Mode of Inhibition of Mitochondrial Energy Transduction by Chlorophenoxyisobutyrate

By S. R. PANINI and C. K. RAMAKRISHNA KURUP

Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

(Received 25 January 1974)

1. α -*p*-Chlorophenoxyisobutyric acid, the ethyl ester of which is widely used as an antihypercholesterolaemic drug, is an inhibitor of energy-transfer reactions in isolated rat liver mitochondria. 2. The compound at lower concentrations ($<4.0\text{ }\mu\text{mol/mg}$ of mitochondrial protein) inhibits state 3 oxidation, stimulates state 4 oxidation, abolishes respiratory control and stimulates the latent adenosine triphosphatase activity of mitochondria. The inhibition imposed on state 3 oxidation is relieved by dinitrophenol. 3. At higher concentrations it inhibits coupled phosphorylation as well as dinitrophenol-stimulated adenosine triphosphatase activity. The inhibition of state 3 oxidation under these conditions is not reversed by uncouplers. 4. The three coupling sites of phosphorylation exhibit differential susceptibility to inactivation by this compound. Coupled phosphorylation at the first site is abolished at a drug concentration of $3.0\text{ }\mu\text{mol/mg}$ of protein. The third site is inactivated when the concentration of the drug reaches $5.0\text{ }\mu\text{mol/mg}$ of protein. The second site is the most refractory and drug concentrations of the order of $10.0\text{ }\mu\text{mol/mg}$ of protein are required effectively to inhibit phosphorylation at this site. 5. The compound also inhibits ATP-dependent reversal of electron transport as well as the adenosine triphosphatase activity in submitochondrial particles. 6. The oxidation of NADH and succinate in these particles is not inhibited. 7. These properties indicate that the compound acts as an 'inhibitory uncoupler' of energy-transfer reactions in isolated mitochondria.

The ethyl ester of α -*p*-chlorophenoxyisobutyric acid is now being extensively used as an antihypercholesterolaemic drug under the trade name 'Atromid-S' or 'clofibrate'. Apart from its hypolipidaemic action, administration of the drug has been shown to produce many changes particularly in the liver (Steinberg, 1969). Kurup *et al.* (1970) reported that on administration of clofibrate to the rat, the hepatic content of mitochondria increased by 50–100%. The specific activities of certain mitochondrial enzymes have also been shown to change significantly on administration of the drug (Krishna-kantha & Kurup, 1972). The compound has been shown to be present in the livers of treated animals (Thorpe, 1962; Daae & Aas, 1973). In the light of these observations, which indicated that the drug might influence mitochondrial function *in vivo*, it was of interest to understand its effect on oxidative phosphorylation *in vitro*.

The results presented in this paper indicate that chlorophenoxyisobutyrate (sodium salt) interferes with the energy-transfer reactions in isolated rat liver mitochondria and that in its mode of action it belongs to a rare class of compounds which have been termed 'inhibitory uncouplers' by Racker (1965*b*). Depending on the concentration of the compound the three sites of phosphorylation show

differential susceptibility to inhibition by the compound. A preliminary account of some portions of this work has been published earlier (Panini, 1972).

Experimental

Preparation of rat liver mitochondria

Adult albino rats weighing 200–300 g from the stock colony of this Institute were used. Liver mitochondria were prepared in 0.25M-sucrose as described earlier (Kurup *et al.*, 1970). The mitochondrial pellet was washed once with sucrose and used with minimum delay. Submitochondrial sonic particles were prepared as described by Graven *et al.* (1967).

Assay of oxidative phosphorylation

Respiratory control and ADP/O ratios were determined polarographically with the Gilson KM oxygraph. The reaction medium and conditions of assay were the same as described previously (Kurup *et al.*, 1970). Since chlorophenoxyisobutyrate at concentrations above $3.0\text{ }\mu\text{mol/mg}$ of mitochondrial protein abolished respiratory control, the ADP/O ratios could not be determined polarographically at these concentrations of the drug.

Therefore oxidative phosphorylation was also determined manometrically in an Aminco Warburg apparatus. The main compartment of the Warburg flask contained 30 μ mol of potassium phosphate buffer, pH 7.4, 150 μ mol of Tris-HCl buffer, pH 7.4, 6 μ mol of MgSO_4 , 3 μ mol of EDTA, 50 μ mol of glucose, 5 μ mol of ATP, 3 mg of hexokinase (Sigma, type III) and 4–6 mg of mitochondrial protein. The reaction volume was adjusted to 3 ml with 0.25 M-sucrose. The centre well contained 0.2 ml of 20% (w/v) KOH. After equilibration for 15 min at 30°C, the reaction was started by tipping in from the side arm 50 μ mol of glutamate+50 μ mol of malate, 100 μ mol of succinate or 100 μ mol of ascorbate+750 nmol of *NNN'*-tetramethyl-*p*-phenylenediamine. After measurement of O_2 uptake at 5 min intervals for 20 min, the reaction was stopped by the addition of 2 ml of 10% (w/v) trichloroacetic acid. The P_i remaining was measured by the method of Fiske & SubbaRow (1925). The addition of chlorophenoxyisobutyrate was always made from the side arm along with the substrate. When succinate or ascorbate was used as the electron donor, 5 μ g of rotenone was added to the reaction system.

Phosphorylation coupled specifically to the first and second sites was measured by using ferricyanide as the electron acceptor (Lee *et al.*, 1967). The reaction system contained 10 μ mol of potassium phosphate buffer, pH 7.4, 50 μ mol of Tris-HCl buffer, pH 7.4, 1.5 μ mol of MgSO_4 , 3 μ mol of KCN, 1 μ mol of EDTA, 50 μ mol of glucose, 2 μ mol of ATP, 1.5 mg of hexokinase (Sigma, type III), 10 μ mol of succinate, or 5 μ mol of glutamate+5 μ mol of malate, and 2 mg of mitochondrial protein. When succinate was the electron donor, the reaction system also contained 10 μ g of rotenone. The total reaction volume was 1.0 ml. After preincubation at 30°C for 5 min, the reaction was started by the addition of 10 μ mol of $\text{K}_3\text{Fe}(\text{CN})_6$ and allowed to proceed for 10 min, when the reaction was stopped by the addition of 1 ml of 10% (w/v) HClO_4 . After centrifugation, the ferricyanide remaining was determined by measuring E_{420} in a Beckman DU-2 spectrophotometer. Addition of chlorophenoxyisobutyrate was made before preincubation.

Reversal of electron transport

Energy-linked succinate-mediated reduction of NAD^+ was determined essentially as described by Ernster & Lee (1967). The reaction system contained 150 μ mol of Tris-HCl buffer, pH 7.4, 18 μ mol of MgSO_4 , 9 μ mol of KCN, 3 μ mol of NAD^+ , 3 μ mol of EDTA, 15 μ mol of succinate, 540 μ mol of sucrose and 1–3 mg of submitochondrial-particle protein in a total reaction volume of 3 ml. The reaction was started by the addition of 3 μ mol of ATP and the rate of reduction of NAD^+ was followed by measuring the

increase in E_{340} in a Cary 14 recording spectrophotometer. That the increase in E_{340} was due to NADH formation was confirmed by its reversal on addition of pyruvate and lactate dehydrogenase at the end of the reaction.

Enzyme assays

The activity of ATPase* (ATP phosphohydrolase, EC 3.6.1.3) was determined as described by Veldsema-Currie & Slater (1968). The reaction system contained 50 μ mol of Tris-HCl buffer, pH 7.4, 75 μ mol of KCl, 5 μ mol of MgCl_2 , 6 μ mol of ATP, 1 μ mol of EDTA and 0.5–1.0 mg of mitochondrial protein or 0.2 mg of particle protein in a final volume of 1.0 ml. Whenever indicated, 200 nmol of 2,4-dinitrophenol was added. The reaction was started by the addition of ATP. After 10 min (5 min in the presence of dinitrophenol) at 30°C, the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. The P_i liberated was measured colorimetrically (Fiske & SubbaRow, 1925).

Succinate dehydrogenase [succinate-(acceptor) oxidoreductase, EC 1.3.99.1] activity was assayed by the phenazine methosulphate method (Bernath & Singer, 1962). Protein was measured by the biuret method (Gornall *et al.*, 1949), by using deoxycholate for solubilization. All aqueous solutions were prepared in water, double-distilled in an all-glass apparatus. All solutions were adjusted to pH 7.4 before use. Cyanide and chlorophenoxyisobutyrate solutions were prepared fresh before use.

Chemicals

The sample of tetramethyl-*p*-phenylenediamine obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., was purified before use (Kurup & Sanadi, 1970). Whenever chemicals dissolved in organic solvents were added, the volume was kept to the minimum and an equal amount of the solvent was also added to the control. Rotenone, ATP and ADP were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and phenazine methosulphate was from Calbiochem Co., Los Angeles, Calif., U.S.A. The chlorophenoxyisobutyrate used in these experiments was a generous gift from Dr. J. M. Thorp, Pharmaceuticals Division, Imperial Chemical Industries Ltd., Macclesfield, Cheshire, U.K. The sample of 2,4-dinitrophenol was obtained from E. Merck, Darmstadt, Germany. The uncoupling agents carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazine and 5-chloro-3-*t*-butyl-2'-chloro-4'-nitrosalicylanilide were gift samples from Dr. W. W. Pritchard, E. I. Du Pont Nemours Co., Wilmington, Delaware, U.S.A., and Dr. P. C. Hamm, Monsanto Co., St. Louis, Mo., U.S.A., respectively.

* Abbreviation: ATPase, adenosine triphosphatase.

Results

Respiratory control and ADP/O ratios

Addition of chlorophenoxyisobutyrate to tightly coupled mitochondria affected active oxidation, respiratory control and ADP/O ratio. Some typical polarographic tracings are given in Fig. 1. At concentrations of the compound less than $1.0 \mu\text{mol}/\text{mg}$ of mitochondrial protein, active oxidation was inhibited and state 4 (ADP exhausted; Chance & Williams, 1955) oxidation was stimulated (Fig. 1*b*). When the concentration of the drug was raised to $2.0 \mu\text{mol}/\text{mg}$ of protein, respiratory control was almost completely abolished. The inhibition imposed on state 3 (ADP not limiting; Chance & Williams, 1955) oxidation at these concentrations of the drug was relieved by dinitrophenol (Figs. 1*b* and 1*c*). At

concentrations above $4.0 \mu\text{mol}$ of the drug/mg of protein, dinitrophenol was ineffective in reversing the inhibition of state 3 oxidation (Fig. 1*d*).

The same pattern was observed with both glutamate+malate and succinate (Table 1). Respiratory control was more susceptible to chlorophenoxyisobutyrate when the substrate oxidized was succinate than when glutamate+malate served as substrate. With glutamate+malate, the ADP/O ratio decreased from 3 to 2 at a drug concentration of $2.5 \mu\text{mol}/\text{mg}$ of protein. At this concentration of the drug, the ADP/O ratio with succinate was not affected.

P/O ratios

These results indicated that chlorophenoxyisobutyrate might preferentially uncouple the first site

Fig. 1. Effect of chlorophenoxyisobutyrate on respiratory control in rat liver mitochondria

The reaction system consisted of the mannitol medium (Kurup *et al.*, 1970) and 2.4 mg of mitochondrial protein. The polarographic tracings are given. At 1 min after the addition of mitochondria (Mt), chlorophenoxyisobutyrate was stirred in. Then 2 min later $40 \mu\text{mol}$ of succinate was added. State 3 oxidation (Chance & Williams, 1955) was initiated by the addition of 400 nmol of ADP. After the added ADP had been exhausted (state 4), active oxidation was repeated by fresh addition of ADP. In the end 100 nmol of dinitrophenol was added as indicated. The reaction was carried out at 25°C . The values in parentheses indicate rates of oxygen uptake in ng-atoms/min per mg of mitochondrial protein. (a), Control; (b), (c) and (d), effect of addition of 2, 5 and $10 \mu\text{mol}$ of chlorophenoxyisobutyrate respectively as indicated.

Table 1. *Effect of chlorophenoxyisobutyrate on respiratory control and ADP/O ratio in rat liver mitochondria*

The reaction system and conditions of experiment were the same as in Fig. 1. The reaction volume was 2 ml and contained 2 mg of mitochondrial protein. The respiratory control index was calculated as the ratio of the rates of oxidation in state 3 and state 4 following state 3 (Chance & Williams, 1955). The addition of dinitrophenol was made when the mitochondria were in state 4. The values given are the average of ten different experiments.

Concentration of chlorophenoxyisobutyrate ($\mu\text{mol/mg}$ of mitochondrial protein)	Glutamate+malate					Succinate				
	(ng-atoms of O/min per mg of protein)					(ng-atoms of O/min per mg of protein)				
	State 3	State 4	+Dinitrophenol	Respiratory control index	ADP/O	State 3	State 4	+Dinitrophenol	Respiratory control index	ADP/O
Nil	91	18	119	5.1	2.8	125	38	150	3.6	1.8
2.5	51	26	113	2.0	2.0	79	73	138	1.1	1.5
3.0	47	28	112	1.7	1.9	71	—	112	—	—
7.3	34	—	61	—	—	52	—	65	—	—

Table 2. *Effect of chlorophenoxyisobutyrate on sites of phosphorylation in rat liver mitochondria*

Manometric determination of P/O ratios and spectrophotometric (ferricyanide reduction) determination of $P/2e^-$ ratios are described in the Experimental section. The uninhibited rates of oxygen uptake (ng-atoms/min per mg of protein) were 77, 136 and 128 with glutamate+malate, succinate and ascorbate+tetramethyl-*p*-phenylenediamine respectively (average of five experiments). The uninhibited rates of ferricyanide reduction were 99 and 441 nmol/min per mg of protein with glutamate+malate and succinate respectively (average of four experiments).

Concentration of chlorophenoxyisobutyrate ($\mu\text{mol/mg}$ of protein)	P/O			$P/2e^-$	
	Glutamate+malate	Succinate	Ascorbate+tetramethyl- <i>p</i> -phenylenediamine	Glutamate+malate	Succinate
Nil	2.7	1.8	0.9	1.9	0.6
1.0	—	—	—	1.4	0.5
2.0	2.3	1.4	0.7	1.0	0.3
3.0	1.8	1.4	0.5	0.9	0.2
4.0	—	1.1	0.4	0.6	0.2
5.0	0.9	0.9	0.1	0.6	0.2
7.5	0.3	0.5	0	0	0
10.0	0	0.2	0	0	0

of phosphorylation. The effect of higher concentrations ($>3 \mu\text{mol/mg}$ of protein) of the compound on the ADP/O ratio could not be determined polarographically owing to abolition of respiratory control (Fig. 1). Therefore this was tested manometrically (Table 2). In agreement with the results obtained polarographically (Table 1), at a drug concentration of $3 \mu\text{mol/mg}$ of protein, the first site of phosphorylation was made non-functional. The phosphorylation at the third site was abolished when the drug concentration reached $5 \mu\text{mol/mg}$ of protein. The second site appeared to offer remarkable resistance to the action of the drug. Concentrations of the order of $10 \mu\text{mol}$ of the drug/mg of protein were required effectively to inhibit phosphorylation at this site.

$P/2e^-$ ratios

The effect of chlorophenoxyisobutyrate on the first and second sites of phosphorylation (Racker,

1965a) was further tested by inactivating the cytochrome oxidase region of the respiratory assembly with CN^- and using $\text{K}_3\text{Fe}(\text{CN})_6$ as the electron acceptor (Lee *et al.*, 1967). When glutamate+malate was the substrate, the $P/2e^-$ ratio decreased from 2 to 1 at a drug concentration of $2.0 \mu\text{mol/mg}$ of protein. Phosphorylation at the second site (substrate succinate) was, however, abolished only at drug concentrations exceeding $7.5 \mu\text{mol/mg}$ of protein.

Respiration and phosphorylation

The results in Tables 1 and 2 show that chlorophenoxyisobutyrate exerts inhibitory action on both oxidation and phosphorylation. The effect of increasing concentrations of the compound on the rates of respiration and phosphorylation with different substrates was studied by using tightly coupled mitochondria. The results presented in

Fig. 2. Effect of chlorophenoxyisobutyrate on the rates of oxidation and phosphorylation in rat liver mitochondria

The rates of oxidation (○) and phosphorylation (●) were measured manometrically as described in the Experimental section. (a) Glutamate+malate; (b) succinate; (c) ascorbate+tetramethyl-*p*-phenylenediamine. The values are the mean of four independent determinations.

Fig. 2 show that irrespective of the substrate used, phosphorylation was more sensitive to the drug than was oxidation. Although the oxidation of glutamate+malate or succinate was inhibited 60–70%, no significant inhibition of electron-transport activity could be observed with ascorbate as the electron donor. The concentrations of chlorophenoxyisobutyrate for 50% inhibition (I_{50}) of phosphorylation were 2.5, 3.5 and 2.5 μmol/mg of protein for glutamate+malate, succinate and ascorbate+tetramethyl-*p*-phenylenediamine respectively. The I_{50} values of oxidation were 3.0 and 7.6 μmol of the drug/mg of protein for glutamate+malate and succinate respectively. This would indicate that the inhibition of phosphorylation is independent of the number of coupling sites activated. The oxidation of glutamate+malate, however, is more sensitive to the drug than the oxidation of succinate at lower concentrations (<4.0 μmol/mg of protein) of the drug.

The above pattern was also observed in the inhibition of phosphorylation coupled to ferricyanide reduction. Phosphorylation was more sensitive to the

inhibition than the reduction of ferricyanide with both glutamate+malate and succinate as substrates (results not given).

Oxidation in the presence of uncoupling agents

The results in Fig. 2 raised the possibility that chlorophenoxyisobutyrate, like oligomycin, may act as an inhibitor of coupled phosphorylation. It may be recalled that the inhibition imposed on state 3 oxidation by low concentrations of the drug could be relieved by dinitrophenol (Fig. 1; Table 1). Further similarity may be drawn from the observation that the inhibition of phosphorylation at the third site by this compound (Fig. 2c) or oligomycin (Guillory & Slater, 1965; Huijing & Slater, 1961) is not attended by inhibition of oxidation. Whether the drug has a direct effect on the respiratory chain was tested by observing the effect on oxidation of succinate in the presence of various uncouplers (Fig. 3). Unlike oligomycin (Huijing & Slater, 1961), this compound exerted considerable

Fig. 3. Effect of chlorophenoxyisobutyrate on oxidation of succinate by rat liver mitochondria in the presence of uncouplers

Oxygen uptake was measured manometrically in the presence of $70\mu\text{M}$ -dinitrophenol (Δ), $10\mu\text{M}$ -dicoumarol ($\circ$), $1\mu\text{M}$ -carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazone (Δ) or $0.1\mu\text{M}$ -5-chloro-3-*t*-butyl-2'-chloro-4'-nitrosalicylanilide ($\bullet$). Other details are given in the Experimental section. Each point represents the mean of four separate experiments.

inhibition of oxidation even in the presence of uncouplers. The succinate dehydrogenase activity was not affected by chlorophenoxyisobutyrate in the concentration range indicated in Fig. 3. The I_{50} value of $12.0\mu\text{mol/mg}$ of protein, however, is substantially higher than that observed with tightly coupled mitochondria.

Inhibition of respiration even in the presence of uncouplers is characteristic of inhibitors of the respiratory chain. Whether chlorophenoxyisobutyrate comes in this class was further tested by observing the effect of the compound on respiratory activity in submitochondrial sonic particles. Concentrations of the drug up to $20.0\mu\text{mol/mg}$ of particle protein had no inhibitory effect on the oxidation of either NADH ($312\text{nmol/min per mg}$ of protein) or succinate ($393\text{ng-atoms of oxygen/min per mg}$ of protein). This would rule out any inhibitory effect of the drug on the electron-transport chain itself.

ATPase activity

Addition of the compound stimulated the latent ATPase activity of tightly coupled mitochondria in both the presence and the absence of added Mg^{2+} ions (Fig. 4). This stimulation was totally inhibited

Fig. 4. Effect of chlorophenoxyisobutyrate on the latent ATPase activity of rat liver mitochondria

The enzyme was assayed with ($\bullet$) or without ($\circ$) the addition of MgCl_2 or in the presence of $1\mu\text{g}$ of oligomycin/ml (Δ). Other details are given in the Experimental section. The values are the means of four determinations. The concentrations of chlorophenoxyisobutyrate used in this experiment did not interfere with phosphate determination.

by oligomycin. In these effects chlorophenoxyisobutyrate resembles classical uncouplers such as dinitrophenol. The ATPase activity reached a maximum at a concentration of $10\text{--}12\mu\text{mol}$ of the drug/mg of protein. It is noteworthy that concentrations of this order completely abolished coupled phosphorylation (Table 2). Further increase in the concentration of the compound inhibited the ATPase activity. The fact that the compound stimulates the latent ATPase activity even in the absence of added Mg^{2+} ions indicates that the stimulation was not of the 'fatty acid type' (Wojtczak *et al.*, 1969).

In contrast with the above pattern, the dinitrophenol-stimulated ATPase activity was inhibited by the drug (Fig. 5a). The concentration of chlorophenoxyisobutyrate required to inhibit this activity

Fig. 5. Effect of chlorophenoxyisobutyrate on uncoupler-stimulated mitochondrial ATPase and submitochondrial-particle ATPase activities

The mitochondrial enzyme activity (a) was determined in the presence of 0.2 mM-dinitrophenol with (●) or without (○) the addition of MgCl_2 . The concentration of dinitrophenol used was optimum. The values are the means of five independent experiments. The enzyme activity in submitochondrial particles (b) was assayed in the presence of Mg^{2+} ions. The values are the means of four independent determinations. Other details are given in the Experimental section.

($I_{50} = 22.5 \mu\text{mol}$ of the drug/mg of protein) is much higher than that required for producing optimal effects on oxidative phosphorylation. This appears to be a general property of inhibitors and uncouplers (Hemker, 1964). The ATPase activity of submitochondrial sonic particles was also susceptible to inhibition by the compound (Fig. 5b). The concentration of the drug required to inhibit this activity ($I_{50} = 80 \mu\text{mol/mg}$ of particle protein) is nearly four-fold higher than that required for a similar inhibition of the activity in whole mitochondria. This seems to be due to a greater concentration of ATPase in the particles, since the I_{50} values normalized for the same ATPase activity (100 nmol of P_i released/min per mg of protein) were of the same order for both particles ($5.3 \mu\text{mol}$ of the drug) and whole mitochondria ($8.3 \mu\text{mol}$ of the drug).

ATP-dependent reversal of electron transport

The effect of the compound on energy-dependent reduction of NAD^+ by succinate was studied in submitochondrial particles with ATP as the source of energy. The results presented in Fig. 6 show that this reaction is very sensitive to the compound ($I_{50} = 1.5 \mu\text{mol}$ of the drug/mg of particle protein).

Fig. 6. Effect of chlorophenoxyisobutyrate on energy-linked reversal of electron transport in rat liver submitochondrial particles

The details of the experiment have been given in the Experimental section. The values are the means of five separate determinations.

This agrees with the earlier finding (Tables 1 and 2) of the greater susceptibility of the first coupling site to inhibition by chlorophenoxyisobutyrate.

The fact that the compound inhibits ATP-linked reactions in submitochondrial particles (ATPase, reversed electron transport) would rule out the possibility of interference at the level of adenine nucleotide translocation as the mechanism of its inhibition of oxidative phosphorylation.

Discussion

The results presented in this paper indicate that the three coupling sites of oxidative phosphorylation show differential susceptibility to inactivation by chlorophenoxyisobutyrate. The first site is shown to be the most sensitive and the second the least. Selective susceptibility of mitochondrial coupling sites to inhibitor action has been demonstrated previously. Whole-body irradiation (Alexander *et al.*, 1972), achondroplasia (Bargman *et al.*, 1972), hydroxylamine (Wikström, 1971), alkylguanidines (Pressman, 1963), biguanidine derivatives (Chappell, 1963; Guillory & Slater, 1965), pent-4-enoic acid (Senior & Sherratt, 1968) and organotin compounds (Sone & Hagihara, 1964) may be cited as examples. Katyare *et al.* (1971) have reported that the first and third sites are more sensitive to the uncoupling action of dinitrophenol than is the second site.

The compound behaves both as an uncoupler (stimulation of state 4 oxidation, abolition of respiratory control, stimulation of latent ATPase activity, oligomycin-sensitivity of stimulated ATPase etc.) and as an inhibitor of phosphorylation (inhibition of state 3 oxidation, coupled phosphorylation, uncoupler-stimulated ATPase, submitochondrial-particle ATPase, etc.). In the latter set of properties it resembles oligomycin. However, unlike oligomycin, chlorophenoxyisobutyrate inhibits mitochondrial respiration even in the presence of uncouplers (Fig. 3). Since it is not an electron-transport inhibitor, the compound may have two sites of inhibition in the energy-transfer pathway, one on the ATP side of the dinitrophenol site (like oligomycin) and another on the respiratory-chain side. The latter possibility has been suggested for a similar property shown by alkylguanidines (Pressman, 1963) and organotin compounds (Aldridge & Threlfall, 1961). From the above properties the compound may be said to act as 'inhibitory uncoupler' of oxidative phosphorylation.

Brief attempts to define the effect of the drug on oxidative phosphorylation have been made (Katyare *et al.*, 1972; Yeh & Kabara, 1972; Mackerer *et al.*, 1973). In these studies the ethyl ester of chlorophenoxyisobutyrate was used with contradictory results. The ethyl ester has been found to have

powerful membrane-lysing activity (S. R. Panini & C. K. R. Kurup, unpublished work). This was not taken into account by previous workers. It may be mentioned that even though the drug is administered in the ester form, only the free acid circulates in the serum (Thorp, 1962). Also, the circulating concentration of the drug has been estimated to be in the range 0.5–1.0 mM (Lamb & Fallon, 1972). This suggests that even though millimolar concentrations of the compound are required to inhibit oxidative phosphorylation (Mackerer *et al.*, 1973; this paper) or enzymes involved in lipid metabolism (Walsh *et al.*, 1969; Maragoudakis & Hankin, 1971; Lamb & Fallon, 1972; Daae & Aas, 1973) *in vitro*, these effects may be physiologically meaningful. Administration of the drug has been reported to decrease oxygen consumption by the rat (Thorp, 1963).

We thank Professor T. Ramasarma for his interest in the work and for the many helpful discussions we had with him. S. R. P. is a scholar of the National Council of Educational Research and Training, India.

References

- Aldridge, W. N. & Threlfall, C. J. (1961) *Biochem. J.* **79**, 214–219
- Alexander, K. C., Aiyar, A. S. & Sreenivasan, A. (1972) *Biochim. Biophys. Acta* **283**, 206–216
- Bargman, G. J., Mackler, B. & Shepard, T. H. (1972) *Arch. Biochem. Biophys.* **150**, 136–146
- Bernath, P. & Singer, T. P. (1962) *Methods Enzymol.* **5**, 601–602
- Chance, B. & Williams, G. R. (1955) *J. Biol. Chem.* **217**, 409–427
- Chappell, J. B. (1963) *J. Biol. Chem.* **238**, 410–417
- Daae, L. N. W. & Aas, M. (1973) *Atherosclerosis* **17**, 389–400
- Ernster, L. & Lee, C. P. (1967) *Methods Enzymol.* **10**, 729–738
- Fiske, C. H. & Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
- Gornall, A. G., Bardawill, G. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Graven, S. N., Lardy, H. A. & Estrada-O., S. (1967) *Biochemistry* **6**, 365–371
- Guillory, R. J. & Slater, E. C. (1965) *Biochim. Biophys. Acta* **105**, 221–232
- Hemker, H. C. (1964) *Biochim. Biophys. Acta* **81**, 1–8
- Huijing, F. & Slater, E. C. (1961) *J. Biochem. (Tokyo)* **49**, 493–501
- Katyare, S. L., Saha, J. & Kabara, J. J. (1972) *Biochem. Pharmacol.* **21**, 747–751
- Katyare, S. S., Fatterpaker, P. & Sreenivasan, A. (1971) *Arch. Biochem. Biophys.* **144**, 209–215
- Krishnakantha, T. P. & Kurup, C. K. R. (1972) *Biochem. J.* **130**, 167–175
- Kurup, C. K. R. & Sanadi, D. R. (1970) *Biochemistry* **7**, 4483–4491

- Kurup, C. K. R., Aithal, H. N. & Ramasarma, T. (1970) *Biochem. J.* **116**, 773–779
- Lamb, R. G. & Fallon, H. J. (1972) *J. Biol. Chem.* **247**, 1281–1287
- Lee, C. P., Sottocasa, G. L. & Ernster, L. (1967) *Methods Enzymol.* **10**, 33–37
- Mackerer, C. R., Haettinger, J. R. & Hutsell, T. C. (1973) *Biochem. Pharmacol.* **22**, 513–519
- Maragoudakis, M. E. & Hankin, H. (1971) *J. Biol. Chem.* **246**, 348–358
- Panini, S. R. (1972) *Biochem. J.* **128**, 50P
- Pressman, B. C. (1963) *J. Biol. Chem.* **238**, 401–409
- Racker, E. (1965a) *Mechanisms in Bioenergetics*, pp. 108–115, Academic Press, New York and London
- Racker, E. (1965b) *Mechanisms in Bioenergetics*, pp. 154–155, Academic Press, New York and London
- Senior, A. E. & Sherratt, H. S. A. (1968) *Biochem. J.* **110**, 499–509
- Sone, N. & Hagihara, B. (1964) *J. Biochem. (Tokyo)* **56**, 151–156
- Steinberg, D. (1969) *Atheroscler. Proc. Int. Symp.* **2nd** pp. 500–508
- Thorp, J. M. (1962) *Lancet* **i**, 1323–1326
- Thorp, J. M. (1963) *J. Atheroscler. Res.* **3**, 737–739
- Veldsema-Currie, R. D. & Slater, E. C. (1968) *Biochim. Biophys. Acta* **162**, 310–319
- Walsh, M. R., Teal, S. W. & Gamble, W. (1969) *Arch. Biochem. Biophys.* **130**, 7–18
- Wikström, M. K. F. (1971) *Biochim. Biophys. Acta* **234**, 16–27
- Wojtczak, L., Bogucka, K., Sarzała, M. G. & Załuska, H. (1969) in *Mitochondria, Structure and Function* (Ernster, L. & Drahota, Z., eds.), pp. 79–92, Academic Press, New York
- Yeh, R. & Kabara, J. J. (1972) *Life Sci.* **11**, 709–716