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# Inhibition of the Respiration of *Escherichia coli* by Carbonyl Cyanide *m*-Chlorophenylhydrazone

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The effect of carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP) on the oxidation of various substrates by resting cells of *E. coli* has been investigated. $5 \cdot 10^{-7}$ M *m*-Cl-CCP inhibited the oxidation of succinate by 65 % while only fifty times higher concentration inhibited significantly the oxidation of glucose. Anaerobic $CO_2$ production from glucose was not inhibited. *m*-Cl-CCP had no inhibitory effect on the oxidation of succinate when the bacterial cells were preincubated with either succinate, pyruvate or glucose before the addition of the inhibitor. Glucose suppressed the inhibition of succinate oxidation also when added after *m*-Cl-CCP. The possible significante of the above findings is discussed.

It has been recently shown by Heytler and coworkers[1–3] that certain derivatives of carbonyl cyanide phenylhydrazone (CCP) uncouple oxidative phosphorylation in mitochondria and photophosphorylation in chloroplasts. This new class of uncouplers has, however, no appreciable effect on respiration in the above systems. During a study of the factors which control respiration in bacteria it was noted that low concentrations of carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP) inhibited oxidative processes in resting cells of *Escherichia coli*, strain B. It was thought that a study of the mechanism of this inhibition may further the understanding of the differences which exist between the bacterial and the mammalian respiratory systems (See Ref. 4).

## EXPERIMENTAL

*Materials.* *m*-Cl-CCP was the generous gift of Dr. P. G. Heytler (E. I. du Pont de Nemours and Company). Uniformly labeled D-glucose-$^{14}$C, succinic acid-1,4-$^{14}$C and $Na_2H^{32}PO_4$ were purchased from The Radiochemical Centre, Amersham, England. Other chemicals were of analytical grade.

*Escherichia coli*, Strain B, was grown on nutrient agar (Difco). The cultures were incubated at 37°C for 24 hours. The bacterial cells were collected in 0.25 M sucrose and washed three times with isotonic sucrose solution. Manometric experiments were carried out by the conventional Warburg technique. The nitrogen used in anaerobic experiments was purified by passing it through Fieser's solution and water. In most experiments the assay mixture contained $2 \cdot 10^{-2}$ M Tris buffer, pH 7.4, $5 \cdot 10^{-3}$ M NaCl, $5 \cdot 10^{-3}$ M KCl, bacterial cells in a con-

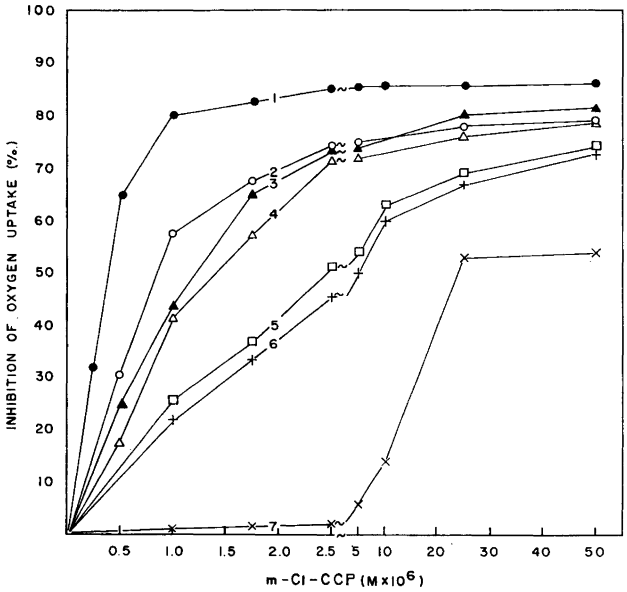


*Fig. 1.* Effect of *m*-Cl-CCP on the oxidation of various substrates. The main compartment of the Warburg flask contained the standard assay mixture and *m*-Cl-CCP in a concentration as indicated. After 10 min of thermoequilibration $3 \times 10^{-3}$ M of the respective substrate was added from the side arm. Duration of the experiment 60 min. Designation of curves: 1, Succinate; 2, Pyruvate; 3, Acetate; 4, L-Glutamate; 5, DL-Glyceraldehyde; 6, D-Fructose; 7, D-Glucose.

centration corresponding approx. to 2.0 mg of protein/ml and water to 3.0 ml (Standard assay mixture). The incubation was carried out at 30°C.

*Radioactivity was measured* in a Tri-Carb Model 314F liquid scintillation counter (Packard Instrument Company). The respiratory $^{14}CO_2$ was trapped on KOH-moistened paper in the Warburg center well and the counting was performed in toluene-ethanol scintillator solution as described by Buhler[5].

*Protein was assayed* according to Lowry *et al.*[6] after solubilizing the bacterial cells by boiling with 1 N NaOH for 20 minutes.

## RESULTS

*Effect of* m-*Cl-CCP on the oxidation of various substrates.* A survey was made of the effect of *m*-Cl-CCP on the oxidation of various substrates. NaCl and KCl were added to the assay mixture since the rate of oxidation of some of the substrates tested was accelerated by $Na^+$ and $K^+$ ions[7]. The $Q_{O_2}$ (N) values measured for glucose varied between 700 and 900 and for the other substrates tested between 300 and 500. As shown in Fig. 1, when succinate was used as the substrate, preincubation of the bacterial cells with $5 \cdot 10^{-7}$ M *m*-Cl-CCP caused 65 % inhibition of the oxygen uptake; maximum inhibition of 85 % was reached at a concentration of $2.5 \cdot 10^{-6}$ M. In contrast to succinate the respiration in the presence of glucose was not inhibited by *m*-Cl-CCP concentrations below $2.5 \cdot 10^{-6}$ M and the maximum inhibition which could be obtained ($2.5 \cdot 10^{-5}$ M *m*-Cl-CCP) was only 53 %. The sensitivity to the inhibition of oxidation of the other substrates

*Table 1.* Effect of m-Cl-CCP on anaerobic $CO_2$ production from glucose. *m*-Cl-CCP pre-incubated with bacterial cells for 10 min at 30°C before the addition of 0.15 $\mu$C randomly labeled glucose-$^{14}$C diluted by $3.3 \times 10^{-3}$ M carrier glucose.

| | *m*-Cl-CCP ($M \times 10^6$) | | | |
|---|---|---|---|---|
| | None | 1.0 | 2.5 | 25.0 |
| $^{14}CO_2$ produced (c.p.m.) in 60 min | 1535 | 1425 | 1380 | 1483 |

tested was intermediate between that of succinate and that of glucose; pyruvate, acetate and glutamate were closer to succinate in this respect while fructose and glyceraldehyde were more similar to glucose. In some experiments $^{14}$C-labeled substrates were used and $^{14}CO_2$ production was measured in addition to oxygen uptake. Since the two methods revealed the same inhibition pattern these experiments will not be described in detail.

The effect of *m*-Cl-CCP on $CO_2$ production from glucose was determined also under anaerobic conditions. Randomly labeled glucose-$^{14}$C was used in this experiment and the radioactivity in the collected $CO_2$ was measured. As seen from Table 1, *m*-Cl-CCP had no inhibitory effect in this case.

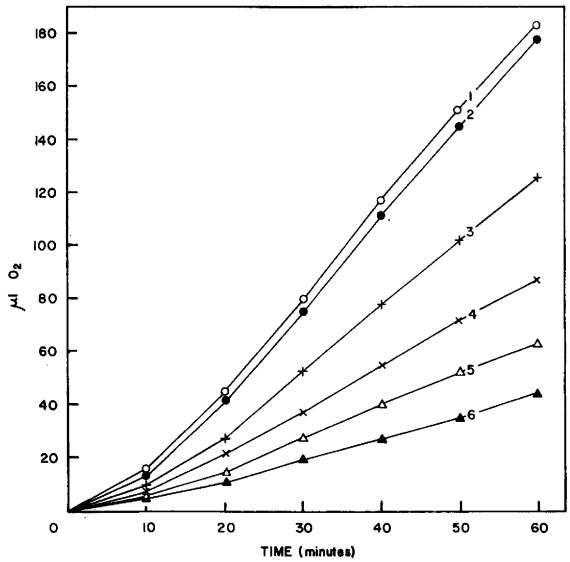


*Fig. 2.* Oxygen uptake as a function of the sequence of addition of substrate and inhibitor. The main compartment of the Warburg flask contained the standard assay mixture. Succinate ($3.3 \times 10^{-3}$ M) and *m*-Cl-CCP ($1 \times 10^{-6}$ M) were added as indicated. In all experiments the gas phase was air with the exception of the experiment shown in curve 5, where nitrogen was the gas phase during preincubations and it was changed to air at zero time. Curve 1: Succinate added at zero time; Curve 2: Succinate added at zero time, *m*-Cl-CCP five min after succinate; Curve 3: Succinate added at zero time, *m*-Cl-CCP one min after succinate; Curve 4: Succinate and *m*-Cl-CCP added simultaneously; Curve 5: 20 min of anaerobic pre-incubation with succinate, *m*-Cl-CCP added 10 min after succinate; Curve 6: *m*-Cl-CCP added 10 min before succinate.

*Table 2.* Effect of various substrates on the inhibition of succinate oxidation.

| Composition of assay mixture in main compartment | Added from side arm | | $^{14}CO_2$ produced (c.p.m.) in 60 min |
|---|---|---|---|
| | After 10 min | After 20 min | |
| S.M.[a] | – | Succinate[b], Succinate-$^{14}$C[c] | 2505 |
| S.M. + *m*-Cl-CCP[d] | – | Succinate, Succinate-$^{14}$C | 600 |
| S.M. + Succinate | *m*-Cl-CCP | Succinate-$^{14}$C | 2450 |
| S.M. + *m*-Cl-CCP | Pyruvate[b] | Succinate, Succinate-$^{14}$C | 866 |
| S.M. + Pyruvate | *m*-Cl-CCP | Succinate, Succinate-$^{14}$C | 1847 |
| S.M. + *m*-Cl-CCP | Glucose[b] | Succinate, Succinate-$^{14}$C | 2366 |
| S.M. + Glucose | *m*-Cl-CCP | Succinate, Succinate-$^{14}$C | 2150 |
| S.M. + *m*-Cl-CCP | Fructose[b] | Succinate, Succinate-$^{14}$C | 1350 |

*a* Standard assay mixture.
*b* $3.3 \times 10^{-3}$ M.
*c* 0.016 $\mu$C Succinate-1,4-$^{14}$C.
*d* $1 \times 10^{-6}$ M.

*The effect, on inhibition, of preincubation with substrate.* During these experiments it was noted that the inhibition of the respiration by *m*-Cl-CCP depends on the sequence in which substrate and inhibitor were added. In Curve 6 of Fig. 2 we see the striking inhibition of succinate oxidation by $1 \cdot 10^{-6}$ M *m*-Cl-CCP when the inhibitor was added 10 min before succinate. However, the inhibition decreased when inhibitor and substrate were added simultaneously (Fig. 2; Curve 4). When the substrate was added one minute before the inhibitor, the inhibition by *m*-Cl-CCP decreased still further (Fig. 2; Curve 3) and when the preincubation with the substrate was extended to five min (Fig. 2; Curve 2) the rate of oxygen uptake was the same as in the control experiment (Fig. 2; Curve 1). The time of preincubation with succinate which was necessary to obtain full protection varied from batch to batch of bacteria 2—10 min). It was essential for the protection that the preincubation with succinate should be carried out under aerobic conditions. If the preincubation with succinate was carried out under anaerobic conditions as in the experiment shown by Curve 5 of Fig. 2, no diminution in the inhibition was observed.

*Effect of various substrates on the inhibition of succinate oxidation.* An experiment was carried out in order to determine whether the preincubation with succinate is specifically required to prevent the inhibition of succinate oxidation

by *m*-Cl-CCP. Therefore, the bacteria were preincubated with other respiratory substrates before the addition of the inhibitor and only then was succinate added. Interference by the added substrates in measurement of succinate oxidation was avoided by using $^{14}C$-labeled succinate and determining $^{14}CO_2$ production instead of oxygen uptake. It can be seen from Table 2 that preincubation with (unlabeled) succinate abolished the decrease in counts which was observed in a control experiment in which *m*-Cl-CCP was added to bacteria without previous preincubation with the substrate. Moreover, it is apparent that when the cells were preincubated with pyruvate or glucose instead of succinate the drop in the counts due to the effect of the inhibitor was largely reversed. Pyruvate, the oxidation of which is inhibited by *m*-Cl-CCP, was found to be effective only when added before the inhibitor. In contrast, glucose which itself is not affected by an $1 \cdot 10^{-6}$ M *m*-Cl-CCP protected succinate oxidation whether it was added before or after the inhibitor. Similarly since fructose oxidation is less sensitive to inhibition than that of pyruvate it was more effective than the latter in the reversal of inhibition of succinate oxidation when added after the inhibitor.

*Effect of preincubation with succinate on the bacterial cells.* In order to elucidate further the nature of possible changes which take place in the bacterial cell on preincubation with an oxidizable substrate and which make the cell more resistent to *m*-Cl-CCP, the experiment shown in Table 3 was carried out. A larger sample of bacterial cells was incubated for 30 min under the conditions of the experiment described in Fig. 1, using $1 \cdot 10^{-2}$ M succinate. In a control experiment succinate was omitted from the medium. The cells were harvested by centrifugation, washed once with 0.15 M NaCl and resuspended in the same medium. The two suspensions obtained designated *Coli* (Succinate) and *Coli* (Control) were added to an assay medium which contained succinate ($3.3 \cdot 10^{-3}$ M) and *m*-Cl-CCP as indicated. Their sensitivity to *m*-Cl-CCP was compared with that of untreated *E. coli*. As seen from the table, incubation with succinate caused some change in the cells, since after washing, the *Coli* (Succinate) cells proved to be significantly less sensitive to *m*-Cl-CCP than either *Coli* (Control) or the untreated cells.

## DISCUSSION

The inhibitory effect of *m*-Cl-CCP on the respiration of *E. Coli* is comparable to that of the most potent inhibitors reported in the literature[8]. The extent of inhibition depended, however, to a large measure on the sequence of the addition of the components of the reaction mixture. It became apparent that the

*Table 3.* Sensitivity of *Coli* (Succinate) and *Coli* (Control) to *m*-Cl-CCP.

| | *Coli* (Succinate)[a] | | *Coli* (Control)[a] *m*-Cl-CCP ($M \times 10^6$) | | *Coli*[a] | |
|---|---|---|---|---|---|---|
| | none | 1.0 | none | 1.0 | none | 1.0 |
| $\mu l$ $O_2$/h[b] | 240 | 190 | 260 | 90 | 247 | 105 |

*a* 2.0 mg protein per Warburg flask.
*b* average from three experiments.

addition of various substrates to the reaction mixture whose oxidation, under the conditions of the test, was not inhibited by *m*-Cl-CCP, abolished also the effect of this inhibitor on the oxidation of succinate, which would have been inhibited most pronouncedly by *m*-Cl-CCP in the absence of the added oxidizable substrate.

This raises the question of the identity of the substance which could have been formed during the oxidation of the various substrates in the bacterial cell and which would make it resistant to the *m*-Cl-CCP. In the present state of this investigation we can offer no definite answer to this question. Considering, that in all systems in which the effect of *m*-Cl-CCP has been studied heretofore, this inhibitor acted on ATP formation and not on respiration, it is likely that in bacteria too its primary site of action is oxidative phosphorylation. The formation of the protective factor might then depend on ATP synthesis. In this context it is noteworthy that succinate oxidation, which in mammalian mitochondria is connected with two phosphorylation steps, was most sensitive to inhibition, whilst that of the pyridine nucleotide-linked substrates with one more phosphorylation step was less affected. The most resistant to inhibition was the oxidation of the glycolytic substances, in particular glucose, which can generate ATP also by substrate-level phosphorylation. Although very little is yet known on the phosphorylation steps in bacteria[8], a difference in the sensitivity of the various sites of phosphorylation to inhibition could account for the differences observed in the extent of respiratory inhibition.

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