Citreoviridin, a Specific Inhibitor of the Mitochondrial Adenosine Triphosphatase

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1. Citreoviridin was a potent inhibitor of the soluble mitochondrial ATPase (adenosine triphosphatase) similar to the closely related aurovertins B and D. 2. Citreoviridin inhibited the following mitochondrial energy-linked reactions also: ADP-stimulated respiration in whole mitochondria from ox heart and rat liver; ATP-driven reduction of NAD$^+$ by succinate; ATP-driven NAD transhydrogenase and ATPase from ox heart submitochondrial particles. 3. The dissociation constant (K_D) calculated by a simple law-of-mass-action treatment for the citreoviridin–ATPase complex was 0.5–4.2 μM for ox-heart mitochondrial preparations and 0.15 μM for rat liver mitochondria. 4. Monoacetylation of citreoviridin decreased its inhibitory potency ($K_D = 2$–25 μM, ox heart; $K_D = 0.7$ μM, rat liver). Diacetylation greatly decreased the inhibitory potency ($K_D = 60$–215 μM, ox heart). 5. Hydrogenation of citreoviridin monoacetate diminished its inhibitory potency considerably. 6. No significant enhancement of fluorescence was observed when citreoviridin interacted with the mitochondrial ATPase.

Citreoviridin is a toxic secondary metabolite isolated from several *Penicillium* species (Uraguchi, 1971; Nagel *et al.*, 1972*a*). Its structure was elucidated by Sakabe *et al.* (1964). The absolute configuration has recently been determined (see structure Ia; L. J. Mulheirn, unpublished work).

Citreoviridin has a close structural similarity to the aurovertins (Mulheirn *et al.*, 1974), which have been shown to be potent inhibitors of the mitochondrial ATP synthetase system (Lardy *et al.*, 1964; Lenaz, 1965; Lee & Ernster, 1968; Roberton *et al.*, 1968; Osselton *et al.*, 1974; Ebel & Lardy, 1975).

In the present paper we show that citreoviridin and, to a lesser extent, citreoviridin monoacetate (structure Ib), inhibit various activities of the mitochondrial energy-conservation system in a manner similar to that of the aurovertins. Citreoviridin diacetate (structure Ic) is an ineffective inhibitor of the mitochondrial ATPase and its associated activities.

A simple model for the interaction of citreoviridin with the ATPase is presented to resolve the apparently anomalous differential sensitivity of the ATPase and the ATP synthetase to citreoviridin.

Abbreviations used: ATPase, adenosine triphosphatase (EC 3.6.1.3); h.p.l.c., high-performance liquid chromatography.

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Experimental

Citreoviridin

A sample of citreoviridin was obtained from Dr. D. W. Nagel and Dr. P. S. Steyn, C.S.I.R., Pretoria, South Africa. After recrystallization from methanol, the citreoviridin sample used for inhibition studies gave only a single peak on analysis by h.p.l.c. with chloroform/acetone (7:1, v/v) (see below). Crude samples of citreoviridin and solutions stored for a long time showed two poorly resolved peaks on h.p.l.c. The major peak, which was eluted first, corresponded to pure citreoviridin; it is probable that the second peak represented isocitreoviridin, which has not been well characterized but is considered to have a *cis*-double bond in the conjugated tetraene system (Nagel *et al.*, 1972*a*).
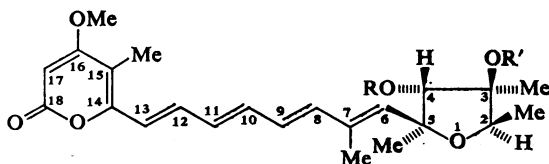
Structures: (Ia) R = R' = H
(Ib) R = MeCO, R' = H
(Ic) R = R' = MeCO

Citreoviridin can be purchased from Cambrian Chemicals Ltd., Croydon CR9 3QL, U.K. A sample from this source analysed by h.p.l.c. contained approx. 94% (by A_{380} measurements) of a 7:4 (w/w) citreoviridin/isocitreoviridin mixture.

Citreoviridin monoacetate

A sample was obtained from Dr. T. Goto, University of Nagoya, Nagoya, Japan, through Dr. D. Shitabsuji, Shell Kagaku, Tokyo, Japan. Its structure was confirmed by ^{1}H n.m.r.; δ (in p.p.m.; 100MHz, $C^2H_3O^2H$) 1.13 and 1.29 (3H each, s, CH_3 at C-3 and C-5), 1.17 (3H, d, $J = 6Hz$, CH_3 at C-2), 1.90 (3H, d, $J = 1Hz$, CH_3 at C-7), 2.00 (3H, s, CH_3 at C-15), 2.15 (3H, s, CH_3CO at C-4), 3.81 (1H, q, $J = 6Hz$, H at C-2), 3.89 (3H, s, CH_3O at C-16), 5.19 (1H, s, H at C-4), 5.60 (1H, s, H at C-17), 5.90 (1H, s, H at C-6), 6.3–7.3 (complex multiplet, 6H at C-8 to C-13); u.v.-absorption maxima in MeOH 400 (shoulder), 385, 294, 285 (shoulder), 238, 204nm; mass spectroscopy, m/e 444 (8%, M^+, $C_{25}H_{32}O_7$), 384 (1, M-CH_3CO_2H), 313 (12), 297 (11), 296 (14), 259 (20), 258 (27), 187 (26), 169 (50), 154 (20), 139 (100%, pyrone moiety, $C_7H_7O_3$), 127 (66) [values in parentheses are percentage intensities of peaks in relation to the most intense (100%) in mass spectrum]. Citreoviridin monoacetate has been briefly described by Sakabe et al. (1964).

Citreoviridin diacetate

The procedure of Steglich & Höfle (1969) for acetylation of tertiary hydroxyl groups was adopted. A citreoviridin sample obtained from Dr. Y. Ueno, University of Tokyo, Tokyo, Japan (4.5mg, 11.2 μmol) was treated with NN-dimethylpyridin-4-amine (1.2mg, 10 μmol), redistilled acetic anhydride (54mg, 0.96mmol) and triethylamine (22mg, 0.22mmol; stored over KOH pellets). After 2.5 days at room temperature (20°C) in the dark, in a stoppered flask, the solution was diluted with 1ml of chloroform, washed with 1ml of water and twice with 1ml of satd. NaCl. The chloroform layer was dried over anhydrous $MgSO_4$ and then applied to two preparative 2mm-thick silica-gel plates (20cm×20cm; Merck no. 5717, BDH Chemicals, Poole, Dorset, U.K.). The plates were eluted with chloroform/acetone (5:1, v/v). The yellow fluorescent band (irradiated at 366nm) corresponding to citreoviridin diacetate (R_F 0.68–0.89) was scraped from the plates and extracted with acetone. After filtration, the acetone solution was evaporated to dryness and the residue was dissolved in methanol. The yield of citreoviridin diacetate was 2.3mg, 4.7 μmol; 42% of initial citreoviridin, by using $\varepsilon = 44000$ litre·mol^{-1}· cm^{-1} at 383nm established for citreoviridin by Nagel et al. (1972a). Two other yellow fluorescent

bands at R_F 0.33–0.49 and R_F 0.57–0.68 were unchanged citreoviridin and impure citreoviridin monoacetate respectively. The citreoviridin diacetate sample gave a single peak on h.p.l.c. with elution by chloroform/acetone (15:1, v/v), and so was used without further purification. Its ^{1}H n.m.r. spectrum showed δ (100MHz, $C^2H_3O^2H$) 1.23 (d, $J = 7Hz$, CH_3 at C-2), 1.30 and 1.44 (s, CH_3 at C-3 and C-5), 1.90 (s, CH_3CO at C-3), 1.95 (s, CH_3 at C-7), 2.01 (s, CH_3 at C-15), 2.19 (s, CH_3CO at C-4), 3.91 p.p.m. (s, CH_3O at C-16); u.v.-absorption maxima in MeOH 399 (shoulder), 383, 293, 285 (shoulder), 230 (shoulder), 204nm; m/e 486 (8%, M^+, $C_{27}H_{34}O_8$), 366 (11), 273 (15), 259 (30), 258 (29), 169 (91), 139 (61, pyrone moiety, $C_7H_7O_3$), 127 (100%).

Hydrogenation of citreoviridin monoacetate

Citreoviridin monoacetate (2.5mg) in 2ml of ethyl acetate was hydrogenated over 10% (w/w) Pd/charcoal at 20°C for 20min at atmospheric pressure. The product was purified by t.l.c. on silica gel with ethyl acetate/hexane (7:3, v/v), giving two compounds. The non-crystalline major component (1.8mg) was identified as octahydrocitreoviridin monoacetate (structure IIa): u.v.-absorption maxima in MeOH 288nm; m/e (M^+ not detected), 322 (33%), 309 (100%), 252 (30), 251 (41), 187 (33), 169 (56), 167 (46), 154 (57), 139 (18), 127 (70). The minor component, which became the predominant product on prolonged hydrogenation, was identified as decahydrocitreoviridin monoacetate (structure IIb): u.v.-absorption max. in MeOH 233nm; m/e (M^+ not detected), 384 (6%), 324 (47), 311 (83), 293 (32), 254 (42), 253 (43), 187 (81), 169 (86), 141 (64), 127 (100%).

Solutions of citreoviridin and its derivatives

Ethanolic solutions were stored in the dark at −20°C. The concentrations of citreoviridin and its acetates were estimated by using the published value of $\varepsilon_{383} = 44000$ litre·mol^{-1}·cm^{-1}.

Mitochondria

Rat liver mitochondria were prepared from young adult Wistar rats, starved overnight (Johnson & Lardy, 1967). They were suspended in 0.25M-sucrose and were stored at 0°C. They remained coupled up to about 5h from preparation.

Ox heart mitochondria were prepared from minced ox heart treated with Nagarse (proteolytic enzyme from Digby Chemical Service, London SW1W 9QU, U.K.) (Beinert, 1964). The mitochondria were isolated by differential centrifugation and then washed and resuspended in 0.25M-sucrose/

10mM-Tris/H_2SO_4, pH7.7. Ox heart mitochondria stored at 0°C could be used for coupled respiration studies for up to 1 week.

Submitochondrial particles

The method used was derived from that described by Hansen & Smith (1964). Suspensions of ox heart mitochondria stored at −20°C were thawed and made 4mM in $MgSO_4$, 1mM in sodium succinate and 1mM in ATP. After two cycles of freezing and thawing, the suspension was centrifuged at 10000g for 10min. The pellet was resuspended at 10mg of protein/ml in 0.25M-sucrose/1mM-ATP/1mM-sodium succinate/ 15mM-$MgSO_4$/20mM-Tris, adjusted to pH7.7 with

sucrose/10mM-KCl/25mM-sodium succinate/20mM-Tris/H_2SO_4/2mM-$MgCl_2$/5mM-KH_2PO_4, final pH7.4, for rat liver mitochondria or (b) 0.15M-KCl/5mM-sodium L-glutamate/5mM-sodium L-malate/3mM-EGTA/10mM-Na_2HPO_4/NaH_2PO_4 buffer, pH6.8, for ox heart mitochondria. A suitable volume of mitochondrial suspension (about 1–2mg of protein) was added, followed by a portion of a solution of the inhibitor in ethanol, or ethanol for the control. This gave a State-4 respiration rate (Chance & Williams, 1955). After at least 1min, sufficient ADP (25–40μl of 0.1M-ADP) was added to give a stimulated respiration rate (State 3) which remained constant until the mixture became anaerobic. Percentage inhibition of ADP-stimulated respiration was defined as:

$$100 - \left[\frac{(\text{State-3 rate with inhibitor}) - (\text{State-4 rate with inhibitor})}{(\text{control State-3 rate}) - (\text{control State-4 rate})} \times 100 \right]$$

H_2SO_4. This mitochondrial suspension cooled in ice was pumped at 20ml/min once through the flow cell attached to the probe of a Rapidis F400 flow-through ultrasonic disintegrator (Ultrasonics Ltd., Shipley, Yorks. BD18 2BN, U.K.) set at an amplitude of 22 μm and power of 110W. The flow cell (internal vol. 1ml) was cooled by water circulation at 0°C. The suspension was exposed to ultrasonic power for an average of 3s. After centrifuging at 10000g for 10min, the supernatant was centrifuged at 100000g for 30min. The pellet was washed by centrifugation with 0.25M-sucrose/10mM-Tris/H_2SO_4, pH7.7, and finally suspended in that buffer at 30–40mg of protein/ml. Portions (1ml) were stored at −20°C and used immediately after thawing.

Soluble ATPase

Soluble ATPase as prepared from ox heart submitochondrial particles by the chloroform method (Beechey et al., 1975) was dissolved in 0.25M-sucrose/ 10mM-Tris/H_2SO_4 (pH7.7)/1mM-EDTA. This buffer was changed to 25mM-Tris/H_2SO_4 (pH7.7)/1mM-EDTA by continuous filtration through an XM-100A membrane in an Amicon pressure ultrafiltration cell (Amicon Ltd., High Wycombe, Bucks., U.K.). Samples (0.15ml) of the resulting solution at 1.7mg of protein/ml were freeze-dried and stored over silica gel at −15°C. Addition of water at 20°C gave solutions of ATPase without loss of activity. These were stored at 20°C and used within 8h.

Assay procedures for energy-linked reactions

ADP-stimulated respiration. Respiration rates were measured at 30°C with a Clark-type oxygen electrode (Chappell, 1964). The oxygen-electrode compartment contained 1.4ml of aerated solutions of (a) 0.25M-

ATP-driven reduction of NAD^+ by succinate. The reaction mixture contained 0.25M-sucrose, 50mM-Tris/H_2SO_4, 10mM-$MgSO_4$, 5mM-sodium succinate, 1μM-antimycin A (Kyowa Fermentation Industry Co., Tokyo, Japan), 0.2mM-NAD^+, 10mM-sodium phosphoenolpyruvate, 50μg of pyruvate kinase [type III; Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.]/ml and submitochondrial particles (0.9mg of protein), pH7.7, in a 1cm-path-length polystyrene cuvette [W. Sarstedt (U.K.) Ltd., Leicester, U.K.], thermostatically maintained at 32°C in a final volume of 1.0ml. The inhibitor or an equivalent volume of ethanol (control) were mixed into the cuvette. After 1min, ATP to a final concentration of 2mM was mixed into the cuvette and the change in A_{340} was monitored in a Beckman Acta M VI spectrophotometer (Beckman-RIIC Ltd., Glenrothes, Scotland). An ATP-regenerating system has been included in this assay to straighten the curved traces usually caused by production of ADP and rapid removal of ATP.

ATP-driven NAD transhydrogenase. The reaction mixture contained 0.25M-sucrose, 50mM-Tris/H_2SO_4, 5mM-$MgSO_4$, 66μM-NAD^+, 0.66μM-rotenone, 0.4M-ethanol, 10mM-sodium phosphoenolpyruvate, 50μg of pyruvate kinase (type II)/ml, 50μg of alcohol dehydrogenase (from yeast, Sigma)/ml and submitochondrial particles (0.66mg of protein), pH8.0, in a final volume of 1.0ml at 32°C. The inhibitor or ethanol (control) was mixed into the cuvette and then $NADP^+$ (40μM final concn.) was added to initiate the non-energy-linked transhydrogenase activity measured at 340nm as above. After about 2min, ATP was added to 2mM final concentration and the new reaction rate was measured. The non-energy-linked rate was subtracted from this new rate to give the ATP-driven transhydrogenase activity.

ATPase. The ATPase activity was assayed by a coupled enzyme system involving ATP regeneration (Pullman *et al.*, 1960). The reaction mixture contained 0.25 M-sucrose, 25 mM-Tris/H_2SO_4, 5 mM-$MgSO_4$, 0.35 mM-NADH, 0.6 μM-rotenone, 2.5 mM-sodium phosphoenolpyruvate, 50 μg of pyruvate kinase (type II)/ml, 50 μg of lactate dehydrogenase (type II, Sigma)/ml and the ATPase preparation under test [either submitochondrial particles (0.3 mg of protein) or soluble ATPase (8.3 μg of protein)], pH 8.2, in a final volume of 1.0 ml was preincubated at 32°C with the inhibitor or ethanol (control). ATP was added to a final concentration of 2 mM to start the reaction. Oxidation of NADH was followed as above.

Protein determination

The protein concentrations of particulate preparations were estimated by the biuret method in the presence of 0.27% (w/v) sodium deoxycholate (Dawson *et al.*, 1969). Soluble ATPase concentrations were estimated by a modified Lowry technique (Beechey *et al.*, 1975).

H.p.l.c.

A stainless-steel column (25 cm × 0.95 cm) packed with 10 μm-diameter silica gel (Partisil 10; Whatman, Maidstone, Kent, U.K.) was used as described by Webber & McKerrell (1976). A pressure of about 3.8 MPa (400 lb/in²) gave a flow rate of 4–5 ml/min. Chloroform solutions (up to 500 μl) were injected on to the column. The eluent was monitored at 380 nm in an 8 μl spectrophotometric flow cell (model CE212; Cecil Instruments Ltd., Cambridge, U.K.).

Physical methods

N.m.r. spectra were obtained at 100 MHz on a Varian HA-100 spectrometer (Varian Associates Ltd., Walton-on-Thames, Surrey, U.K.). The spectra of citreoviridin and its monoacetate have been confirmed at 270 MHz on a Bruker WH-270 spectrometer (Bruker Physik A.G., Karlsruhe, W. Germany).

Mass spectra were obtained by electron impact with a direct insertion probe at 70 eV and 4 kV accelerating voltage on an A.E.I. MS-30 spectrometer (Kratos-A.E.I. Scientific Instruments, Manchester, U.K.).

Results

Inhibitory effects of citreoviridin and its acetates on reactions associated with the mitochondrial ATPase

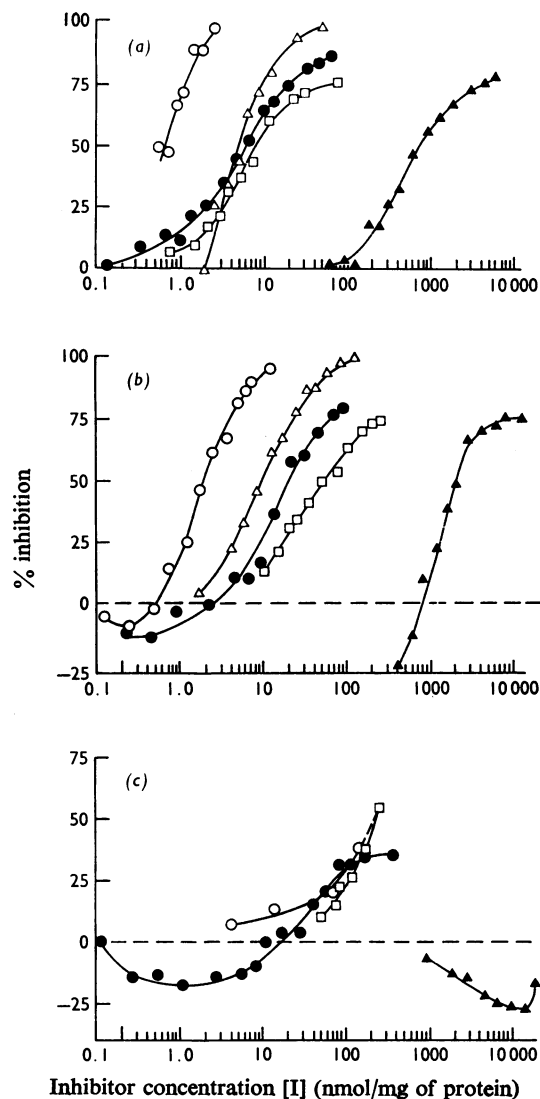Pure samples of citreoviridin (structure Ia), citreoviridin monoacetate (structure Ib) and citreo-

Fig. 1. *Inhibition curves for citreoviridin and its acetates against energy-linked enzymes activities from ox heart mitochondria*

Enzyme activities were assayed as described in the Experimental section. The effects of citreoviridin, citreoviridin monoacetate and citreoviridin diacetate are shown in (*a*), (*b*) and (*c*) respectively against: ADP-stimulated respiration in mitochondria (○); ATP-driven reduction of NAD^+ by succinate in submitochondrial particles (△); ATP-driven NAD transhydrogenase in submitochondrial particles (□); ATPase in submitochondrial particles (●); and soluble ATPase (▲). Negative inhibition points represent stimulation of enzyme activity. Note the logarithmic scale for the horizontal axes. Results from single experiments have been used to construct these curves. Similar $i_{\frac{1}{2}max.}$ and K_D values have been obtained in other experiments.

Table 1. *Inhibitory activities of citreoviridin and its acetates against energy-linked enzyme activities from ox heart mitochondria*

The values of $I_{\frac{1}{2}max.}$ and $i_{\frac{1}{2}max.}$ were estimated directly from the curves of Fig. 1. $I_{\frac{1}{2}max.}$ is the concentration of inhibitor causing half-maximal inhibition of the enzyme activity expressed relative to the total amount of protein in the assay. The actual concentration of inhibitor in the assay at half-maximal inhibition is represented by $i_{\frac{1}{2}max.}$. The dissociation constant K_D (μM) for the inhibitor–enzyme complex was calculated from the expression $K_D = E(I_{\frac{1}{2}max.} - 0.5C)$ (cf. Easson & Stedman, 1936; Straus & Goldstein, 1943; Linnett *et al.*, 1977), where E is the concentration of total protein in the assay (mg of protein/ml), $I_{\frac{1}{2}max.}$ is defined as above (nmol of inhibitor/mg of total protein) and C is the concentration of ATPase molecules in the preparation (assuming C = 0.2, 0.3 or 2.8 nmol of ATPase/mg of total protein for mitochondria, submitochondrial particles or soluble ATPase respectively).

Enzymic activity	E (mg of total protein/ml)	Citreoviridin				Citreoviridin monoacetate				Citreoviridin diacetate			
		$I_{\frac{1}{2}max.}$ (nmol/mg of protein)	$i_{\frac{1}{2}max.}$ (μM)	Maximum inhibition (%)	K_D (μM)	$I_{\frac{1}{2}max.}$ (nmol/mg of protein)	$i_{\frac{1}{2}max.}$ (μM)	Maximum inhibition (%)	K_D (μM)	$I_{\frac{1}{2}max.}$ (nmol/mg of protein)	$i_{\frac{1}{2}max.}$ (μM)	Maximum inhibition (%)	K_D (μM)
ADP-stimulated respiration in mitochondria	0.90	0.67	0.61	100	0.51	1.9	1.8	100	1.7	215*	190*	(100)	190
ATP-driven reduction of NAD+ by succinate in submitochondrial particles	0.40	5.1	2.1	100	2.0	9.9	4.0	100	3.9				
ATP-driven NAD(P) transhydrogenase in submitochondrial particles	0.66	5.9	3.9	80	3.8	38	25	85	25	200	120	(85)	130
ATPase in submitochondrial particles	0.30	4.8	1.4	87	1.4	16	4.6	85	4.5	200*	59*	(85)	59
Soluble ATPase	0.0083	500	4.1	80	4.1	1700	14	80	14				

*Estimated by extrapolation.

viridin diacetate (structure Ic) were tested for their inhibitory activity against (a) ADP-stimulated respiration in intact mitochondria (this inhibition was relieved by 2,4-dinitrophenol), (b) ATP-driven reduction of NAD^+ by succinate in submitochondrial particles, (c) ATP-driven NAD transhydrogenase in submitochondrial particles, (d) ATPase in submitochondrial particles and (e) soluble ATPase, all derived from ox heart mitochondria. Typical titration curves for each compound are shown in Fig. 1. The inhibitor concentrations required to give half-maximal inhibition in terms of nmol of inhibitor/mg of total protein ($I_{\frac{1}{2}max.}$) are collected in Table 1. Included in Table 1 are values of the dissociation constant for the inhibitor–enzyme complex calculated by the simple treatment of Easson & Stedman (1936) and Straus & Goldstein (1943) as described in Linnett et al. (1977). The assumptions made for this calculation include (a) a single inhibitory binding site for the inhibitor on the enzyme, (b) reversible binding, (c) the binding equilibrium to be reached before the assay of enzyme activity and (d) no effect of binding of substrates or products on the binding of inhibitor (i.e. non-competitive inhibition).

Table 1 shows that, for the inhibition of the ATP synthetase and the ATPase, the $I_{\frac{1}{2}max.}$ values for citreoviridin and its monoacetate range over almost three orders of magnitude. In contrast, the calculated dissociation constants for citreoviridin, citreoviridin monoacetate or citreoviridin diacetate against the range of enzyme activities are reasonably similar. The differences in the $I_{\frac{1}{2}max.}$ values for each inhibitor appear mainly to be due to differences in the protein concentrations used in the various assays.

The values of the calculated dissociation constants, K_D (Table 1), indicate that the affinity of the ATPase for citreoviridin is greater than that for citreoviridin monoacetate and much greater than that for citreoviridin diacetate. The concentrations of citreoviridin diacetate required to give detectable inhibition

were so great as to cause technical problems owing to its lack of solubility in aqueous systems and its absorbance at 340 nm.

Table 2 shows that citreoviridin and its monoacetate also inhibited ADP-stimulated respiration in rat liver mitochondria. Citreoviridin was a more potent inhibitor than the monoacetate ($K_D = 0.15$ and $0.68 \mu M$ respectively). The inhibitions were relieved by 2,4-dinitrophenol. The affinity of citreoviridin was higher for the rat liver mitochondrial ATPase molecule than for the ox heart enzyme.

Fig. 1 shows that complete inhibition of ADP-stimulated respiration and of ATP-driven reversed electron transport could be achieved with citreoviridin and citreoviridin monoacetate. Inhibition of the membrane-bound and soluble ATPases and of the ATP-driven transhydrogenase, however, reached only 80–87% even at high inhibitor concentrations.

Stimulation of the ATPase activity

Reproducible stimulation of the soluble ATPase activity up to 26% higher than controls was found with citreoviridin diacetate (see Fig. 1c). Less pronounced, but probably significant, stimulation was noted with citreoviridin mono- and di-acetate on the ATPase of submitochondrial particles and with citreoviridin monoacetate on the soluble ATPase. No such stimulation of ATPase activity was found in controls to which only ethanol had been added. This stimulation occurs at lower concentrations of the citreoviridin acetates than those required to inhibit the ATPase.

### Effect of hydrogenation on the inhibitory potency of citreoviridin monoacetate

Hydrogenation of citreoviridin monoacetate yielded the octahydro and decahydro derivatives (structures IIa and IIb respectively) depending on the time of reaction, in an analogous fashion to the hydrogenation of aurovertin B (Mulheirn et al., 1974). Table 2 shows that the octahydro derivative was a much less effective inhibitor than citreoviridin monoacetate when assayed against ADP-stimulated respiration in rat liver mitochondria. Hydrogenation to decahydrocitreoviridin monoacetate further decreased the inhibitory potency. For each derivative, 2,4-dinitrophenol was able to relieve the inhibition of ADP-stimulated respiration.

Fluorescence properties of citreoviridin

In ethanol or aqueous ethanolic solutions, citreoviridin fluoresced weakly with an excitation maximum at 380 nm and a broad emission maximum at 530 nm. There was no significant enhancement of fluorescence

Table 2. *Inhibitory activities of citreoviridin and of hydrogenated citreoviridin monoacetate derivatives against ADP-stimulated respiration in rat liver mitochondria*

See Table 1 for definitions. The calculation for K_D assumes that $C = 0.1$ nmol of ATPase/mg of mitochondrial protein.

	$I_{\frac{1}{2}max.}$ (nmol/mg of protein)	$i_{\frac{1}{2}max.}$ (μM)	K_D (μM)
Citreoviridin	0.18	0.21	0.15
Citreoviridin monoacetate	0.40	0.78	0.68
Octahydrocitreoviridin monoacetate	12	15	15
Decahydrocitreoviridin monoacetate	73	93	93
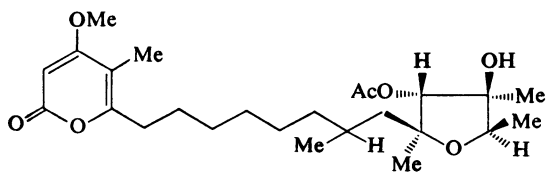
Structure (IIa)
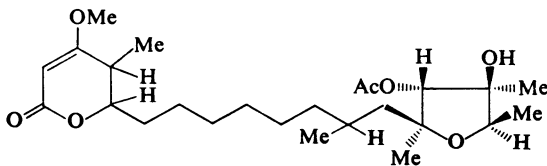
Structure (IIb)

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when citreoviridin was added to ox heart submitochondrial particles or soluble ATPase in buffer.

Discussion

Citreoviridin has been shown to inhibit ADP-stimulated respiration in mitochondria. The inhibition is relieved by uncouplers. Citreoviridin also inhibits both the membrane-bound and soluble ATPases and two partial reactions of mitochondrial energy conservation, the ATP-driven reduction of NAD^+ by succinate and the ATP-driven NAD transhydrogenase (Fig. 1). The inhibition of the soluble ATPase by citreoviridin localizes its site of action and distinguishes it from the class of membrane-directed ATPase inhibitors exemplified by oligomycin (Linnett & Beechey, 1978). The mode of action of citreoviridin appears to be similar to that of aurovertins B and D (Kagawa & Racker, 1966; Roberton et al., 1967; Lambeth & Lardy, 1971; Chang & Penefsky, 1973; Catterall & Pedersen, 1974; Beechey et al., 1975; Ebel & Lardy, 1975), as might be expected from the similarities in their structures (Mulheirn et al., 1974). Citreoviridin and the aurovertins all contain pyrone–polyene moieties. The remaining features of each molecule are closely related both stereochemically and biosynthetically (Nagel et al., 1972b; L. J. Mulheirn, A. D. Mitchell & P. E. Linnett, unpublished work).

It has been observed frequently that the mitochondrial ATP synthetase is apparently much more sensitive to the aurovertins than are those activities that rely on the hydrolysis of ATP (Lardy et al., 1964; Lenaz, 1965; Lee & Ernster, 1968; Roberton et al., 1968; Bertina et al., 1973; Conover & Schneider, 1976). The present data show that the inhibition by citreoviridin of the forward and reverse reactions of the ATPase molecule also appears anomalous. The simple treatment described by Linnett et al.

(1977) resolves most of the apparent anomaly. The confusion has been mainly due to the method of expressing inhibition data (e.g. $I_{\pm max.}$ in terms of nmol of inhibitor/mg of protein) and to large differences in the enzyme concentration in the assays being compared. Values of the absolute, inhibitory citreoviridin concentration ($i_{\pm max.}$) are comparable with the derived dissociation constants (K_D) (see Table 1), although this would not necessarily be the case for inhibitors with higher affinity for the ATPase. Small differences in the calculated K_D values for citreoviridin still remain, as would be expected from the simplicity of the model used. The equation used for calculating K_D (see legend of Table 1) can be modified to account for the effects of n inhibitor molecules binding at identical, non-interacting sites on each enzyme molecule (Straus & Goldstein, 1943). The dissociation constants calculated in Table 1 for $n = 1$ are not changed significantly for $n \leqslant 3$.

Monoacetylation of citreoviridin decreases the inhibitory potency somewhat, but further acetylation to citreoviridin diacetate greatly decreases the affinity for the inhibitory site on the ATPase molecule. Beechey et al. (1974) previously reported that 'citreoviridin diacetate' was a reasonably potent inhibitor of ADP-stimulated respiration in rat liver mitochondria and of the ATPase of ox heart submitochondrial particles. We have found out subsequently that the sample used for this work was in fact a mixture of citreoviridin monoacetate (structure Ib) and an as yet unidentified citreoviridin derivative obtained in the acetylation procedure (P. E. Linnett, unpublished work).

It is clear that the hydrogenation of citreoviridin monoacetate decreases its inhibitory potency dramatically. This suggests that the conjugated polyene system in citreoviridin (C-6 to C-13) is an important component in the binding of the inhibitor to the ATPase.

The reason for the stimulation of ATPase activity by citreoviridin diacetate and to a lesser extent by the monoacetate is not well understood. The present results do not enable us to determine whether the stimulatory and inhibitory citreoviridin acetate-binding sites are identical.

The lack of fluorescence enhancement when citreoviridin is bound to the ATPase is in direct contrast with the pronounced enhancement seen when aurovertin D associates with the ATPase (Lardy & Lin, 1969; Bertina et al., 1973; Chang & Penefsky, 1973, 1974; Layton et al., 1973; Van de Stadt & Van Dam, 1974; Van de Stadt et al., 1974). The precise mechanism by which the fluorescence of aurovertin becomes less quenched when tightly bound to the ATPase is not known. Presumably the conformation in which citreoviridin is bound to the ATPase is such that an increased quantum yield is not obtained.

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