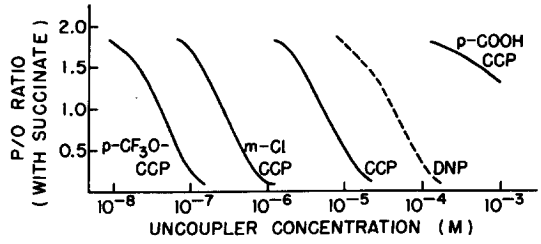
The possible inhibition of either hexokinase or glucose-6-phosphate dehydrogenase by the CCP derivatives was carefully checked and eliminated as a source of error.

Spinach chloroplasts were isolated, and the PMS catalyzed photophosphorylation determined by the methods of Jagendorf and Avron (3).

Results

Figure 1 illustrates the sensitivity of succinoxidase-coupled phosphorylation of mouse liver mitochondria to several CCP uncouplers and to 2,4-dinitrophenol. Almost identical results were obtained with freshly prepared mitochondria from several animal and plant sources. Frozen-thawed preparations (e.g. beef heart mitochondria) were somewhat more sensitive to all these agents.

Figure 1



The profound influence of ring substituents on the uncoupling efficiency of the CCP is seen clearly in this graph. About eighty such derivatives, with activities throughout the range illustrated, have been synthesized and a study of the relation of structure to activity is being made.

CCP uncoupling of phosphorylation in DPN-linked oxidations is demonstrated in Table 1. The sensitivities of the three phosphorylation sites of the electron transport chain were essentially equal in our experiments.

Photophosphorylation by isolated spinach chloroplasts in the presence of phenazine methosulfate is also strongly inhibited by CCP uncouplers (Table 2). In this system, dinitrophenol causes no inhibition and, in fact, can show cofactor activity (5).

The mechanism of action of the CCP derivatives on these phosphorylation processes is being investigated and results will be published in greater detail elsewhere.

Table 1

Uncoupling of Frozen-Thawed Beef Heart Mitochondria

	Δ Aceto-acetate	ΔG1-6-P	P/O
Control (β-OH-butyrate as substrate)	.44μmols	1.24μmols	2.8
"	.42	1.24	2.9
+ 0.1 x 10^{-6}M m-Cl-CCP	.42	.60	1.4
+ 0.3 " "	.40	.20	0.5
+ 0.01 " p-CF_3O-CCP	.44	.72	1.6
+ 0.03 " "	.48	.38	0.8
+ 0.1 " "	.46	.08	0.2

Table 2
Photosynthetic Phosphorylation

	P/Chl
Control	88
"	82
+ 0.16 x 10^{-6}M m-Cl-CCP	73
+ 0.5 x 10^{-6}M m-Cl-CCP	67
+ 1.6 x 10^{-6}M m-Cl-CCP	38
+ 5.0 x 10^{-5}M m-Cl-CCP	17
+ 1.0 x 10^{-4}M DNP	108

(P/Chl = μmols P_i esterified/hr./mg chlorophyll)

We wish to thank Dr. C. W. Todd for suggestions and encouragement in the course of this work.

References

1. Paper in preparation.
2. D. E. Green, Advances in Enzymology Vol XXI, p. 17 (1959).
3. A. T. Jagendorf & M. Avron, J. Biol. Chem. 231, 277 (1958).
4. P. G. Walker, Biochem. J. 58, 699 (1954).
5. J.S.C. Wessels, Biochim. Biophys. Acta 36, 264 (1959).