Roland, J. F., and Grove, A. M. (1954), *Anal. Chem. 26,* 502.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Spies, J. R., and Chambers, D. C. (1949), *Anal. Chem. 21,* 1249.
Stein, W. H., and Moore, S. (1949–50), cited by Craig, L. C. *in Fortschr. Chem. Forsch. 1,* 312.

Uncoupling of Oxidative Phosphorylation by Carbonyl Cyanide Phenylhydrazones. II. Effects of Carbonyl Cyanide *m*-Chlorophenylhydrazone on Mitochondrial Respiration

R. A. GOLDSBY AND P. G. HEYTLER

From the Central Research Department, E. I. du Pont de Nemours and Company*

Received March 25, 1963

The effects of carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP) on respiration in rat liver mitochondria has been studied polarographically and manometrically. Experiments with the oxygen electrode have shown that 2 μM *m*-Cl-CCP abolishes respiratory control when β-hydroxybutyrate, a glutamate-malate mixture, or succinate is used as a substrate. A similar concentration of *m*-Cl-CCP reverses the inhibition of respiration by oligomycin. The depression by *m*-Cl-CCP of succinate oxidation has been shown to be prevented by addition of either cysteine sulfinic acid or lipoic acid. However, neither of these agents protects against uncoupling by *m*-Cl-CCP. The ability of a fixed amount of *m*-Cl-CCP to abolish respiratory control and lower the P/O ratio is dependent on the amount of mitochondrial protein in the system. However, respiratory control is somewhat more sensitive to *m*-Cl-CCP than is phosphate esterification. The ATP-P_i^{32} exchange reaction is also inhibited by *m*-Cl-CCP. The possible site of carbonyl cyanide phenylhydrazone action is discussed in relation to these data.

The phenomenon of respiratory control as defined and studied by Chance and his collaborators (Chance and Williams, 1956) has been of great value in the study of oxidative phosphorylation. Through studies of respiratory control, it is possible to discriminate between substances that inhibit oxidative phosphorylation, such as guanidines (Pressman, 1963; Hollunger, 1955), and true uncouplers of this process, i.e., 2,4-DNP. Polarographic observation of respiration provides useful information about the mode of action of inhibitory substances known to prevent phosphate esterification; e.g., Estabrook's (1961) studies with oligomycin.

With these considerations in mind, a study of the effects of *m*-Cl-CCP on mitochondrial respiration and respiratory control has been conducted and is reported here. In addition to studies of CCP effects on respiration, it was felt that useful information might be obtained by a study of the ATP-P_i^{32} exchange reaction described by Boyer *et. al.* (1956). An earlier paper (Heytler, 1963) reported the effect of *m*-Cl-CCP on several other aspects of mitochondrial oxidative phosphorylation.

Experimental

Mitochondria used in these studies were obtained from the livers of young adult male rats. The method of isolation is outlined in the previous paper (Heytler, 1963).

Oxidation of substrate was measured either by direct determination of oxygen uptake or in some cases by colorimetric measurements of acetoacetate (Walker, 1954). Direct measurements of oxygen uptake were obtained manometrically by standard Warburg technique, or polarographically with a modified Clark electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio).

Determinations of phosphate uptake were made by measuring the disappearance of inorganic phosphate from the medium (Taussky and Schoor, 1953). Protein determinations were made according to the method of Lowry *et al.* (1951).

The ATP-P_i^{32} exchange was conducted under a nitrogen atmosphere at 20°. Incorporation of labeled phosphate into ATP was measured after removal of inorganic phosphate by solvent extraction (Nielson and Lehninger, 1955). Radioactivity determinations were performed by drying aliquots of P^{32}-containing solutions on aluminum planchets and counting with a thin-window GM tube.

The carbonyl cyanide *m*-chlorophenylhydrazone used in this study was synthesized by Dr. W. W. Prichard of this laboratory by the method described in a previous report (Heytler and Prichard, 1962). The oligomycin was the generous gift of Dr. H. A. Lardy. All other chemicals used in these investigations were obtained from commercial sources in the highest purity routinely available.

Results

Polarographic Studies of Coupled Mitochondria.— The polarographic tracing of Figure 1 shows that the mitochondria used in these studies exhibit respiratory control[1] with respect to both ADP and P_i. Figure 2 shows that the respiratory control of β-hydroxybutyrate oxidation by ADP is abolished by 2 μM *m*-Cl-CCP. As shown in Figure 3, the respiration of a coupled mitochondrial system deficient in ADP and P_i is markedly stimulated by 2 μM *m*-Cl-CCP. The stimulation of respiration is seen immediately (<2 seconds) after the introduction of *m*-Cl-CCP to the electrode chamber.

The antibiotic oligomycin A has been shown to block the respiration of coupled mitochondria (Lardy *et al.*, 1958). This block is not removed by arsenate (Estabrook, 1961), an uncoupler which acts at the level of phosphate. However, 2,4-DNP, which ap-

* Contribution No. 839.

[1] Respiratory control is defined as that physiological state in which ADP and P_i are rate limiting for mitochondrial respiration.

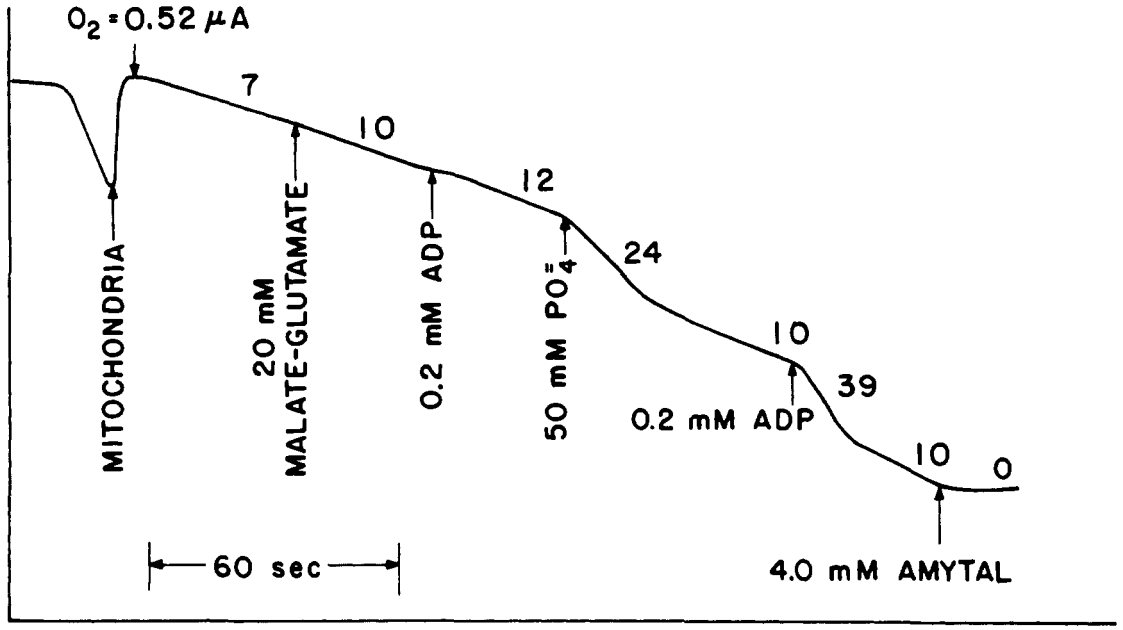

FIG. 1.—Polarographic tracing of malate-glutamate oxidation by mitochondria. The 1-cc electrode chamber contained the following reaction medium: 5 mM $MgCl_2$; 100 mM Tris, *p*H 7.4; and 80 mM KCl. Other components were added as indicated and 2.8 mg rat liver mitochondrial protein was added. The rates of oxygen uptake indicated are in μatoms oxygen/min × 10^2. The temperature was 25°.

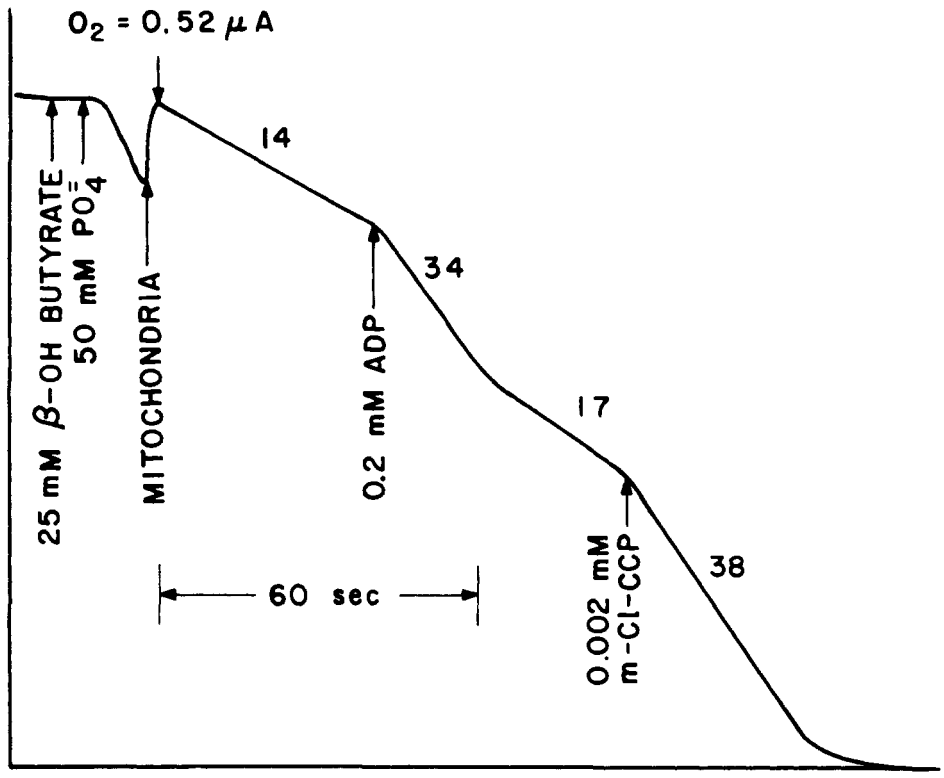

FIG. 2.—Effect of *m*-Cl-CCP on β-hydroxybutyrate oxidation. Rat liver mitochondrial protein (2.0 mg) was added to the system. Other conditions as in Figure 1.

pears to act prior to phosphate (Lardy and McMurray, 1959) in the sequence of reactions comprising oxidative phosphorylation, relieves the oligomycin-induced inhibition of respiration. Figure 4 shows that 2.4 × 10^{-6} M *m*-Cl-CCP similarly releases the oligomycin block and gives maximal stimulation of respiration.

Figure 5 shows that under the conditions of these experiments succinate oxidation exhibits respiratory control with respect to ADP even in the presence of 4 mM Amytal. The figures also show that addition of 0.002 μmole of *m*-Cl-CCP to the system gives maximal stimulation of succinate oxidation in the presence or absence of Amytal. This stimulation of succinate oxidation by *m*-Cl-CCP was consistently observed during polarographic runs lasting 2 or 3 minutes. However, in runs of longer duration it was noticed that addition of *m*-Cl-CCP to mitochondria-oxidizing succinate gave a transitory stimulation of respiration followed by a progressive inhibition of succinate oxidation.

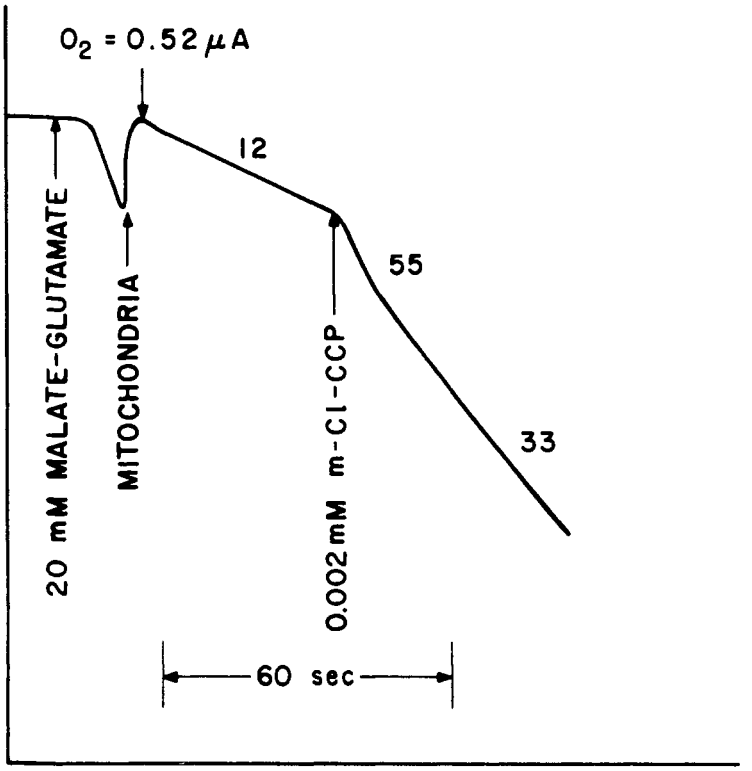

FIG. 3.—Effect of *m*-Cl-CCP on malate-glutamate oxidation. Rat liver mitochondrial protein (2.2 mg) was added to the system. Other conditions were the same as described for Figure 1.

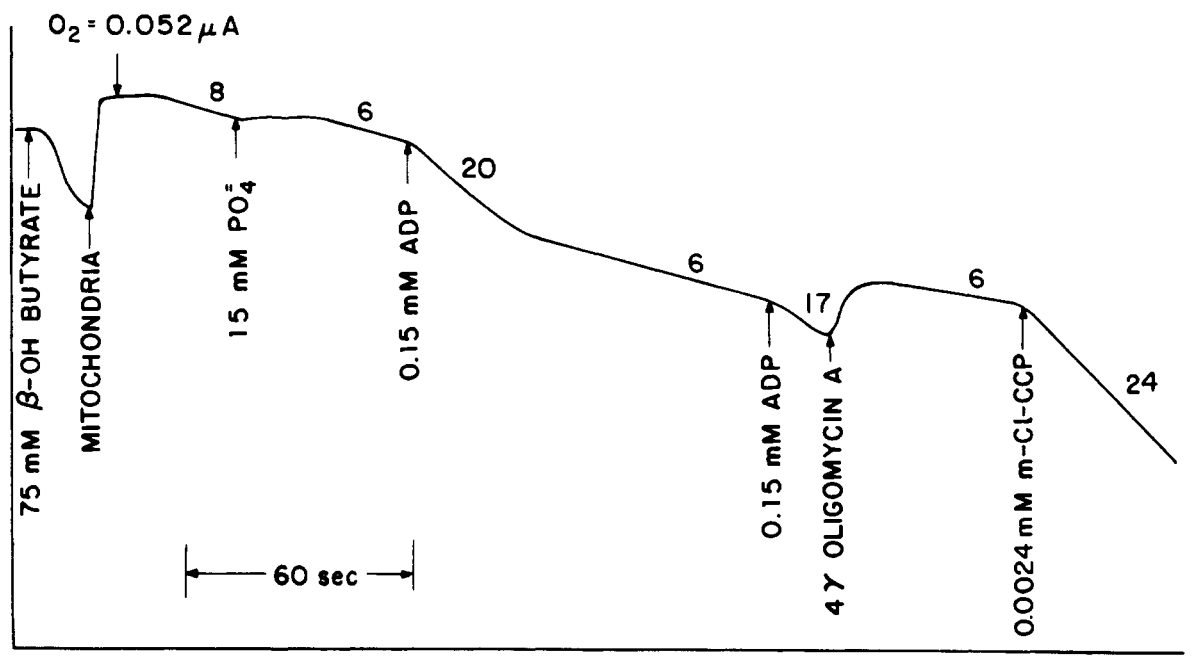

FIG. 4.—Effect of *m*-Cl-CCP on oligomycin-blocked respiration. Rat liver mitochondrial protein (2.3 mg) was added to the system. Other conditions were as in Figure 1.

In order to investigate the longer term effects of *m*-Cl-CCP on succinate oxidation, manometric studies were carried out.

Manometric Studies of Succinate Oxidation.—Table I shows that in a 27-minute manometric determination of succinate oxidation 1.9×10^{-6} M *m*-Cl-CCP caused a 36% decrease in oxygen uptake as well as complete uncoupling. The *m*-Cl-CCP inhibition of succinate oxidation was completely abolished by 3.8×10^{-3} M cysteine sulfinic acid, which did not protect the mitochondria from the uncoupling action of *m*-Cl-CCP. Addition of 1×10^{-3} M lipoic acid also protected against the inhibition of succinoxidase activity by *m*-Cl-CCP but not against uncoupling by this reagent.[2]

Effect of m-Cl-CCP on the ATP-P_i^{32} Exchange.—In phosphorylating mitochondria, the ATP-P_i^{32} exchange reaction has been interpreted as a reversal of the reactions of oxidative phosphorylation. The data in Table II demonstrate that this exchange is inhibited by 4.8×10^{-6} M *m*-Cl-CCP. Cysteine (10^{-3} M)

[2] P. G. Heytler, unpublished results.

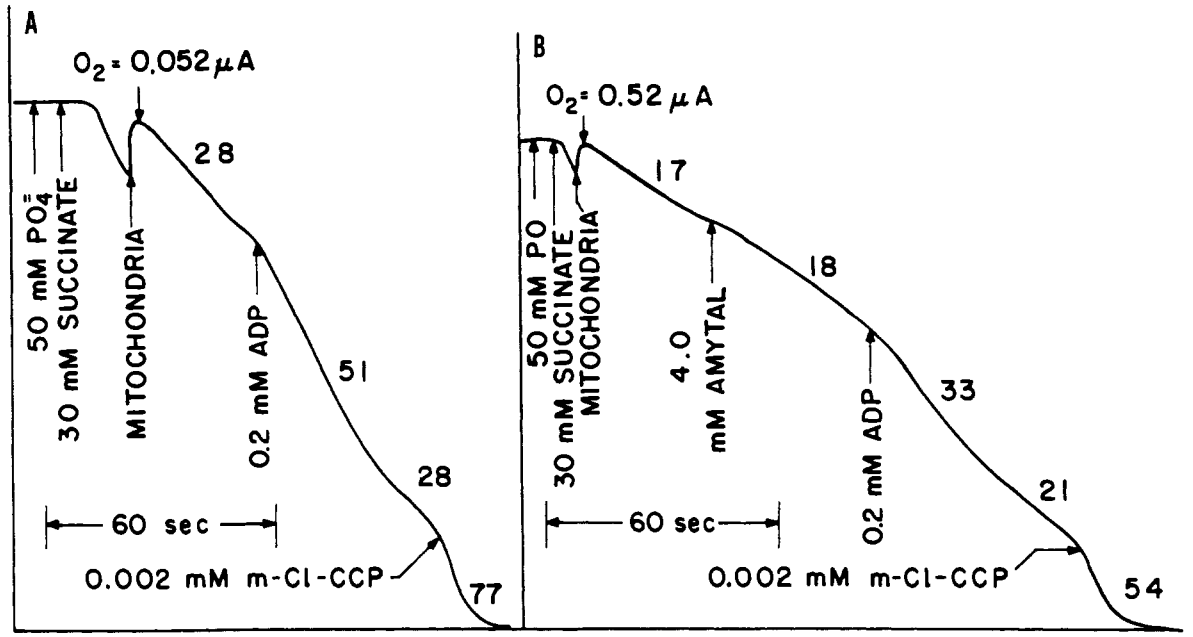

FIG. 5.—Respiratory control of succinate oxidation. Rat liver mitochondrial protein (A, 1.7 mg; B, 1.1 mg) was added. Other conditions as in Figure 1.

partially protects the ATP-P_i^{32} exchange against the inhibitory action of *m*-Cl-CCP. The protection is not complete because at this level cysteine itself is inhibitory to the exchange reaction. This observation is consistent with previous reports (Maley and Lardy, 1954) that at concentrations above 1×10^{-3} M cysteine begins to uncouple oxidative phosphorylation.

Effect of Mitochondrial Protein Concentration on the Efficiency of m-Cl-CCP Uncoupling.—Using the same mitochondrial preparation, parallel determinations were made of the effect of mitochondrial protein concentration on the ability of *m*-Cl-CCP to abolish respiratory control and lower the P/O ratio. Figure 6 shows that the capacity of *m*-Cl-CCP to stimulate oxygen uptake, in the absence of ADP and P_i, is dependent on the amount of mitochondrial protein in the system. Table III demonstrates that the effect of a fixed amount of *m*-Cl-CCP on the P/O ratio is also a function of mitochondrial protein concentration. Comparison of Table III with Figure 6 shows that the P/O ratio is less sensitive to *m*-Cl-CCP than is respiratory control. This is especially true at the lower concentrations of mitochondrial protein.

TABLE I

EFFECT OF *m*-Cl-CCP AND CSA[a] ON SUCCINATE OXIDATION

Each vessel contained: 1.4 mg of rat liver mitochondrial protein; 5 mM $MgCl_2$; 100 mM KCl; 16 mM KH_2PO_4; 0.5 mM ADP; 0.015 mM cytochrome c; 50 mM Tris; 10 mM NaF; 45 K.M. units[b] hexokinase (Sigma Type III); 100 mM glucose; 10 mM succinate; other additions were as indicated. The total volume of liquid in each Warburg vessel was 2.6 ml and the *p*H was 7.4. The experiment was run at 30° for 27 minutes, then stopped by addition of 0.1 ml of 50% TCA.

No.	*m*-Cl-CCP ($\times 10^{-6}$ M)	CSA[a] ($\times 10^{-3}$ M)	P/O	μAtoms of Oxygen Consumed	Oxygen Uptake (%)
1			1.4	16.3	100
2	1.9		0	10.5	64
3		3.8	1.7	23	140
4	1.9	3.8	0	20.7	126

[a] Cysteine sulfinic acid.
[b] K.M. units = Kunitz-MacDonald units.

DISCUSSION

Oxidative phosphorylation may be considered to involve three stages (Racker, 1961): (*a*) Formation of a configuration of high chemical potential as a result of electron transport from a carrier of low to a carrier of higher electrode potential. (*b*) Reaction of the high-energy configuration with phosphate ion to form a high-energy phosphate. (*c*) Reaction of precursor high-energy phosphate with ADP to form ATP.

These stages, which proceed sequentially and may involve more than one reaction at each stage, may be schematized as follows:

$$\overset{2e^-\quad 2e^-}{\text{X} \rightleftharpoons \text{X}^*} \qquad (a)$$

$$\text{X}^* + \text{P}_i \rightleftharpoons \text{X} \sim \text{P} \qquad (b)$$

$$\text{ADP} + \text{X} \sim \text{P} \rightleftharpoons \text{ATP} + \text{X} \qquad (c)$$

Reaction (*a*) involves the link which establishes communication between the electron transport and phosphorylation systems. It represents the mechanism by which oxidation or reduction in the electron transport chain is utilized to energize X to X*. The need

TABLE II

EFFECT OF *m*-Cl-CCP ON ATP-P_i^{32} EXCHANGE

Each vessel contained: 0.71 mg rat liver mitochondrial protein; 5.72 mM K_2HPO_4 (4.2×10^6 cpm/μmole); 2.85 mM ATP; 19 mM Tris; 5.71 mM $MgCl_2$; 197 mM sucrose. Other additions were as indicated. The *p*H was 7.4. The exchange was conducted for 15 minutes at 20°. The gas phase was nitrogen.

No.	Additions	ATP^{32} (cpm)	Control ATP-P_i^{32} Exchange (%)
1	None	5.83×10^5	100
2	1×10^{-4} M 2,4-DNP	6.17×10^4	10.6
3	4.8×10^{-6} M *m*-Cl-CCP	1.14×10^5	19.6
4	4.8×10^{-6} M *m*-Cl-CCP 1×10^{-3} M cysteine	3.78×10^5	64.8
5	1×10^{-3} M cysteine	3.1×10^5	53.0

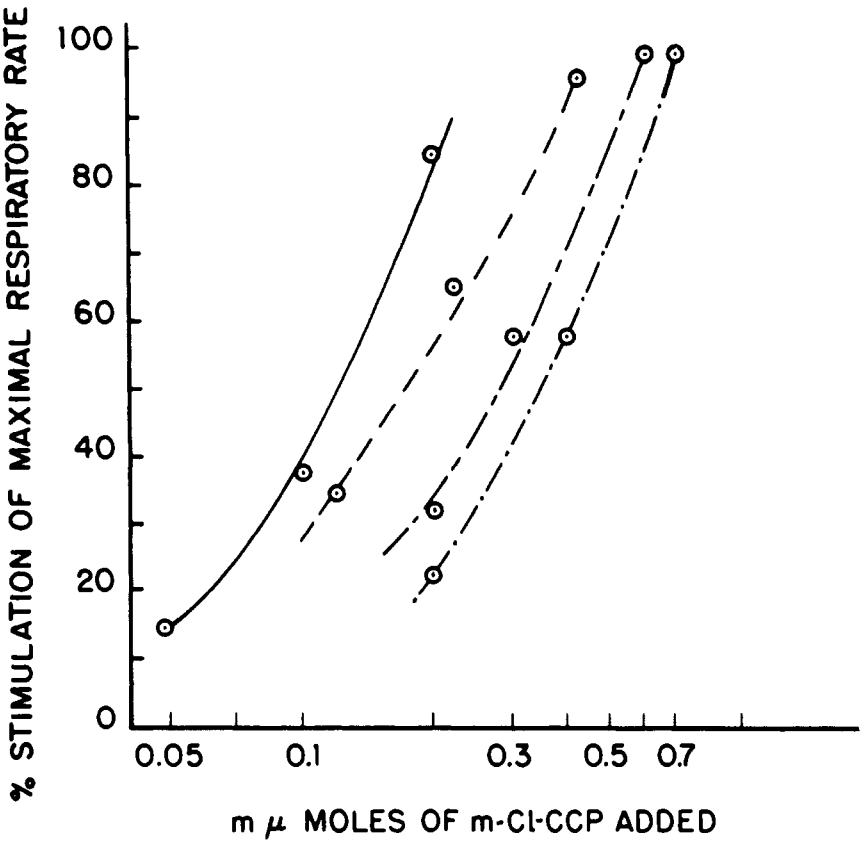

FIG. 6.—Effect of mitochondrial protein on ability of *m*-Cl-CCP to stimulate respiration. Mitochondrial suspension was added to the buffered isotonic medium described in Figure 1. *m*-Cl-CCP was then added in successive small increments until a maximal rate of respiration was obtained. The curves were obtained at the indicated amounts of mitochondrial protein: —— 1.8 mg; — — — 3.6 mg; — - — 5.7 mg; —·—· 7.2 mg.

for postulating step (*a*) has been made more apparent by observations in submitochondrial systems. During oxidative phosphorylation in certain submitochondrial systems, soluble, nonphosphorylated protein intermediates capable of synthesizing ATP from ADP and P_i are formed during electron transport (A. Smith and G. Webster, 1962, personal communication). However, these high-energy intermediates are not an obligatory part of the electron transport chain.

One of the objectives of our studies of CCP uncoupling has been to determine which stage of oxidative phosphorylation is directly affected by CCP. The data presented in the Results section of this paper have shown that *m*-Cl-CCP can abolish respiratory control in mitochondria showing a dependency on ADP or P_i. Since the respiratory effects of *m*-Cl-CCP are seen in the presence or absence of added P_i or ADP, it does not seem likely that the primary action of this uncoupler is on the terminal (*c*) stage of oxidative phosphorylation. An effect at the (*b*) stage would imply interference with the formation of X ~ P, or as with arsenate, competition with P_i resulting in an unstable X ~ inhibitor complex (Racker, 1961). There is evidence (Lardy and McMurray, 1959; Suelter *et al.*, 1961) that oligomycin prevents the breakdown of the X ~ P intermediate or its arsenate analog. Arsenate is thus unable to evoke respiration in mitochondrial systems inhibited by oligomycin (Estabrook, 1961). However, low concentrations of *m*-Cl-CCP are able to elicit maximal respiration in oligomycin-blocked mitochondria. This indicates that *m*-Cl-CCP does not act by forming an unstable X ~ CCP analog of X ~ P; rather, *m*-Cl-CCP, like 2,4-DNP, must act early in the mitochondrial phosphorylation process at stage (*a*). This conclusion is supported by the previously mentioned stimulation by CCP of P_i-limited respiration in tightly coupled mitochondria. It may disrupt the coupling link between electron transport and the X → X* transformation or it may effect a rapid breakdown of X* to X.

Whether the degree of uncoupling is determined by direct measurement of the P/O ratio or by the more indirect techniques of polarography, the uncoupling efficiency of a fixed amount of *m*-Cl-CCP goes down as the amount of mitochondrial protein in the system increases. Such behavior indicates that *m*-Cl-CCP is readily bound by mitochondrial sites and is consistent

TABLE III

EFFECT OF MITOCHONDRIAL CONCENTRATION ON *m*-Cl-CCP REDUCTION OF P/O RATIO

Each vessel contained the following components: β-OH butyrate, 16 mM; KH_2PO_4, 25 mM; $MgCl_2$, 5 mM; Tris, 50 mM; KCl, 100 mM; glucose, 100 mM; 45 K.M.[a] units hexokinase (Sigma, Type III); NaF, 10 mM; ADP, 0.5 mM; cytochrome c, 0.015 mM; mitochondria and *m*-Cl-CCP were added to the levels indicated. The *p*H was 7.4 and the volume was 3.0 ml. The experiment was run at 25° for 30 minutes, then stopped by addition of 0.1 ml of 50% TCA.

No.	Mitochondrial Protein (mg)	*m*-Cl-CCP (mμmoles)	Control P/O (%)
1	1.8	0.4	15
2	3.6	0.4	25
3	5.4	0.4	38
4	7.2	0.4	51

[a] K.M. units = Kunitz-MacDonald units.

with the notion that *m*-Cl-CCP exerts its uncoupling action by reaction with mitochondrial sites. However, nonspecific binding or solubility in the lipid matrix may contribute significantly to the concentration dependencies shown here.

Data presented in the Results section of this paper show that *m*-Cl-CCP is a much less potent inhibitor of the ATP-P_i^{32} exchange reaction than of oxidative phosphorylation. In the presence of 4.86×10^{-6} M *m*-Cl-CCP, an order of magnitude higher concentration than needed for complete uncoupling, 20% of ATP-P_i^{32} exchange activity remained. Studies with potassium actractylate in rat liver mitochondria (Vignais *et al.*, 1961) have shown that this reagent will cause 90% inhibition of the ATP-P_i^{32} reaction with only 10% inhibition of oxidative phosphorylation. Work in certain submitochondrial systems (Webster, 1962) strongly suggests that the ATP-P_i^{32} exchange reaction may be ancillary but not central to oxidative phosphorylation. Viewed in light of these findings, the differential sensitivities of oxidative phosphorylation and ATP-P_i^{32} excange to *m*-Cl-CCP may be accommodated.

During polarographic investigations of succinate oxidation, we observed a dependence of succinate oxidation on ADP which was abolished by addition of *m*-Cl-CCP. Abolition of respiratory control of succinate oxidation has been reported (Hatefi *et al.*, 1961) upon addition of 3 mM Amytal, with the conclusion that respiratory control of succinate oxidation is an artifact resulting from the utilization of pyridinoprotein-linked substrates present or produced during the oxidation of this substrate. We have observed no diminution in the respiratory control of succinate oxidation in the presence of 4 mM Amytal. This level of Amytal completely abolished respiration when a malate-glutamate mixture was used as substrate. Our results indicate that in rat liver mitochondria respiratory control is associated with succinate oxidation. This is consistent with Packer's observation (Packer and Denton, 1962) that in antimycin-blocked mitochondria the oxidation of cytochrome c shows respiratory control.

The results reported here show depression of the oxidation of succinate by *m*-Cl-CCP unless cysteine sulfinic acid is added to the system. In the presence of cysteine sulfinic acid, *m*-Cl-CCP no longer inhibits respiration but still uncouples oxidative phosphorylation. This behavior is analogous to the inhibitory effect of 2,4-DNP on succinate oxidation, which has been attributed to the accumulation of oxlaoacetate, an inhibitor of succinic dehydrogenase (Krebs, 1961). In mitochondria, oxaloacetate is rapidly converted, via transamination, to aspartate when cysteine sulfinic acid is present (Singer and Kearney, 1955). Such an accumulation of oxaloacetate in the presence of *m*-Cl-CCP would suggest inhibition of the condensing enzyme complex by this uncoupler. Since lipoic acid relieves the inhibition of succinate oxidation by *m*-Cl-CCP one might suppose that it is the lipoate segment of the condensing enzyme complex that is affected by this inhibitor. The demonstration that *m*-Cl-CCP is able to inhibit enzymatic processes other than those of oxidative phosporylation suggests that at higher concentrations CCP's may show a broader pattern of activity. This possibility is being examined in a number of different enzyme systems.

References

Boyer, P. D., Luchsinger, W. W., and Falcone, A. B. (1956), *J. Biol. Chem. 223*, 405.

Chance, B., and Williams, G. R. (1956), *Advan. Enzymol. 16*, 65.

Estabrook, R. W. (1961), *Biochem. Biophys. Res. Commun. 4*, 89.

Hatefi, Y., Jurtshuk, P., and Haavik, A. G. (1961), *Arch. Biochem. Biophys. 94*, 148.

Heytler, P. G. (1963), *Biochemistry 2*, 357.

Heytler, P. G., and Prichard, W. W. (1962), *Biochem. Biophys. Res. Commun. 7*, 272.

Hollunger, G. (1955), *Acta Pharmacol. Toxicol. (Suppl. 1 11 (1955)*, 84.

Krebs, H. A. (1961), Presentation to Robert A. Welch Foundation Conference on Chemical Research, December 4, 1961.

Lardy, H. A., Johnson, D., and McMurray, W. C. (1958), *Arch. Biochem. Biophys. 78*, 587.

Lardy, H. A., and McMurray, W. C. (1959), *Fed. Proc. 18*, 269.

Lowry, O. H., Rosebrough, H. J., Farr, L. A., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Maley, G. F., and Lardy, H. A. (1954), *Recent Progr. Hormone Res. 10*, 129.

Nielson, S. O., and Lehninger, A. L. (1955), *J. Biol. Chem. 215*, 555.

Packer, L., and Denton, M. D. (1962), *Fed. Proc. 21*, 53.

Pressman, B. C. (1963), *J. Biol. Chem. 238*, 401.

Racker, E. (1961), *Advan. Enzymol. 23*, 373.

Singer, T. P., and Kearney, E. B. (1955), *in* Amino Acid Metabolism, McElroy, W. D., and Glass, B., eds., Baltimore, Johns Hopkins Press, 558.

Suelter, C. H., DeLuca, M., Peter, J. B., and Boyer, P. D. (1961), *Nature 192*, 43.

Taussky, H. H., and Shoor, E. (1953), *J. Biol. Chem. 202*, 675.

Vignais, P. V., Vignais, P. M., and Stanislas, E. (1961), *Biochim. Biophys. Acta 51*, 394.

Walker, P. G. (1954), *Biochem. J. 58*, 699.

Webster, G. (1962), *Biochem. Biophys. Res. Commun. 7*, 245.