Biochemical Pharmacology, Vol. 18, pp. 73–83. Pergamon Press. 1969. Printed in Great Britain

EFFECT OF A POLYACETYLENIC FISH POISON ON THE OXIDATIVE PHOSPHORYLATION OF RAT LIVER MITOCHONDRIA

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(*Received* 26 *April* 1968; *accepted* 24 *July* 1968)

Abstract—The effects of a C_{14} polyacetylenic tetrahydropyranyl alcohol and its acetate ester—the active principles of the leaves of *Clibadium sylvestre* which are used by South American Indians as a fish poison—have been studied on the oxidative phosphorylation of rat liver mitochondria.

Preincubation of rat liver mitochondria with these compounds caused a fall in the P/O ratio and respiratory control of the mitochondria when oxidizing β-hydroxybutyrate, succinate, pyruvate and malate, and isocitrate.

With all substrates, the state III (stimulated) respiration fell by at least 20 per cent after a few minutes preincubation with the polyacetylenic compounds. By contrast the state IV (resting) respiration was not markedly affected except when β-hydroxybutyrate was the substrate.

Both compounds were found to inhibit, linearly with concentration, the ATP-Mg^{2+} induced contraction of mitochondria swollen by inorganic phosphate. Complete inhibition of contraction occurred at a final concentration of 15×10^{-5}M for the polyacetylenic alcohol and 12×10^{-5} M for the acetate ester.

Derivatives of these compounds in which the polyacetylenic chain had been partially or fully saturated did not uncouple oxidative phosphorylation. These derivatives also only partially inhibit the ATP-Mg^{2+} induced contraction of swollen mitochondria.

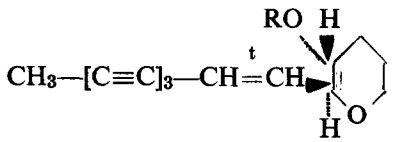
The results are discussed with respect to configuration of these compounds and the structure and function of the mitochondrial membrane.

CONSIDERABLE advances in the understanding of oxidative phosphorylation have been made by using specific, naturally occurring, toxic compounds as scientific probes, e.g. rotenone, oligomycin, atractylate. This paper is concerned with the effects on the oxidative phosphorylation of rat liver mitochondria of a new naturally occurring polyacetylenic fish poison.

The isolation and characterisation of the active components of the leaves of the shrub *Clibadium sylvestre*—used by the South American Indians as a fish poison—and subsequent preliminary pharmacological investigations (1) indicated two compounds—a C_{14} polyacetylenic tetrahydropyranyl alcohol and its acetate ester (Fig. 1)—which had marked activity on fish (Guppies), mice and isolated spinal cord preparations. The polyacetylenic alcohol was named Cunaniol since the shrub is known by the indigenous peoples as 'Cunani'.

To test the hypothesis that these compounds may act by disturbing normal membrane function, their effect on a membrane dependent system was studied. The system chosen was the mitochondrion since much is known about the interelationship between its metabolic activity and the integrity of its membrane structure.[2] The work

described here indicates that Cunaniol and its acetate ester uncouple oxidative phosphorylation and also inhibit the ATP-Mg^{2+} induced contraction of mitochondria swollen by inorganic phosphate. Further on partially or fully saturating the polyacetylenic chain, the uncoupling activity is lost and complete inhibition of contraction is no longer possible. Preliminary reports of some of this work have already been presented.[3, 4]



1. R=H
2. R=Ac

FIG. 1. Structure of Cunaniol and Cunaniol acetate.

MATERIALS AND METHODS

Animals

Male Albino rats weighing between 180–220 g. and fed *ad libitum* on a stock diet were used.

Chemicals

DL-β-hydroxybutyrate, L-Malate, DL-Isocitrate (all sodium salts), ADP, ATP and Tris buffer were supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A.; succinate and pyruvate (sodium salts) by C. F. Boehringer und Soehne B.m.b.H., Mannheim, Germany. All other reagents were of standard *AnalaR* grade and all solutions were made up in doubly distilled water. ADP was assayed and used as reported previously.[5]

Preparation of mitochondria (see ref. 6).

After removal of the liver from the rat into ice-cold 0·25 M sucrose—1mM EDTA, the liver was washed free of blood, chopped up finely, rewashed and finally homogenised in a Potter–Elvehjem homogeniser (Teflon pestle; clearance 0·015 in.) to give a 1:10 (w/v) suspension.

The nuclei and cell debris were removed and the mitochondria sedimented at 80,000 *g*-min in a refrigerated centrifuge. The mitochondrial pellet was freed of the 'fluffy' layer and resuspended in the sucrose-EDTA medium. After centrifuging at 1500 *g* for 10 min to remove any traces of cell debris, the mitochondria were resedimented as described above. The mitochondria were washed twice more and the pellet from the final sedimentation was resuspended in the sucrose-EDTA medium and used as the mitochondrial source.

Estimation of P/O ratio and respiratory control

This was carried out with an oxygen electrode apparatus as outlined previously.[5] When mitochondria were preincubated with Cunaniol or its acetate the final concentration (in the preincubation medium) of mitochondrial protein was about 20 mg/ml and that of the Cunaniol (or acetate) approximately 5×10^{-4} M i.e. Cunaniol/mitochondrial protein ratio of approximately 5 μg/mg protein. 0·1 ml of the preincubation medium of Cunaniol and mitochondria were used for each oxygen electrode assay (F.V. = 2 ml.) Mitochondrial protein was estimated by the method of Lowry, Rosebrough, Farr and Randall.[7]

Swelling–contraction cycles

Swelling was followed at 25° and 520 mμ in 0·125 M KCl—0·02 M Tris pH 7·4.[8,9] In all cases, swelling was initiated by the addition of 0·1 ml (0·8 mg mitochondrial protein) of a mitochondrial suspension in the KCl-Tris medium, to a cuvette containing 10 mM (final concentration) potassium phosphate pH 7·4. Reversal of swelling was brought about by the addition of a mixture giving the following final concentrations: ATP, 7·5 mM; $MgCl_2$, 5 mM; bovine plasma albumin, 0·03 mg/ml (see ref. 9).

Use of polyacetylene solutions

As Cunaniol and its derivatives are not very soluble in aqueous solutions they were dissolved in a small amount of ethanol and then made up to the required concentration in 0·25 M sucrose for oxidative phosphorylation experiments and in 0·125 M KCl-0·02 M Tris for swelling experiments in order to avoid osmotic dilution. In both types of assay control experiments containing in addition the amount of ethanol normally required to solubilize the polyacetylenes, showed no significant difference from the normal control experiments.

RESULTS

Calculation of results

P/O ratios and respiratory control values were calculated as outlined by Chance and Williams.[10] Each assay allowed at least two estimations of the various mitochondrial parameters and the values were averaged. Results of the P/O ratios and respiratory control were expressed as a percentage of the zero time value because it was not possible to perform a preincubation experiment with each different substrate on one preparation of mitochondria. For the swelling—contraction cycles the amount of contraction induced 4 minutes after the addition of the ATP mixture was noted and taken as a measure of the contraction rate. Each dose–response curve is the average of 2 experiments.

Effect on P/O ratios and respiratory control

No obvious effects were seen on State I or II respiration (i.e. respiration in the absence of substrate and phosphate acceptor, and in absence of phosphate acceptor only) either with or without preincubation with Cunaniol or its acetate. If Cunaniol was injected directly into a respiring mitochondrial preparation, only very slight effects were observed if a similar final concentration (25 μM) was used to that employed in the preincubation experiments. However, if a much higher final concentration was used in the oxygen electrode assay (125 μM) then with both β-hydroxybutyrate and succinate as substrates a decrease in respiratory control was observed.

The most marked and obvious effects were seen after the mitochondria had been preincubated with Cunaniol or its acetate at 0° for up to 4 hr. The conditions of the preincubation were as stated in the experimental section, i.e. Cunaniol/mitochondrial protein ratio of 5 μg/mg. protein Figs. 2–5 show the effect of Cunaniol on the P/O ratio and respiratory control of rat liver mitochondria oxidizing β-hydroxybutyrate, succinate, pyruvate and malate, and isocitrate respectively as compared with a control sample of mitochondria under similar conditions but without the Cunaniol. Figs 6, 7, show similar effects of Cunaniol acetate on mitochondria oxidizing β-hydroxybutyrate and succinate.

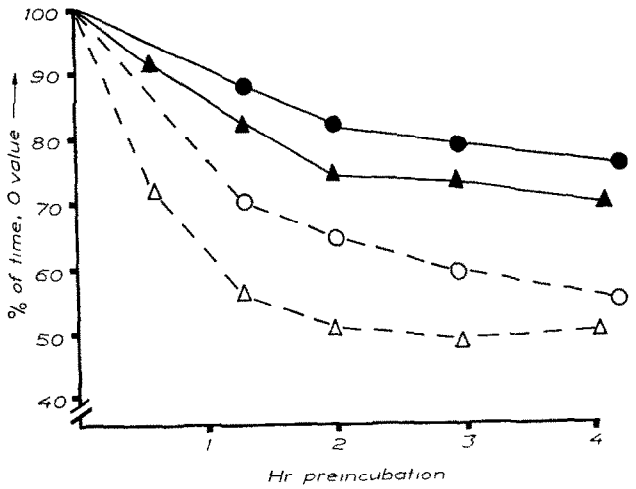


FIG. 2. Effect of preincubation with Cunaniol on DL-β-hydroxybutyrate oxidation by rat liver mitochondria. The results are expressed as a % of the zero time control values which were—P/O ratio = 2·75; Respiratory control = 5·25. The conditions of preincubation and assay and method of calculating P/O ratios and respiratory control were as described in the methods section. ●—● control P/O; ○ - - - ○ control respiratory control (R.C.); ▲ - - - ▲ experimental P/O; △—△ Experimental respiratory control (R.C.).

Several trends in these results are common to all substrates. Firstly, the respiratory control was affected more than the P/O ratio suggesting that Cunaniol and its acetate affect more markedly the rate of oxidative phosphorylation rather than the overall stoichiometry (although this too fell at later stages). Next a significant part of this fall in respiratory control appeared to occur during the first part of the preincubation (cf. Figs. 4–7 in particular, where a large fall in respiratory control occurred in a matter of minutes). Further, after a preincubation period of about 1 hr the mito-

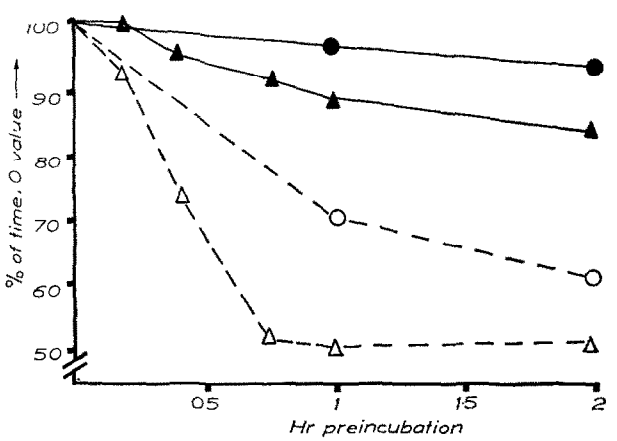


FIG. 3. Effect of preincubation with Cunaniol on succinate oxidation by rat liver mitochondria. The results are expressed as a % of the zero time control values which were—P/O ratio = 2·0; respiratory control = 8·2. All other conditions were as in Fig. 2. ●—● control P/O; ○ - - - ○ control respiratory control (R.C.); ▲—▲ experimental P/O; △ - - - △ experimental respiratory control (R.C.).

chondria preincubated with Cunaniol (or acetate) and the control mitochondria followed much the same rate of loss of efficiency of oxidative phosphorylation. Hence in this respect Cunaniol and its acetate appear to accelerate the normal 'ageing' process of mitochondria under these conditions, at least with respect to P/O ratios and respiratory control. There are, however, differences between the rates and extents of the loss of oxidative phosphorylation efficiency of different substrates. With mito-

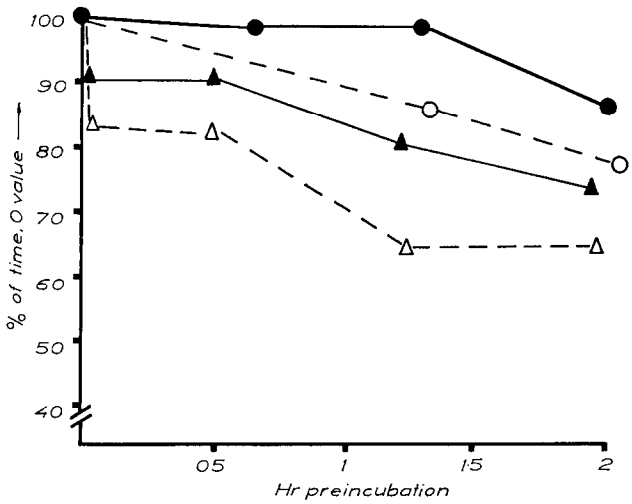


FIG. 4. Effect of preincubation with Cunaniol on pyruvate + L-malate oxidation by rat liver mitochondria. The results are expressed as a % of the zero time control values which were—P/O ratio = 2·74; respiratory control = 2·79. All other conditions as in Fig. 2. ●——● control P/O; ○ - - - ○ control respiratory control (R.C.); ▲—▲ Experimental P/O; △ - - - △ Experimental respiratory control (R.C.).

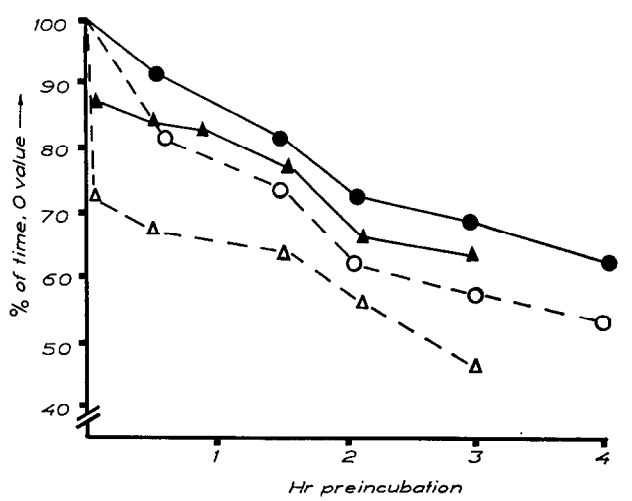


FIG. 5. Effect of preincubation with Cunaniol on DL-isocitrate oxidation by rat liver mitochondria. The results are expressed as a % of the zero time control values which were P/O ratio = 2·68; respiratory control = 3·50. All other conditions as in Fig. 2. ●—● control P/O; ○ - - - ○ control respiratory control (R.C.); ▲—▲ experimental P/O; △ - - - △ experimental respiratory control (R.C.).

chondria preincubated with Cunaniol oxidizing β-hydroxybutyrate or succinate (Figs. 2, 3), the respiratory control fell to 50 per cent of the Time 0 value whereas with pyruvate and malate (Fig. 4) it only fell to 65 per cent. With isocitrate as substrate, however, the mitochondria did not exhibit respiratory control after 3 hr preincubation with Cunaniol (Fig. 5). These differences may well reflect the different locations of each of the enzyme systems involved in the mitochondrial membrane.

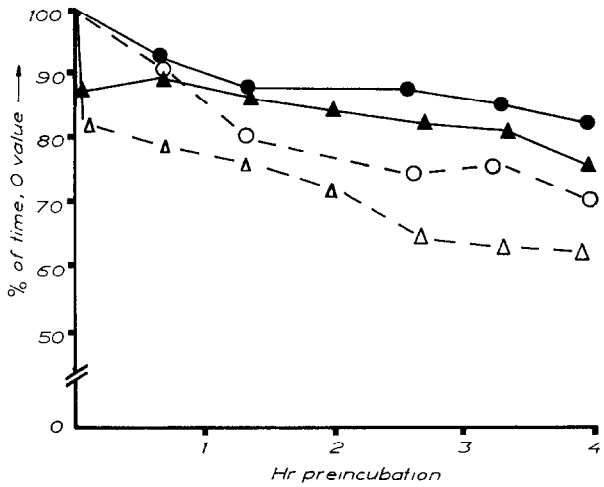


FIG. 6. Effect of preincubation with Cunaniol acetate on DL-β-hydroxybutyrate oxidation by rat liver mitochondria. The results are expressed as a % of the zero time control values which were P/O ratio = 2·95; respiratory control = 5·00. All other conditions as in Fig. 2. ●—● control P/O; ○---○ control respiratory control (R.C.); ▲—▲ experimental P/O; △---△ experimental respiratory control (R.C.).

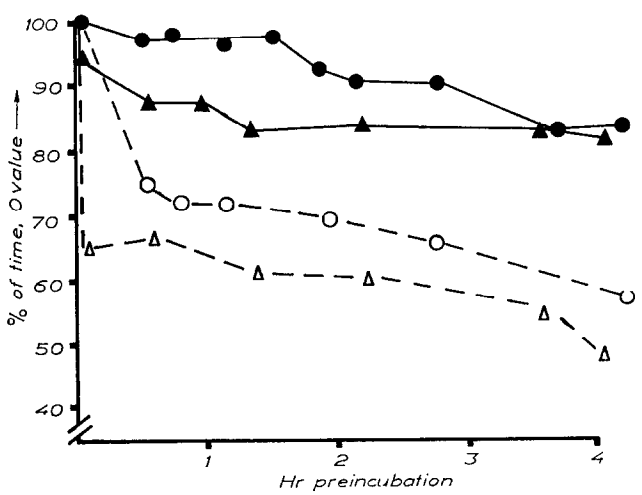


FIG. 7. Effect of preincubation with Cunaniol acetate on succinate oxidation by rat liver mitochondria. The results are expressed as a % of the zero time control values which were—P/O ratio = 1·94; respiratory control = 6·46. All other conditions as in Fig. 2. ●—● control P/O; ○—○ control respiratory control (R.C.); ▲—▲ experimental P/O; △—△ experimental respiratory control (R.C.).

Inspection of the state III (stimulated) and state IV (resting) respiration for each of the substrates after preincubation with Cunaniol or its acetate leads to the general conclusion that the fall in respiratory control as compared with the control was due to an inhibited state III (stimulated) respiration (Fig. 8 and Fig. 10). In both figures in the interests of clarity, the experimental respiratory quotients have been corrected for coincident changes in the control respiratory quotients and the points represent the difference between the experimental and control values. In all cases the state III respiration fell by at least 20 per cent after only a few minutes. Corresponding values for the changes in state IV (resting) respiration (similarly corrected) are shown in Fig. 9 (Cunaniol) and Fig. 10 (Cunaniol acetate). In both cases there was an increase in State IV (resting) respiration with β-hydroxybutyrate as substrate and a slight increase with succinate as substrate with mitochondria preincubated with Cunaniol. However, with the other substrates the state IV values did not vary ± 10 per cent from the time 0 value.

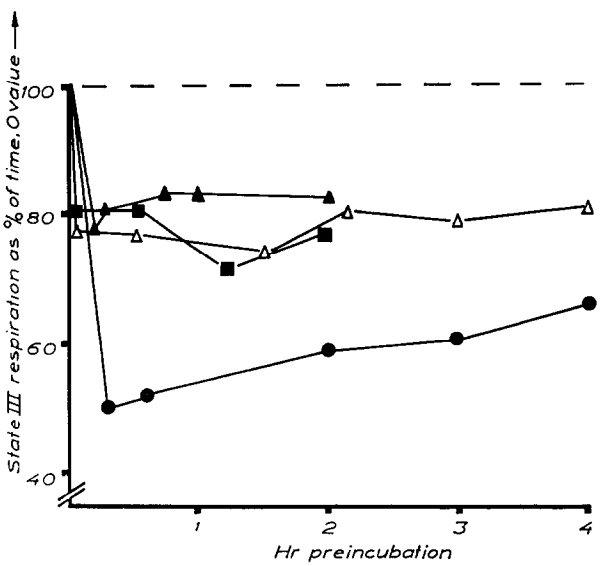


FIG. 8. Effect of preincubation with Cunaniol on state III or 'stimulated' respiration of rat liver mitochondria. The results are expressed as a % of zero time control values. Each point represents the mean of at least two experimental values ± the corresponding mean of at least two control values (as appropriate) and hence represents an experimental value corrected for any change in the control value. State III is as defined by Chance and Williams.[10] The zero time control values expressed as $10^3 \times \mu$ atom O/mg. protein/min. ± S.E.M. were: ●—● 40·8 ± 3·1 for DL-β-hydroxybutyrate; ▲—▲ 119·5 ± 4·6 for succinate; ■—■ 23·0 ± 0·7 for pyruvate + L-malate; △—△ 60·4 ± 4·2 for DLIsocitrate.

If the partially hydrogenated (hexahydro) or fully hydrogenated (perhydro) derivative of Cunaniol or its acetate is incubated with mitochondria under similar conditions, no differences to the controls are apparent with respect to P/O ratios and respiratory control. Thus the polyacetylenic chain appears to be necessary for this activity on oxidative phosphorylation.

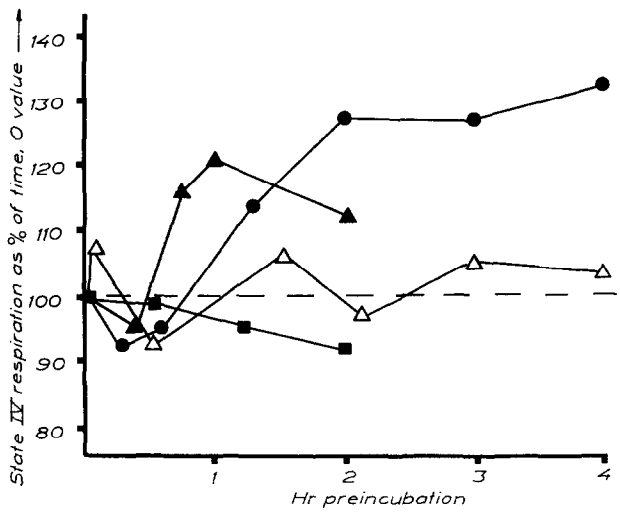


FIG. 9. Effect of preincubation with Cunaniol on State IV or 'resting' respiration of rat liver mitochondria. The results are expressed as a % of zero time control values. Each point has been corrected as in Fig. 8. State IV is as defined by Chance and Williams.[10] The zero time control values expressed as $10^3 \times \mu$ atom 0/mg. protein/min. ± S.E.M. were: ●—● 10·2 ± 0·5 for DL-β-hydroxybutyrate; ▲—▲ 20·9 ± 1·0 for succinate; ■—■ 8·5 ± 0·0 for pyruvate + L-malate; △—△ 17·4 ± 0·5 for DL-Isocitrate.

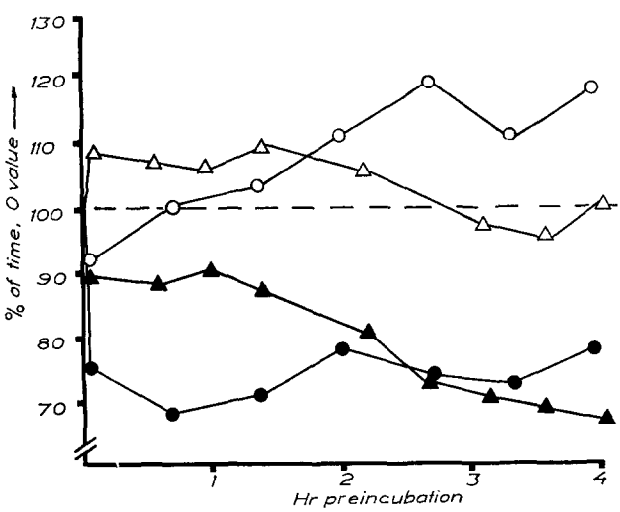


FIG. 10. Effect of preincubation with Cunaniol acetate on state III and state IV respiration of rat liver mitochondria. The results are expressed as a % of zero time control values. Each point has been corrected as in Fig. 8. States III and IV are as defined by Chance and Williams.[10] The zero time control values expressed as $10^3 \times \mu$ atom 0/mg. protein/min. ± S.E.M. were: State III, ●—● 43·1 ± 2·9 for DL-β-hydroxybutyrate; ▲—▲ 92·8 ± 3·8 for succinate. State IV, ○—○ 8·5 ± 0·4 for DL-β-hydroxybutyrate; △—△ 18·5 ± 0·9 for succinate.

Effect on the swelling–contraction cycle

Cunaniol itself and its two derivatives (hexahydro- and perhydro-) did not appear to affect the rate or time of onset of swelling, although Cunaniol acetate has been shown to stimulate the rate of swelling induced by phosphate.[3] Both Cunaniol and its acetate linearly inhibited the ATP-induced contraction of phosphate swollen mitochondria until complete inhibition of contraction occurred at final concentrations of $15{\cdot}5 \times 10^{-5}$ M for Cunaniol and 12×10^{-5} M for its acetate (Figs. 11, 12). Higher concentrations up to 17×10^{-5} M produced no further effects. When the effects of the partially (hexahydro-) or fully saturated (perhydro) derivatives of Cunaniol or its acetate were studied on the ATP- induced contraction of swollen mitochondria it was

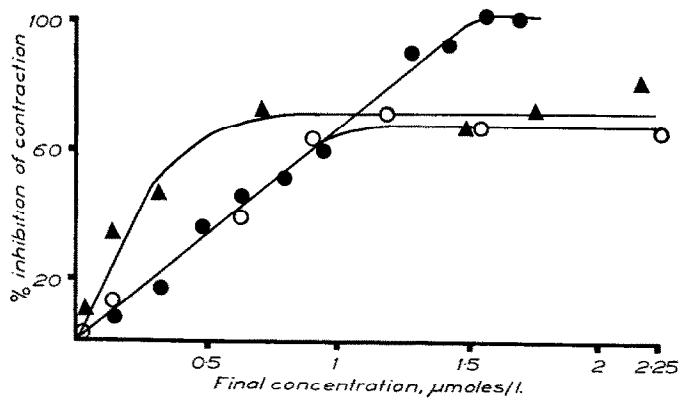


FIG. 11. Effect of Cunaniol and derivatives on ATP-induced contraction of rat liver mitochondria. The results are expressed as a % inhibition of the total contraction of control mitochondria. The mean contraction of control mitochondria was 0·175 ± 0·009 optical density units (6 experiments). The assay conditions were as described in the text. ●—● Cunaniol; ▲—▲ perhydro-derivative of Cunaniol ○—○ hexahydro-derivative of Cunaniol.

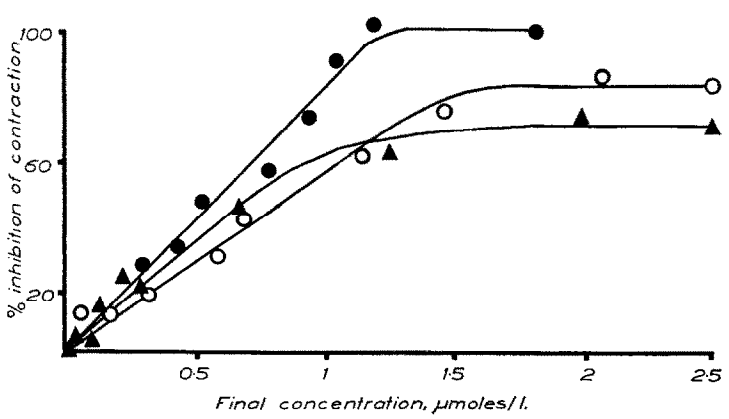


FIG. 12. Effect of Cunaniol acetate and derivatives on ATP-induced contraction of rat liver mitochondria. The results are expressed as a % inhibition of the total contraction of control mitochondria. All other conditions as in Fig. 11. ●—● Cunaniol acetate; ▲—▲ perhydro-derivative of Cunaniol acetate ○—○ hexahydro-derivative of Cunaniol acetate.

found that 100 per cent inhibition of contraction could not be achieved even with concentrations of the derivatives of between 20–25 $\times$ 10^{-5} M (Figs. 11, 12). The two derivatives of Cunaniol were only able to inhibit approximately 70 per cent of the control contraction, although the perhydro derivative was able to inhibit the contraction maximally at about half the concentration of the hexahydro derivative or Cunaniol itself (Fig. 11). The derivatives of Cunaniol acetate gave similar results (Fig. 12), inhibiting contraction only to the extent of 70–80 per cent.

DISCUSSION

These results should be considered from two aspects. Firstly, why should these polyacetylene compounds affect mitochondrial metabolism and how is this related to present knowledge of the mitochondrial membrane and its functional complexes. Secondly, why on partial or full saturation of the polyacetylenic chain is the activity on oxidative phosphorylation lost but only part of the activity on contraction. With this second aspect it is also important to consider the relationship between oxidative phosphorylation and the swelling–contraction cycle.

The ability to uncouple oxidative phosphorylation and inhibit ATP-dependent contraction is shared with such compounds as oligomycin and atractylate. Neubert and Lehninger[11] demonstrated that oligomycin linearly inhibits mitochondrial contraction to a saturation level and suggested that it titrated 'sensitive sites' on the mitochondrial membrane. More recent work[12] suggests that it is more likely to be associated with an inhibition of a phosphorylation step in the formation of a phosphorylated high energy intermediate. Whether these polyacetylenes similarly inhibit a reaction in the phosphorylation sequence or actually titrate sensitive sites on the mitochondrial membrane is not apparent. However, in view of the requirement for a slight preincubation period and also since the decrease in efficiency of oxidative phosphorylation is due for the greater part to an inhibited state III respiration it is possible that Cunaniol and its acetate restrict access of a vital substrate or intermediate to the mitochondrial oxidative phosphorylation machinery, probably that part which is common to oxidative phosphorylation and mitochondrial contraction.

When the effects of the partially or fully saturated derivatives are considered the situation becomes more complex, since whilst the saturated derivatives are inactive as far as uncoupling of oxidative phosphorylation is concerned they are still capable of inhibiting about 70 per cent of the total control contraction. Thus the loss of activity with respect to oxidative phosphorylation appears to be associated with only 30 per cent of the 'contraction sites'. In other words, only approximately one third of the sites associated with swelling–contraction activity are also associated with oxidative phosphorylation. If the partially/fully saturated derivatives cause their effects by a non-specific binding then these results might be interpreted as meaning that there are a number of very specific and unique sites on the mitochondrial membrane whose functional integrity is obligatory to oxidative phosphorylation. That these sites have a very characteristic and specific chemical geometry is apparent from considerations of the configuration of these polyacetylenes and derivatives. At this point it is worth noting the suggestions of Sjöstrand,[13] Lehninger[14] and O'Brien[16] that the mitochondrial membrane may not contain a continuous phospholipid bilayer but a series of globular bilayers.

The exact relationship between oxidative phosphorylation and swelling–contraction

cycles is still unknown. However, the report of the presence of a contractile protein in the mitochondrial membrane[16] leads to the suggestion that ATP-induced mitochondrial contraction and ATP-induced actomyosin contraction might have certain similarities.[2] As muscle 'relaxing factor' has been shown to inhibit mitochondrial contraction[17] it was of interest to see if Cunaniol affected actomyosin contraction–relaxation properties. Neither Cunaniol nor its acetate had any effect upon the contraction–relaxation properties of actomyosin. It seems therefore that Cunaniol acts on mitochondrial contraction by some means other than on the contractile protein of the mitochondrial membrane. Alternatively, this result may be explained by the fact that mitochondrial contraction is not associated with this contractile protein.

These polyacetylenic compounds have two particular properties which are probably associated with their effects on the mitochondrial membrane. Firstly, they are lipophilic but by virtue of the tetrahydropyranyl alcohol they have a relatively hydrophilic or polar endgroup. In this respect the Cunaniol would be more polar than its acetate. They may therefore be compared with a phospholipid in as far as this has a relatively polar end group and lipophilic 'tail'. Secondly, Cunaniol and its acetate have a particularly unique configuration which appears to be the most important of their properties since on losing it they lose their activity. This configuration is the particularly rigid, straight and inflexible polyacetylenic chain which on partial or full saturation becomes spatially larger, less rigid and more flexible. This change in configuration from a needle-like to a fairly flexible and spatially larger compound is associated with loss of uncoupling activity on oxidative phosphorylation and the inability to achieve more than two-thirds inhibition of contraction. Thus whilst all the derivatives may be able to achieve some inhibition of contraction by a non-specific binding onto the membrane, only the polyacetylenic alcohol and its acetate with their unique needle-like configuration may intrude into what must be very small specific sites or pores associated with oxidative phosphorylation.

Acknowledgements—I am indebted to Professor E. M. Crook for encouragement and advice and Dr. S. A. H. Zaidi and Mr. C. Gorinsky for discussion and supplies of the polyacetylenic compounds.

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