Effect of Carbonyl Cyanide *m*-Chlorophenylhydrazone on Respiration and Respiration-Dependent Phosphorylation in *Escherichia coli*

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1. The interference mechanism of carbonyl cyanide *m*-chlorophenylhydrazone with the respiratory process and with phosphorylation coupled to respiration has been investigated in resting cells of *Escherichia coli*. 2. Preincubation of the cells with carbonyl cyanide *m*-chlorophenylhydrazone in the absence of substrate caused strong inhibition of succinate oxidation. The inactivation of the respiratory system proved to be time-dependent and temperature-dependent and could be arrested by adding the substrate. Inhibition of incorporation of ^{32}P into acid-soluble organic phosphate esters exceeded the inhibition of oxygen uptake. 3. In contrast with succinate, the rate of oxidation of glucose was increased by carbonyl cyanide *m*-chlorophenylhydrazone. The sensitivity of other substrates to the inhibitor was less than that of succinate. 4. Various observations are described in support of the view that respiratory inhibition induced by carbonyl cyanide *m*-chlorophenylhydrazone is a result of its interference with ATP synthesis. The capacity of a given substrate to increase intracellular ATP concentration appeared to be directly related to its resistance to inhibition. In cell-free extracts carbonyl cyanide *m*-chlorophenylhydrazone still suppressed ^{32}P incorporation but had no effect on respiration. 5. Carbonyl cyanide *m*-chlorophenylhydrazone-induced stimulation of glucose oxidation and the acceleration of succinate oxidation by ADP or AMP in cells rendered permeable to nucleotides are tentatively interpreted as an indication that a certain part of respiration in *E. coli* is under phosphate-acceptor-mediated control.

Several workers have reported that, in mammalian mitochondria, uncouplers exert biphasic action on electron transfer, i.e. they stimulate respiration at low concentrations but inhibit this function at higher concentrations (Runeberg, 1963; Hemker, 1963; Vázquez-Colón, Ziegler & Elliott, 1966). Overlapping of uncoupling and respiratory inhibition was also demonstrated in bacteria. CCC-P,* a powerful uncoupling agent in mammalian mitochondria (Heytler, 1963; Goldsby & Heytler, 1963) and in photophosphorylation (Heytler & Prichard, 1962), inhibits oxidation of different substrates in resting cells of *Escherichia coli* (Avi-Dor, 1963). Uncoupling agents, including CCC-P, were reported to inhibit succinate and NADH oxidation in resting cells of *Haemophilus parainfluenzae* (White & Smith, 1964) and that of various other substrates by cell-free extracts of *Mycobacterium phlei* (Asano & Brodie, 1965).

* Abbreviation: CCC-P, carbonyl cyanide *m*-chlorophenylhydrazone.

The present paper deals with certain aspects of the interference mechanism of CCC-P with respiratory processes. Evidence is presented in support of the view that this interference is a result of its effect on ATP synthesis, rather than a direct effect on electron transfer.

MATERIALS

The CCC-P used was a generous gift from Dr P. G. Heytler (E. I. Du Pont de Nemours and Co., Wilmington 98, Del., U.S.A.).

[3,4- $^{14}\text{C}_2$]Glutamic acid, [2,2',3,3'- $^3\text{H}_4$]succinic acid and $\text{Na}_2\text{H}^{32}\text{PO}_4$ were the products of The Radiochemical Centre, Amersham, Bucks. Other chemicals were of analytical grade.

METHODS

Bacterial suspensions. *E. coli*, strain B, was grown on nutrient broth (Difco). The cultures were incubated at 37° for 15 hr. and the cells collected in redistilled water and washed three times with water. For preparing cell-free

extracts and for EDTA treatment the organisms were grown on a synthetic medium as described by Kashket & Brodie (1963) and harvested by centrifugation in the middle of the exponential phase of growth.

Preparation of cell-free extract and particles. The harvested bacteria were washed twice with water and resuspended in water to yield a suspension equivalent to approx. 20 mg. of protein/ml. The suspension was brought with tris-chloride to pH 7.3 and then treated with ultrasound in a MSE model 60 w ultrasonic disintegrator for 2 min. After readjusting the pH to 7.3, cellular debris and whole cells were removed by centrifugation in a Servall centrifuge model RC-2 (rotor SS-34) at 16000g for 20 min. at 0°. The supernatant is referred to as the cell-free extract. To obtain respiratory particles the extract was centrifuged in a Spinco model L preparative ultracentrifuge (rotor 50) for 2½ hr. at 140 000g and the sedimented pellet resuspended in 50 mM-tris-chloride buffer, pH 7.3.

EDTA treatment of bacterial cells. The harvested cells were washed with 120 mM-tris-chloride buffer, pH 7.8, and resuspended in the same buffer. To increase the permeability of the cells to nucleotides the procedure employed by Leive (1965) was adapted. EDTA was added to the cell suspension to a final concentration of 5 mM. After 2 min. incubation at 37°, the suspension was centrifuged at 16000g for 10 min. at 0°. As a rapid test for a change in permeability, the increase in NADH-oxidase activity was determined by spectrophotometric measurement of the rate of NADH disappearance. The permeability change was considered satisfactory when the NADH-oxidase activity reached 30–50% of the level obtained in an ultrasonic extract. Cells not exposed to EDTA were incapable of oxidizing NADH.

Assay conditions. Manometric experiments were carried out by the conventional Warburg technique. When not indicated otherwise the temperature of incubation was 30°. Manometric readings were made every 10 min. With ¹⁴C-labelled glutamate as substrate the respiratory ¹⁴CO₂ was trapped on KOH-moistened paper in the Warburg centre well and counting was carried out in toluene-ethanol scintillation liquid as described by Buhler (1962). For measuring the ³H₂O formed during oxidation of [2,2',3,3',3'-³H₄]succinate a 0.4 ml. portion of the reaction mixture was withdrawn and the water isolated by the method of Lee, Simard-Duquesne, Ernster & Hoberman (1965); 0.2 ml. of the isolated water sample was diluted with Bray's (1960) scintillation liquid to 10 ml. and counted.

Since in intact cells the standard methods were not applicable for the measurement of oxidative phosphorylation, the phosphorylation capacity of the bacteria was estimated by measuring incorporation of ³²P into acid-soluble phosphate esters and the concomitant oxygen uptake both in the presence and in the absence of an oxidizable substrate. $\Delta^{32}\text{P}$ (in counts/min.) and ΔO (in $\mu\text{g. atoms}$) were calculated from the differences in phosphate incorporation and oxygen uptake respectively. The phosphorylating capacity of the cells was expressed as the ratio $\Delta^{32}\text{P}/\Delta\text{O}$.

For determination of ³²P incorporation into acid-soluble phosphates, a 1.0 ml. sample of the assay mixture was withdrawn and added to 1.0 ml. of ice-cold 10% (w/v) trichloroacetic acid. The precipitate was removed by centrifuging at 6000g for 10 min. and the organic phosphates were separated from the supernatant according to

Avron (1960); 0.5 ml. of the organic phase was diluted with Bray's scintillation liquid to 10 ml. and counted.

ATP was determined according to Strehler & Totter (1952).

Protein was assayed according to Lowry, Rosebrough, Farr & Randall (1951) with the bacterial cells solubilized by boiling with N-NaOH for 20 min. Radioactivity was measured in a Packard Tri-Carb model 3003 scintillation spectrometer.

RESULTS

Effect of preincubation time on degree of respiratory inhibition. It was known from a previous investigation (Avi-Dor, 1963) that preincubation with CCC-P in the absence of substrate is essential for inhibition of oxygen uptake. The experiment described below was undertaken to gain more information on the velocity of the process leading to impairment of the respiratory rate. *E. coli* cells were incubated with 1 μM -CCC-P for different time-intervals after which succinate was added. Oxygen uptake was measured with the aid of an oxygen electrode and the rate calculated from the slope of the polarographic trace. In Fig. 1 the respiratory rate is plotted against preincubation time. It is apparent that at 20° inactivation of the respiratory system is a relatively slow process. When succinate and CCC-P were added simultaneously to the

Fig. 1. Effect of preincubation of *E. coli* cells with CCC-P on the rate of succinate oxidation. The assay mixture contained 50 mM-tris-chloride buffer (pH 7.4), 5 mM-NaCl, 5 mM-KCl and washed *E. coli* cells equivalent to 1 mg. of protein/ml. At zero time CCC-P was added to give a final concentration of 1 μM . The final volume was 20 ml. The incubation temperature was 20°. Every 10 min. a 2 ml. sample of the reaction mixture was withdrawn, mixed rapidly with succinate (final concn. 10 mM) and transferred to the reaction vessel of the Oxygraph. Oxygen uptake was measured for 5 min. at 30° and the rate of respiration calculated from the slope of the polarographic trace. ○, Oxidation rate in the presence of CCC-P; ●, rate in the absence of CCC-P.

Table 1. *Effect of carbonyl cyanide m-chlorophenylhydrazone on succinate oxidation and on respiration-dependent phosphorylation*

Each Warburg vessel contained tris-chloride, KCl and NaCl as in Fig. 1 and bacterial cells equivalent to approx. 2mg. of protein/ml. The total volume was 3ml. In Expts. 1, 2 and 3, CCC-P (1 μ M), when added, was in the main compartment. In Expts. 4, 5 and 6 succinate (10mM) was added in the main compartment. After 10min. of thermoequilibration either 30 μ moles of succinate (in Expts. 1, 2 and 3) or 3 μ moles of CCC-P (in Expts. 4, 5 and 6 when indicated) was added from one of the side arms. After an additional 20min. of incubation 30 μ moles of K_2HPO_4 brought to pH 7.4 with HCl and carrier-free [^{32}P]phosphate (equivalent to 10^5 counts/min.) were tipped into the main compartment from the other side arm. Incubation was then continued for a further 20min. Incorporation of ^{32}P in acid-soluble organic phosphate esters was determined as described in the Methods section. $\Delta P/\Delta O$ is defined in the Methods section.

Expt. no.	Oxygen uptake (μ g.atoms/20min.)				Inhibition of oxygen uptake (%)	$\Delta P/\Delta O$		Inhibition of ^{32}P incorporation (%)
	No CCC-P added	CCC-P added before the substrate	No CCC-P added	CCC-P added after the substrate		No CCC-P added	CCC-P added	
1	4.5	1.2	—	—	73	66	0	100
2	2.9	1.4	—	—	52	65	0	100
3	3.7	1.1	—	—	70	80	15	81
4	—	—	3.0	2.0	33	65	30	54
5	—	—	3.9	3.0	23	67	45	33
6	—	—	3.2	2.5	22	70	48	31

medium, the decrease in the respiratory rate was smaller than that observed in the absence of CCC-P. At 37° maximum inhibition was reached in less than 5min.

Effect of carbonyl cyanide m-chlorophenylhydrazone on ^{32}P incorporation into acid-soluble organic phosphate esters. CCC-P was found to uncouple phosphorylation in cell-free bacterial extracts (Asano & Brodie, 1965). It was decided to test whether phosphorylation is more sensitive to CCC-P than is respiration in whole *E. coli* cells. Respiration-dependent phosphate incorporation (as defined in the Methods section) was taken as a measure of oxidative phosphorylation. In a preliminary experiment it was found that in the time-interval of 30min. after addition of the labelled phosphate both the rate of ^{32}P incorporation and oxygen uptake were nearly constant. All the experiments were therefore carried out within this time-interval. In the control experiments, where no substrate was added, oxygen uptake was negligibly small, but phosphate incorporation was significant and reached approx. 25–30% of the value obtained in the presence of substrate. The presence of phosphate did not change the respiratory rate. Table 1 shows that in all circumstances inhibition of phosphorylation exceeded that of oxygen uptake and that succinate provided equal protection to both the respiratory system and the respiration-dependent phosphorylation. A comparison of the data presented in Table 1 and Fig. 1 also reveals that the initial rate of respiration as recorded with the oxygen electrode is much less sensitive to CCC-P

than oxygen uptake measured in a longer time-interval by the manometric method.

Effect of carbonyl cyanide m-chlorophenylhydrazone on glucose oxidation and on respiration-dependent phosphorylation. Under certain conditions, uncoupling of phosphorylation in tightly coupled mammalian mitochondrial systems is associated with respiratory stimulation as a result of the relief of respiratory control (Lardy & Wellman, 1952). Respiratory control in bacteria has not yet been demonstrated (cf. Smith, 1961; Asano & Brodie, 1965). It was therefore noteworthy that, when *E. coli* cells were incubated with succinate and 0.1 μ M-CCC-P was added to the assay mixture after 2–5min., a small but still significant increase (up to 18%) in the rate of oxygen uptake resulted occasionally, as indicated by a change in the slope of the polarographic trace. The lack of effect of CCC-P in many otherwise similar experiments remains unexplained. Consistent results were obtained with glucose as respiratory substrate. The results of a manometric experiment are summarized in Table 2. CCC-P partially inhibited the respiration-dependent esterification of ^{32}P but increased oxygen uptake by approx. 30%. Similar respiratory stimulation was also recorded in polarographic experiments.

Relief of carbonyl cyanide m-chlorophenylhydrazone-induced inhibition of succinate or glutamate oxidation by other respiratory substrates. Relief of CCC-P-induced inhibition of succinate oxidation by subsequent addition of glucose has been reported (Avi-Dor, 1963). These experiments have now

Table 2. *Effect of carbonyl cyanide m-chlorophenylhydrazone on glucose oxidation and on respiration-dependent phosphorylation*

Conditions were as in Expts. 1, 2 and 3 in Table 1, except that 30 μ moles of glucose were added from the side arm instead of succinate. $\Delta P/\Delta O$ is defined in the Methods section.

Expt. no.	Oxygen uptake (μ g.atoms/20 min.)		Stimulation of oxygen uptake (%)	$\Delta P/\Delta O$		Inhibition of ^{32}P incorporation (%)
	No CCC-P added	CCC-P added		No CCC-P added	CCC-P added	
1	4.7	6.0	28	61	39	36
2	3.7	4.8	30	95	67	29
3	2.7	3.6	33	89	60	33
4	3.3	4.3	30	44	37	16

been extended by showing that relief of the inhibition of succinate oxidation is also obtainable by other substrates, provided they are less sensitive than succinate to inhibition by CCC-P. To measure the rate of succinate oxidation while another substrate was being simultaneously oxidized labelled [2,2',3,3'- $^3\text{H}_4$]succinate was used in conjunction with an unlabelled substrate. Preliminary experiments showed that the degree of CCC-P-induced inhibition is of the same order, whether obtained from the rate of $^3\text{H}_2\text{O}$ formation associated with the succinate-fumarate oxidation step, or from the oxygen uptake associated with multi-step oxidation of succinate (Fig. 2).

Table 3 shows the effect of different CCC-P concentrations on oxidation of succinate and also presents results of experiments in which unlabelled glutamate, pyruvate or glucose was added simultaneously with labelled succinate. It is seen that CCC-P stimulated glucose oxidation in the range of concentrations tested. Oxidation of the other three substrates was inhibited, with sensitivity towards the inhibitor decreasing in the following order: succinate > glutamate $\gg$ pyruvate.

Examination of the results obtained for a pair of substrates undergoing simultaneous oxidation reveals a characteristic pattern of interactions: (1) Glutamate stimulated the rate of succinate oxidation in the absence of CCC-P and partially relieved its inhibition at CCC-P concentrations in which glutamate oxidation was not too strongly suppressed. (2) Both pyruvate and glucose stimulated succinate oxidation in the presence of low CCC-P concentrations and relieved inhibition of succinate oxidation over a much wider concentration range as compared with glutamate.

The experiment summarized in Table 4 differed from that shown in Table 3 in the labelled substrate ([3,4- $^{14}\text{C}_2$]glutamate) used as well as in that, in the simultaneous oxidation of a pair of substrates, the rate of glutamate oxidation was measured by determining the rate of $^{14}\text{CO}_2$ evolution. In this

Fig. 2. Comparison of manometric and isotopic methods for the measurement of succinate and glutamate oxidation. Each Warburg vessel contained tris-chloride buffer, KCl and NaCl as in Fig. 1, CCC-P as indicated and bacterial cells equivalent to 2 mg. of protein/ml. The substrate, added from the side arm after 10 min. of thermoequilibration, was either 10 μ moles of [2,2',3,3'- $^3\text{H}_4$]succinate giving 2.5×10^5 counts/min./ μ mole or 10 μ moles of [3,4- $^{14}\text{C}_2$]glutamate giving 10^4 counts/min./ μ mole. The total volume was 3 ml. The incubation temperature was 30°. $\circ$ and $\bullet$, Succinate; $\triangle$ and $\blacktriangle$, glutamate. $\circ$ and $\triangle$, Oxygen uptake; $\bullet$ and $\blacktriangle$, $^3\text{H}_2\text{O}$ or $^{14}\text{CO}_2$ formed.

case the degree of inhibition was again found to be independent of the method of assay (Fig. 2).

It is apparent from Table 4 that inhibition of CCC-P-induced glutamate oxidation was partially relieved by glucose, as with succinate as the respiratory substrate.

Change in intracellular ATP concentration. Azzone & Ernster (1961) and Suranyi & Ernster (1964) demonstrated an ATP requirement for succinate

Table 3. *Reversal of carbonyl cyanide m-chlorophenylhydrazone inhibition of succinate oxidation by glutamate, pyruvate and glucose*

The composition of the reaction mixture in the main compartment of the Warburg vessel was as in Fig. 2. The content of the side arms was as indicated in the Table, and it was tipped into the main compartment after 10 min. of preincubation. The amount of substrates in the side arm was 10 μ moles. The amount of labelled [2,2',3,3'- $^3\text{H}_4$]succinate was so as to give 2.5×10^5 counts/min./ μ mole. The rate of succinate oxidation was estimated from that of $^3\text{H}_2\text{O}$ formation, and that of the other substrates by manometric measurement. Stimulation (+) or inhibition (–) of pyruvate, glutamate or glucose oxidation is expressed in percentages of the rate of oxidation of the respective substrate in the absence of CCC-P.

Concn. of CCC-P (μM)	Succinate	Glutamate	Succinate + glutamate	Pyruvate	Succinate + pyruvate	Glucose	Succinate + glucose
0	0	0	+27	0	+4	0	+4
0.1	–28	–10	–10	0	+29	0	+14
0.2	–50	–23	–18	0	+18	0	+18
0.5	–75	–69	–62	0	–28	+10	–28
1.0	–85	–85	–65	–28	–44	+30	–46

Table 4. *Relief of carbonyl cyanide m-chlorophenylhydrazone-induced inhibition of glutamate oxidation by glucose*

The composition of the reaction mixture in the main compartment of the Warburg vessel was as in Fig. 2. Glutamate or glucose (or both) was added from the side arm in an amount of 10 μ moles. The rate of glutamate oxidation was estimated from the rate of $^{14}\text{CO}_2$ formation from [3,4- $^{14}\text{C}_2$]glutamate (10^4 counts/min./ μ mole). All other conditions were as in Table 3. Stimulation (+) or inhibition (–) of glutamate oxidation is expressed in percentages of the rate of oxidation of this substrate in the absence of CCC-P.

Concn. of CCC-P (μM)	Glutamate	Glucose	Glutamate+ glucose
0	0	0	0
0.1	–14	0	+5
0.2	–35	–2	–1
0.5	–75	+8	–17
1.0	–84	+21	–30

oxidation after preincubation of mitochondria with 2,4-dinitrophenol. It was conceivable that, as in the case described by Ernster and his team, substrates found to be particularly sensitive to the action of CCC-P may require ATP for their oxidation. An indication of such a requirement was sought, accordingly, by measuring the change in the steady-state concentration of intracellular ATP during incubation. Kinetic studies (not reported in detail) showed that, on adding the substrate to the cell suspension, the intracellular ATP concentration reached a steady state within a few minutes. In a large number of experiments in which the change in the intracellular ATP concentration was assayed in relation to the respiratory substrate used, the absolute values varied within a range of 30%. However, in all experiments the pattern of change was the same as presented in the experiment shown in Table 5, i.e. succinate and glutamate decreased the intracellular ATP concentration below that found in the absence of

substrate; by contrast, pyruvate and glucose caused a significant rise in the ATP concentration. It therefore appears that there is an inverse relation between sensitivity to CCC-P on the one hand and the ability to increase the intracellular ATP concentration on the other.

Effect of exogenous adenine nucleotides on succinate oxidation. Azzone & Ernster (1961) found that ATP relieves 2,4-dinitrophenol-induced inhibition of succinate oxidation in mitochondria. The question arose whether ATP and other nucleotides would have a similar effect on CCC-P-induced inhibition. To render the cells permeable to nucleotides they were pretreated with EDTA as described in the Methods section and incubated with CCC-P alone or together with ATP, ADP or AMP in the absence of a substrate. Preincubation was terminated by adding labelled succinate, and the rate of oxidation followed both manometrically and by measuring the rate of $^3\text{H}_2\text{O}$ formation. Table 6 shows that all three nucleotides proved

Table 5. *Effect of different oxidizable substrates on intracellular ATP concentration in relation to their sensitivity to carbonyl cyanide m-chlorophenylhydrazone*

The main compartment of the Warburg vessel contained tris-chloride buffer, KCl and NaCl as in Fig. 1 and *E. coli* cells equivalent to 4mg. of protein/ml. The substrates were added from the side arm after 10min. of thermoequilibration in an amount of 30 μ moles. The experiment was terminated after 20min. by boiling a 2.0 ml. sample of the reaction mixture in a water bath for 10min. ATP determination was carried out as described in the Methods section. Data on CCC-P concentrations causing 30% inhibition are quoted from other experiments.

Substrate	Concn. of ATP (μ g./10 mg. of protein)		Concn. of CCC-P needed for 30% inhibition of respiration (μ M)
	At zero time	At 20 min.	
—	0.85	0.73	—
Succinate	—	0.54	0.1
Glutamate	—	0.46	0.2
Pyruvate	—	1.22	1.0
Glucose	—	1.52	20.0

Table 6. *Effect of exogenous adenine nucleotides on succinate oxidation*

The main compartment of the Warburg vessel contained tris-chloride buffer, KCl and NaCl as in Fig. 1 and EDTA-treated *E. coli* cells (see the Methods section) equivalent to 2mg. of protein/ml. 1 μ M-CCC-P or 5 μ moles of adenine nucleotides (or both) were added as indicated. The side arm contained 10 μ moles of labelled [2,2',3,3'- 3 H₄]succinate giving 2.5×10^5 counts/min./ μ mole, tipped into the main compartment after 10min. of thermoequilibration. The rate of succinate oxidation was measured both manometrically and by estimation of the rate of 3 H₂O formation (see the Methods section).

	No nucleotides		ATP		ADP		AMP	
	- CCC-P	+ CCC-P	- CCC-P	+ CCC-P	- CCC-P	+ CCC-P	- CCC-P	+ CCC-P
Oxygen uptake (μ g.atoms/20 min.)	7.3	2.3	6.7	5.7	7.8	7.2	8.0	6.9
Succinate (μ moles oxidized/ 20 min.)	1.20	0.64	1.32	1.00	1.86	1.31	1.49	1.15

Table 7. *Effect of carbonyl cyanide m-chlorophenylhydrazone on succinate oxidation and respiration-dependent 32 P incorporation in cell-free systems*

The main compartment of the Warburg vessel contained 50 mM-tris-chloride buffer (pH 7.4), 10 mM-MgCl₂, 10 mM-mannose, 200 K.M. units (Kunitz & McDonald, 1946) of hexokinase, CCC-P as indicated and ultrasonic extract (equivalent to 10mg. of protein) or particles (equivalent to 3mg. of protein) respectively. Then 10 μ moles of [2,2',3,3'- 3 H₄]succinate giving 2.5×10^5 counts/min./ μ mole, 10 μ moles of K₂H³²PO₄ giving 1×10^5 counts/min./ μ mole, 100 μ moles of KF and 3 μ moles of AMP were added after 10min. of thermoequilibration. After additional 20min. of incubation at 30°, a 0.4 ml. sample was taken for determination of the 3 H₂O formed, and a 1 ml. sample for estimation of 32 P incorporated into phosphate ester. $\Delta P/\Delta O$ is defined in the Methods section.

Source of enzyme	Oxygen uptake (μ g.atoms/10 mg. of protein/20 min.)			Succinate oxidized (μ moles/10 mg. of protein/20 min.)			$\Delta P/\Delta O$		
	No CCC-P	1 μ M- CCC-P	5 μ M- CCC-P	No CCC-P	1 μ M- CCC-P	5 μ M- CCC-P	No CCC-P	1 μ M- CCC-P	5 μ M- CCC-P
Ultrasonic extract	1.5	1.6	1.4	0.23	0.24	0.23	29.800	7.100	0
Particles	3.8	4.7	—	0.39	0.41	—	5.700	1.130	—

effective in preventing inhibition. In addition, ADP and AMP (but not ATP) caused an increase in the rate of succinate conversion into fumarate, as measured by the rate of 3 H₂O formation. No

stimulatory effect of the two nucleotides was observed on the total oxygen consumption. Other tests, not reported in detail, showed that in non-pretreated cells neither ADP nor AMP caused

stimulation of succinate oxidation, nor could the nucleotides relieve CCC-P-induced respiratory inhibition. Moreover, it was demonstrated that ATP can also relieve CCC-P-induced inhibition in EDTA-treated cells when glutamate is the respiratory substrate.

Effect of carbonyl cyanide m-chlorophenylhydrazine on succinate oxidation and ^{32}P incorporation in cell-free systems. The effect of CCC-P on ultrasonic extracts and on the particulate fraction derived from the ultrasonically treated suspension was tested in experiments summarized in Table 7. It is seen that $1\text{ }\mu\text{M}$ -CCC-P, which strongly inhibited phosphorylation associated with succinate oxidation, had no effect on respiration in the ultrasonic extract and a slightly stimulating effect on oxygen uptake in a particulate fraction. Even $5\text{ }\mu\text{M}$ -CCC-P, which completely suppressed phosphorylation, caused no inhibition of succinate oxidation. Other experiments showed that NADH oxidation is similarly insensitive to $5\text{ }\mu\text{M}$ -CCC-P irrespective of whether the oxidation was catalysed by ultrasonic extract, particulate fraction or by cells pretreated with EDTA.

DISCUSSION

Bacterial energy conversions have been studied in general in cell-free systems prepared by vigorous disintegration (see Asano & Brodie, 1965). Some of the systems show a P/O ratio exceeding unity, but none possess respiratory control. Hence it is significant that both ADP and AMP accelerated succinate oxidation in EDTA-pretreated cells. In non-pretreated cells, not permeable to nucleotides, pronounced stimulation of glucose oxidation was obtained by CCC-P and this stimulating effect was associated with inhibition of respiration-dependent esterification of inorganic phosphate (Table 2). All these observations support the contention that phosphate-acceptor-mediated control of respiration may occur in *E. coli*. However, the stimulation obtained in *E. coli* is relatively small (approx. 30%) as compared with the respiratory control values for mitochondria (see Ernster & Luft, 1964).

Succinate oxidation was stimulated by low concentrations of CCC-P only under specific conditions, i.e. when either pyruvate or glucose was undergoing simultaneous oxidation. Under the usual conditions of the experiment CCC-P acted as an inhibitor of the oxidation of succinate. In the absence of CCC-P, however, succinate oxidation could be stimulated by adding glutamate to the assay mixture. These findings suggest an additional limiting factor (apart from energy transfer), with regard to the rate of succinate oxidation in resting cells of *E. coli*. It is conceivable that this additional factor is the oxaloacetate formed in the process and known to act as feedback inhibitor of succinate

oxidation in certain mammalian systems (Azzone & Ernster, 1961; Suranyi & Ernster, 1964). In mammalian mitochondria the inhibitory effect of oxaloacetate may be prevented by acetate or glutamate (Slater & Hülsmann, 1961; Chappell, 1964) and even reversed by ATP (Azzone & Ernster, 1961; Papa, Lofrumento & Quagliariello, 1965). It is thus likely that in *E. coli* oxaloacetate is removed by glutamate (through transamination) or by pyruvate or glucose (through formation of acetyl-CoA or ATP or both) whereby the energy transfer coupled to oxidation becomes the rate-limiting process.

In *E. coli*, an apparent ATP requirement for succinate oxidation is indicated by a fall in the intracellular ATP concentration below the endogenous level during incubation with succinate. As ATP is formed during succinate oxidation, a likely explanation for the above is that ATP is continuously removed by a side reaction, such as conversion of the oxaloacetate into phosphoenolpyruvate. It can be expected that the amount of ATP available for this side reaction will be limited in the presence of the uncoupler, CCC-P, so that unless ATP is provided by simultaneous oxidation of a CCC-P-insensitive substrate or by adding exogenous ATP (after EDTA pretreatment) oxaloacetate will act as feedback inhibitor and respiration will be arrested. The higher effectiveness of glucose (compared with other substrates) as an accessory source of ATP is probably associated with the lack of effect of uncoupling agents on ATP formation through the glycolytic cycle. The ability of AMP and ADP, as well as of ATP, to relieve CCC-P-induced respiratory inhibition is attributable to rapid conversion of the former two nucleotides in the bacterial cell into ATP.

Of the respiratory substrates tested, glutamate was closest to succinate in its sensitivity to CCC-P and its lack of ability to increase the intracellular ATP pool. It is conceivable that in this case the ATP-consuming side reaction is the conversion of glutamate into glutamine (McIlwain, Roper & Hughes, 1948), whereas in the presence of CCC-P the lack of ATP for introducing the glutamate into the cells (Gale, 1953; Davies, Folkes, Gale & Bigger, 1953) is the limiting factor for the rate of glutamate oxidation.

It could be argued that the CCC-P-induced inhibition of succinate oxidation might be due to an interference with the accumulation of the substrate, as suggested for glutamate. However, we could find no evidence for the contention that succinate transport in *E. coli* is an energy-dependent process.

The lack of effect of CCC-P on the oxidation of succinate and NADH in cell-free extracts, as well as its lack of effect on that of pyruvate or glucose

in the concentration range which is inhibitory for succinate or glutamate, strongly indicate that this uncoupling agent has no appreciable direct effect on the respiratory system. It is thus tentatively suggested that the effect of CCC-P on respiration is a result of inhibition of ATP synthesis. Relief of the respiratory inhibition effected by simultaneous oxidation of a CCC-P-insensitive substrate or by ATP is probably not a reversal of the damage to the mechanism of energy conversion, but rather an offsetting of secondary effects (removal of oxaloacetate etc.). By contrast, prevention of CCC-P-induced inhibition by the presence of a respiratory substrate is apparently a manifestation of a protection of the CCC-P-sensitive site of the enzyme system responsible for coupling respiration and ATP synthesis.

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