The Effects of Acetylcolletotrichin on the Mitochondrial Respiratory Chain

By BERNARD FOUCHER,* J. B. CHAPPELL and J. D. MCGIVAN

Department of Biochemistry, Medical School, University of Bristol, Bristol BS8 1TD, U.K.

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1. Acetylcolletotrichin is a phytotoxic compound that has been isolated from the culture medium of the fungus *Colletotrichum capsici* (Grove *et al.*, 1966). 2. With isolated liver and kidney mitochondria acetylcolletotrichin markedly inhibited the oxidation of succinate and those substrates with NAD-linked dehydrogenases, but did not inhibit the oxidation of ascorbate in the presence of tetramethyl-*p*-phenylenediamine. In this respect its action was similar to that of antimycin A. 3. Acetylcolletotrichin differed from antimycin in that, even at high concentrations which produced a maximal inhibitory effect, its action was partially reversed by uncoupling agents. Also acetylcolletotrichin had no detectable effect on the oxidative activity of blowfly flight-muscle mitochondria and was not very effective with heart mitochondria. 4. Acetylcolletotrichin inhibited the oxidative activity of liver mitochondria more markedly when respiration was stimulated by ADP together with phosphate and was less effective when respiration was stimulated by uncoupling agents. 5. There was an unusual interaction between the succinate oxidation system and the oxidation of glutamate together with malate. Thus, glutamate together with malate, even in the presence of rotenone, markedly decreased the effectiveness of acetylcolletotrichin in inhibiting succinate oxidation. 6. These effects were paralleled in the observed redox changes of cytochrome *c*. 7. The unusual behaviour of the cytochromes *b* in the presence of acetylcolletotrichin is described, and it is suggested tentatively that this inhibitor acts between cytochromes *b* with absorption maxima at 30°C of approximately 560 and 565 nm.

Acetylcolletotrichin has been extracted and purified from the culture medium of *Colletotrichum capsici*, a pathogenic fungus which attacks a number of plants. Only the partial chemical structure is known (Grove *et al.*, 1966), but the compound used in the investigations reported in the present paper was a white crystalline compound, which showed only one component on g.l.c. (J. MacMillan, personal communication).

The action of acetylcolletotrichin on the mitochondrial respiratory chain was discovered during the screening of a number of natural products which were known or thought to be toxic to animals or plants. Most of these compounds either were without effect on mitochondria or were uncoupling agents. Only acetylcolletotrichin showed any interesting inhibitory effects. The related compound colletodiol, also a fermentation product of *Colletotrichum capsici* (Grove *et al.*, 1966), did not mimic any of the effects of acetylcolletotrichin.

In some respects the action of acetylcolletotrichin resembled that of antimycin A, but there were impor-

tant differences, which are reported in the present paper.

Experimental

Methods

Preparation of mitochondria. Rat liver, kidney and heart mitochondria, pig kidney-cortex and blowfly (*Calliphora erythrocephala*) flight-muscle mitochondria were prepared as described by Chappell & Hansford (1969).

Oxygen uptake. This was followed by using a Clark-type electrode in a medium (4 ml) containing 0.12 M-KCl and 20 mM-Tris-HCl at pH 7.4 and 30°C (except for those experiments with blowfly mitochondria, which were at 25°C), essentially as described by Chappell (1964). Other additions were made as described in the text and legends.

Spectrophotometric observations. These were made with a dual-wavelength (double-beam) instrument constructed in the Mechanical Workshops of the Medical School of this University, by using the principles outlined by Chance (1957). The cuvette of 1 cm

* Present address: Institut de Biochemie, Université de Paris Sud, Centre d'Orsay, 31 Orsay, France.

light-path contained 2.5 ml of 0.12 M-KCl and 20 mM-Tris-HCl, pH 7.4. The temperature was maintained at 30°C by using a circulating thermostatically controlled water bath.

Redox changes of cytochrome *c*. In addition to spectrophotometric changes, the redox state of cytochrome *c* was followed by using a Pt electrode with calomel reference electrode according to the principle of Caswell & Pressman (1968), except that 5 mM-ferrocyanide was used as mediator rather than tetramethyl-*p*-phenylenediamine. The latter was not used since, under some circumstances, it can cause a by-pass of the antimycin-inhibition site (Kaniuga *et al.*, 1969) and possibly that of acetylcolletotrichin.

Ubiquinone assays. The redox state of ubiquinone was determined as described by Kröger & Klingenberg (1966), by using pig kidney-cortex mitochondria.

ATPase (adenosine triphosphatase) activity. This was followed by monitoring the acid pH change consequent on the hydrolysis of ATP, as described by Chance & Nishimura (1967), in a medium containing 0.5 mM-ATP, 0.15 M-KCl and 0.5 mM-Tris-HCl. The initial pH was 7.4 and the temperature was 30°C.

Assay of mitochondrial protein. This was determined by the method of Gornall *et al.* (1949) with crystalline bovine plasma albumin [fraction V; Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] as a standard.

Materials

All chemicals were of A.R. grade where available. Antimycin A was purchased from Sigma (London) Chemical Co. and acetylcolletotrichin was a generous gift from Dr. J. MacMillan of the School of Chemistry

of this University. Carbonyl cyanide *p*-chlorophenylhydrazone was a kind gift from Dr. P. G. Heytler (Du Pont de Nemours, Wilmington, Del., U.S.A.).

Results

Inhibition of respiration by acetylcolletotrichin and antimycin A

Like antimycin, acetylcolletotrichin inhibited the oxidation of succinate and of those substrates with NAD-linked dehydrogenases (e.g. glutamate, glutamate together with malate, 3-hydroxybutyrate), but did not inhibit the oxidation of ascorbate in the presence of tetramethyl-*p*-phenylenediamine. However, acetylcolletotrichin was considerably less effective (50% inhibition at approx. 2 nmol/mg of mitochondrial protein) than antimycin (approx. 0.02 nmol/mg). Further, the effectiveness of acetylcolletotrichin depended on the substrate used and on whether respiration was stimulated by an uncoupling agent or by ADP together with phosphate (Fig. 1).

Acetylcolletotrichin was significantly more effective with succinate than with those substrates with NAD-linked dehydrogenases or in the presence of the uncoupler carbonyl cyanide *p*-chlorophenylhydrazone. Thus under conditions where acetylcolletotrichin had caused maximal inhibition of ADP-stimulated respiration, the addition of carbonyl cyanide *p*-chlorophenylhydrazone caused a 20–30% stimulation, as shown in Fig. 1. These findings are similar to those reported by Kaniuga *et al.* (1969) for antimycin, except that with this latter inhibitor the partial release of respiration by uncoupler was only observed at a critical suboptimal concentration of antimycin.

Fig. 1. Inhibition of mitochondrial respiration by acetylcolletotrichin

Oxygen uptake was measured as described in the Experimental section. Additions to the medium were: (a) 5 mM-L-malate, 5 mM-L-glutamate and 2 mM-potassium phosphate; (b) 10 mM-succinate plus 2 mM-potassium phosphate. ●, Respiration stimulated by addition of 2 mM-ADP; ○, respiration stimulated by addition of 1 μM-carbonyl cyanide *p*-chlorophenylhydrazone.

Acetylcolletotrichin had similar effects on the respiration of rat and pig kidney mitochondria, was much less effective with rat heart mitochondria, and at a concentration of 60 nmol/mg of protein was totally without effect on the oxidation of glycerol 1-phosphate or pyruvate by well-coupled blowfly flight-muscle mitochondria. This last effect is in marked contrast with the action of antimycin. Most of the subsequent experiments described in this paper were performed with rat liver mitochondria.

That acetylcolletotrichin and antimycin do not act at precisely the same inhibitory site is, perhaps, indicated by the results of experiments in which the possible additive effects of the two inhibitors were investigated (Fig. 2). Thus the antimycin concentrations required to cause 50% and maximal inhibition were the same in the absence and in the presence of sufficient acetylcolletotrichin to cause 50% inhibition of respiration. There is of course the possibility that the high-affinity inhibitor (antimycin) displaced the low-affinity inhibitor (acetylcolletotrichin). However, a number of observations, such as inhibition of respiration and modification of the spectral properties of cytochrome *b* (Lightbown & Jackson, 1956; Chance, 1958) indicate that the effects of 2-alkyl-4-hydroxyquinoline *N*-oxides and antimycin are strictly additive (Nijs, 1967). Also in a parallel situation in which Amytal (low-affinity) and rotenone (high-affinity) were used to block the oxidation of those substrates with NAD-linked dehydrogenases, it was found that the effects of the two inhibitors were strictly additive. A similar situation has been investigated by Garland *et al.* (1969) with rotenone and piericidin A;

again the effects of these two inhibitors was additive. The spectroscopic observations reported below also indicate that acetylcolletotrichin and antimycin have different sites of action.

Binding of acetylcolletotrichin to rat liver mitochondria

Comparison of the binding curve for acetylcolletotrichin to rat liver mitochondria (Fig. 3) with the curve for the inhibition of respiration shows some surprising features. The inhibition curve is hyperbolic, whereas the binding curve is sigmoidal. The binding of 1 nmol of acetylcolletotrichin/mg of mitochondrial protein produced 80% inhibition of respiration, but the binding of a further 3 nmol of acetylcolletotrichin was required to produce maximal effects on respiration (95–98% inhibition). With intact mitochondria both the binding and the inhibitory effects of antimycin show sigmoidal curves (Kaniuga *et al.*, 1969).

Fig. 2. Effect of antimycin on the rate of succinate oxidation in the presence of a fixed amount of acetylcolletotrichin, and in its absence

Various amounts of antimycin were added to a medium containing 5 mM-succinate, 5 mM-phosphate, 0.24 mM-ADP (all as potassium salts) and when present (●) 1.5 nmol of acetylcolletotrichin/mg of mitochondrial protein (○, control). Sufficient time was allowed to elapse so that the antimycin had produced its maximal effect before the rate was determined.

Fig. 3. Inhibition of respiration (●) and the binding (○) of acetylcolletotrichin to rat liver mitochondria

In the oxygen-electrode experiments acetylcolletotrichin was added as an ethanolic solution to a medium containing 5 mM-succinate, 5 mM-phosphate and 0.24 mM-ADP. At all concentrations used acetylcolletotrichin produced an immediate inhibitory effect. This behaviour is in contrast with that with low concentrations of antimycin, where incubation for several minutes is required before maximal effects are observed. In the binding experiments rat liver mitochondria (5.1 mg of protein) were added to a series of centrifuge tubes containing 4 ml of a medium composed of 0.12 M-KCl, 20 mM-Tris-HCl, pH 7.4, and various amounts of acetylcolletotrichin. After incubation for 5 min at 25°C the mitochondria were sedimented by centrifugation at 12000g for 7 min. The acetylcolletotrichin remaining in the supernatant was determined by assaying its inhibitory effect on the oxidation of succinate under the conditions described above. The volume of supernatant used was sufficient to give a 20–30% inhibition. At this concentration the inhibitory effect of acetylcolletotrichin is nearly directly proportional to its concentration.

Fig. 4. Spectral changes observed at various wavelengths (with 575 nm as the reference wavelength) on addition of acetylcolletotrichin to mitochondria in presence of succinate

The upper two traces are recordings obtained at (a) 560 nm and (b) 555 nm on addition of acetylcolletotrichin (8 nmol/mg of protein) to a mitochondrial suspension containing 5 mM-succinate and 2 μ M-rotenone. The lower three spectra were plotted from data obtained in separate experiments (using the same mitochondrial suspension) conducted at different measuring wavelengths at 30°C; (c), spectrum of initial rapid change; (d), spectrum of subsequent slow change; (e), spectrum of final steady state.

The binding of acetylcolletotrichin to rat liver mitochondria was not affected by the metabolic state, i.e. the presence of ADP together with phosphate or of an uncoupling agent (1 μ M-carbonyl cyanide *p*-chlorophenylhydrazine) did not affect the binding curve significantly.

Spectroscopic effects induced by acetylcolletotrichin

The spectroscopic changes that occurred on addition of acetylcolletotrichin were dependent to some extent on the substrate that was present. With succinate (in the presence of rotenone), the addition of

acetylcolletotrichin caused a reduction of cytochrome(s) *b* and an oxidation of cytochrome *c* (Fig. 4). However, the details of the kinetics were more complex. Immediately after addition of acetylcolletotrichin there was a rapid initial spectral change, followed by a slow change with a half-time of 20 s at 30°C. These changes may be interpreted to mean that on first addition of acetylcolletotrichin, cytochromes *b*₅₆₀ and *b*₅₆₄₋₅₆₅ were reduced and cytochrome *c*₅₅₀ was oxidized. In the slow phase cytochrome *b*₅₆₀ and cytochrome *c*₅₅₅ were then oxidized. The final steady state was one in which cytochrome *b*₅₆₄₋₅₆₅ was reduced and the *c* cytochromes and cytochrome *b*₅₆₀

were oxidized. The ultimate effect of acetylcolletotrichin is consistent with the following sequence:

However, this scheme does not account for the anomalous behaviour of cytochrome b_{560} and the component assigned as cytochrome c_{555} (cytochrome c_1 ?) on first addition of acetylcolletotrichin.

A series of experiments carried out under the same conditions as those described above but in which the reaction was stopped by freezing in liquid N_2 were performed. Difference spectra at 77°K revealed in the rapid initial phase after addition of acetylcolletotrichin peaks at 562 and 555 nm and troughs at 558 and 548 nm. When the samples were incubated for 2 min and then frozen in liquid N_2 the peak at 555 nm was considerably diminished, but the trough at 548 nm was considerably deeper. These findings are consistent with the results obtained at 30°C if the following assignments are made. The 77°K peak at 562 nm corresponds to the 30°C peak at 564–565 nm, i.e. the long-wavelength cytochrome b , the low-temperature peak at 555 nm corresponds to the short-wavelength cytochrome b absorbing at approx. 560 nm at 30°C, and the deepening of the 548 nm trough at 77°K corresponds to the oxidation of the cytochromes c .

Fig. 5 shows the spectral changes induced by acetylcolletotrichin at 30°C with glutamate together with

malate as substrates. The changes observed were qualitatively the same as those with succinate as substrate, except that in the initial rapid phase no trough at 550 nm appeared. This was presumably due to the fact that the steady-state reduction of the c cytochromes with glutamate together with malate is not as great as that with succinate (e.g. Fig. 8). The 77°K spectra were similar to those observed with succinate as substrate.

One point to be emphasized is that antimycin caused a spectral shift in the low-temperature band at 555 to 557 nm (Kaniuga *et al.*, 1969), whereas acetylcolletotrichin did not.

Preliminary observations showed that acetylcolletotrichin at concentrations which caused maximal inhibition of respiration, like antimycin, produced extensive reduction of ubiquinone in pig kidney mitochondria. It was not possible to determine the effect of acetylcolletotrichin in rat liver mitochondria because of interference by other pigments.

Interaction of NAD- and succinate-oxidizing systems in the presence of acetylcolletotrichin

A surprising interaction was found between the inhibitory effect of acetylcolletotrichin on succinate oxidation on the one hand and of those substrates with NAD-linked dehydrogenases on the other. Table 1 shows that, at a concentration of 2 nmol of acetylcolletotrichin/mg of mitochondrial protein, the addition of glutamate together with malate in the presence of rotenone (which almost completely abolished the oxidation of these latter substrates) almost halved the effectiveness of acetylcolletotrichin on succinate oxidation. At higher acetylcolletotrichin

Fig. 5. Effect of acetylcolletotrichin on the spectral response of cytochromes with glutamate together with malate as substrates. The experimental procedure was the same as that described in the legend to Fig. 4, except that succinate and rotenone were omitted and 5 mM-L-glutamate, 5 mM-L-malate and 1 mM-malonate were added. (a), Spectrum of initial rapid change; (b), spectrum of slow change; (c), spectrum of final steady state.

Table 1. *Inhibitory effect of acetylcolletotrichin on succinate oxidation in the absence and presence of rotenone with or without the addition of glutamate together with malate*

The incubation medium contained 10mM-succinate and 2mM-phosphate; 0.5 μ M-rotenone, 5mM-L-glutamate and 5 mM-L-malate were present where indicated. Respiration was stimulated by the addition of 2mM-ADP, and acetylcolletotrichin was added after the steady state of respiration was attained.

Additions	Concn. of acetylcolletotrichin (nmol/mg of protein) ...	Inhibition of succinate oxidation (%)	
		2	4
Succinate only		65	75
Succinate with rotenone		55	65
Succinate, rotenone, glutamate and malate		35	50

Fig. 6. *Interaction of the oxidation of succinate and of glutamate together with malate in the presence of acetylcolletotrichin*

The incubation medium contained 5mM-L-glutamate, 5mM-L-malate and 2mM-phosphate; 2mM-ADP, various amounts of acetylcolletotrichin (ACT) as shown in the Figure, 10mM-succinate and 0.5 μ M-rotenone were added as shown.

concentrations (4nmol/mg of protein) the effect was less marked but was still apparent. Thus it appears that the reduction of that segment of the respiratory chain on the substrate side of the rotenone block affects the ability of acetylcolletotrichin to interact with the respiratory chain at the level of cytochromes *b* and *c*.

The converse may be shown to be the case (Fig. 6). With glutamate together with malate as substrates and with an excess of ADP to stimulate respiration, increasing amounts of acetylcolletotrichin caused progressively greater inhibition of oxygen uptake. At certain concentrations of acetylcolletotrichin addition of succinate caused a more extensive inhibition of

Fig. 7. Effect of carbonyl cyanide *p*-chlorophenylhydrazone on the acetylcolletotrichin-induced inhibition of respiration with glutamate together with malate and succinate as substrates

The incubation medium contained 5mM-L-glutamate, 5mM-L-malate, 10mM-succinate and 2mM-phosphate; 2mM-ADP or 1 μ M-carbonyl cyanide *p*-chlorophenylhydrazone (CCCP) were added followed by acetylcolletotrichin (ACT) and 0.5 μ M-rotenone. Note that when carbonyl cyanide *p*-chlorophenylhydrazone was used to stimulate respiration, twice the quantity of acetylcolletotrichin was added; this is because acetylcolletotrichin is less effective in the presence of uncouplers (see Fig. 1 and the text).

respiration, which was partially reversed by the addition of rotenone. This latter increase in rate can only have been due to the release of the inhibitory effect of acetylcolletotrichin on succinate oxidation consequent on the reduction of that portion of the respiratory chain on the substrate side of the rotenone block (cf. Table 1). These effects were much less obvious when uncoupler was used to stimulate respiration, but were still observable (Fig. 7).

Supporting results were obtained by observing the redox behaviour of cytochrome *c* either spectrophotometrically or, as shown in Fig. 8, by following the redox potential of cytochrome *c* indirectly by using a Pt electrode with ferro- and ferri-cyanide as mediators. Thus the addition of succinate led to a reduction of cytochrome *c*, and acetylcolletotrichin largely reversed this. However, with glutamate together with malate which caused a less extensive reduction, the addition of the same amount of acetylcolletotrichin had a relatively small effect. The subsequent addition of succinate resulted in the oxidation of cytochrome *c*, an effect that was reversed by the addition of malonate.

Effects of acetylcolletotrichin on some other mitochondrial functions

At concentrations up to 30nmol/mg of mitochondrial protein acetylcolletotrichin did not elicit mitochondrial ATPase activity, nor did it affect the

Fig. 8. Changes in the redox potential followed with a Pt electrode with ferricyanide and ferrocyanide as mediators

Mitochondria were added to a medium containing 5mM-ferrocyanide, which was then partially oxidized. Addition of either 5mM-succinate or of 5mM-L-glutamate together with 5mM-L-malate caused a reduction and after 2min a steady state was achieved. Acetylcolletotrichin (ACT) (8nmol/mg of mitochondrial protein), 5mM-malonate and 1 μ M-carbonyl cyanide *p*-chlorophenylhydrazone (CCCP) were added as shown. No attempt was made to calibrate the electrode system in an absolute sense, so the potentials given are only approximate. Also the response time of the system was very slow.

ATPase activity observed in the presence of uncoupling agents.

Under a wide variety of conditions in the types of experiments reported in this paper acetylcolletotrichin did not appear to induce mitochondrial volume changes as judged by changes in light-scattering.

Acetylcolletotrichin inhibited the oxidation of NADH (rotenone-sensitive) and of succinate by sonicated rat liver mitochondria. The effect was slightly less than with intact mitochondria, as was to be expected, since acetylcolletotrichin was less effective with uncoupled mitochondria (see Figs. 1 and 7).

Discussion

One certain thing emerges from the studies reported in this paper, which is that acetylcolletotrichin inhibited electron flow between cytochromes *b* and *c* in rat liver and rat and pig kidney mitochondria. At the concentrations tested (up to 60 nmol/mg of mitochondrial protein) acetylcolletotrichin had no observable effect on the oxidative activity of blowfly mitochondria. The action of acetylcolletotrichin with liver and kidney mitochondria is superficially similar to that of antimycin, but quite different with blowfly mitochondria. This latter finding and the fact that the inhibitory effects of acetylcolletotrichin and antimycin were not additive (see Fig. 2) suggest that these two inhibitors do not act at precisely the same site.

Spectroscopically the effects of acetylcolletotrichin and antimycin are similar but not identical. Thus whereas antimycin causes a red-shift in the absorption of the α and γ bands of cytochrome *b*, perhaps indicating the formation of a complex (Kaniuga *et al.*, 1969), no evidence was obtained in the present investigation that this was the case with acetylcolletotrichin.

The series of spectroscopic changes before the final steady state was achieved (Figs. 4 and 5) seem to be peculiar to acetylcolletotrichin. It is difficult to understand what is happening during these slow changes. Several factors may contribute, among which may be:

(i) changes in mid-point potential of the cytochromes *b* consequent on changes in the redox state of the *c* cytochromes (Rieske, 1971; Wilson *et al.*, 1971);

(ii) a slow structural modification of cytochromes *b*₅₆₀ and *c*₁ as a result of the binding of acetylcolletotrichin;

(iii) 'allosteric' changes in the *b* cytochromes induced by changes at the succinate dehydrogenase and NADH dehydrogenase levels (see Figs. 6, 7 and 8) (Singer *et al.*, 1972; Nelson *et al.*, 1972);

(iv) an effect of membrane potential on the mid-point potential of cytochrome *b* similar to those

postulated for cytochromes *a* and *a*₃ (Hinkle & Mitchell, 1970).

Any combination of these or other factors may be involved and may contribute to the observed changes. Obviously more sophisticated techniques than those available to us at the present time are required to solve this problem.

The partial reversal of the action of low (but not high) concentrations of antimycin by uncoupling agents has been observed previously (Kaniuga *et al.*, 1969). This also occurs with acetylcolletotrichin, even at concentrations that cause maximal inhibition of ADP-stimulated respiration. Since acetylcolletotrichin had no effect on mitochondrial ATPase activity, it is unlikely that this compound has an oligomycin-like effect, and an explanation must be sought elsewhere. Differences in the potency of electrically charged inhibitor compounds in the coupled and uncoupled states are well documented, e.g. azide (Palmieri & Klingenberg, 1967) and guanidine and the substituted guanidines (Chappell, 1963). The most likely explanation of these differences is that put forward by Mitchell (1966). In the coupled state the mitochondria have a membrane potential, negative inside, which tends to exclude azide and cause the accumulation of the guanidines. The addition of an uncoupling agent causes the collapse of the membrane potential and a consequent migration of the inhibitor molecules.

However, acetylcolletotrichin is not an electrically charged molecule at anywhere near physiological pH values (J. MacMillan, personal communication) and therefore the above argument cannot apply. This is substantiated by experiments (similar to those of Fig. 3) where no effect of ADP together with phosphate or of uncoupling agents on the binding of acetylcolletotrichin to liver mitochondria could be demonstrated.

This latter finding would also seem to rule out the explanation that Kaniuga *et al.* (1969) have proposed for the partial reversal of the inhibitory action of antimycin by uncoupling agents. They proposed that uncoupling agents displaced antimycin from its site of action. It seems possible that the action of uncoupling agents in this respect will only be explained when it is known which, if any, of the points made in paragraphs (i)–(iv) above is important.

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