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Effect *in vitro* of clofibrate and *trans*-1,4-bis-(2-chlorobenzylaminoethyl)-cyclohexane dihydrochloride (AY 9944) on respiration and adenosine triphosphatase activity of mouse liver mitochondria

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CLOFIBRATE is clinically an important drug because of its hypolipidemic effect. This compound lowers blood triglycerides, cholesterol and phospholipids.[1–3] Clofibrate is also effective in inhibiting the growth of *Tetrahymena* and altering its glycogen and triglyceride content.[4] The mechanism of action of clofibrate on lipid biosynthesis is a controversial one. Though the inhibition of specific steps in the synthesis of cholesterol and fatty acids in liver have been suggested for the drug,[5,6] an interesting aspect of the problem still existed to be explored, i.e. the effect of the drug on energy metabolism of the liver. A study of this aspect would also throw light on the mechanism of action of this drug. The present study was therefore undertaken to investigate the effect of clofibrate [ethyl-2-(*p*-chlorophenoxy)-2-methylpropionate] on some of the aspects of oxidative phosphorylation. There is evidence that some of the hypocholesterolemic drugs can uncouple oxidative phosphorylation.[7,8] To test it further, another hypocholesterolemic drug, AY 9944 [*trans*-1,4 bis-(2-chlorobenzylaminomethyl)-cyclohexane dihydrochloride], was also included in the study.

Freshly prepared mouse liver mitochondria were used in all the experiments. These were prepared by the method of Schneider[9] in a medium composed of 0·25 M sucrose, 0·01 M tris–HCl buffer, pH 7·4, and 1 mM EDTA. The final suspension of mitochondria was made in 0·25 M sucrose containing 0·01 M tris–HCl buffer, pH 7·4. Solutions of AY 9944* were prepared in distilled water and that of clofibrate* (ester form) in ethyl alcohol. Oxygen uptake was measured in a Gilson Oxygraph (Gilson Medical Electronics, Middleton, Wis.) fitted with a Clark oxygen electrode. The air-saturated reaction mixture contained in a final volume of 1·5 ml the following: sucrose, 16·6 mM; sodium glutamate, 8·3 mM, or sodium succinate, 8·3 mM, with 10 μl of a saturated solution of rotenone in ethanol (4·9 mg/ml), or 8·3 mM alpha-ketoglutarate with malonate, 8·3 mM; $MgCl_2$, 8·3 mM; tris–HCl buffer (pH 7·4), 44·4 mM; sodium phosphate buffer (pH 7·4), 3·32 mM; and KCl to 250 milliosmolar. Adenosine triphosphatase (ATPase) activity was assayed by the method of Lardy *et al.*[10] Controls, with and without drug, were also run. Inorganic phosphorus was estimated by the method of Fiske and SubbaRow[11] and protein by the biuret reagent using deoxycholate.[12] Both of these drugs at the levels used in the present study had no effect on phosphorus determination.

Figure 1 shows the effect of AY 9944 on the mitochondrial respiration. Curve A was the normal response of mitochondria to two additions of ADP and one of dinitrophenol (DNP). Glutamate was used as a respiratory substrate. The addition of 50 μg of the drug resulted in stimulation of state 4 respiration (ADP limiting, substrate and oxygen in excess), but state 3 (excess ADP) was eliminated (curve B). When alpha-ketoglutarate plus malonate was used instead of glutamate, similar responses were obtained. When succinate was used as a respiratory substrate (curve D), the inclusion of 100 μg AY 9944 brought about an immediate increase in the respiration rate. This rate was similar to the rate measured after ADP addition, but without drug.

* Gift of Dr. D. Dvornik, Ayerst Research Laboratories, Montreal, Canada.

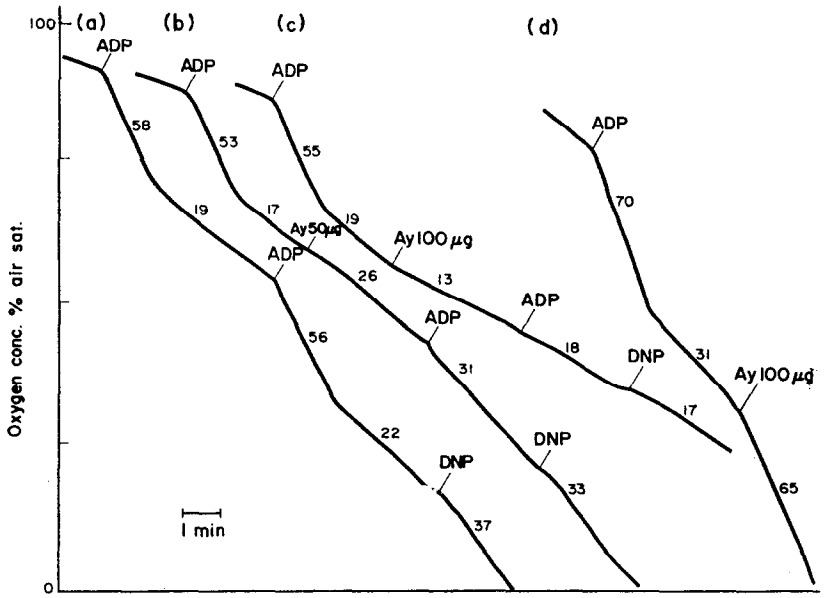

FIG. 1. Effect of AY 9944 (AY) on respiration of mouse liver mitochondria. Mitochondria were incubated at 22°. Oxygen consumption was recorded with Gilson Oxygraph with a Clark oxygen electrode. Glutamate, 8·3 mM (added in A, B and C), or succinate, 8·3 mM (added in D), were the substrates. When added: ADP, 0·2 mM; 2,4-dinitrophenol (DNP), 0·01 mM. Mitochondrial protein (1·5 mg) was added to each incubation. ADP, drug and DNP were added at the points indicated by arrows. The rates of respiration are indicated by the numbers above the oxygen trace in units of millimicroatoms per minute per milligram of mitochondrial protein. For other details, refer to the description of methods.

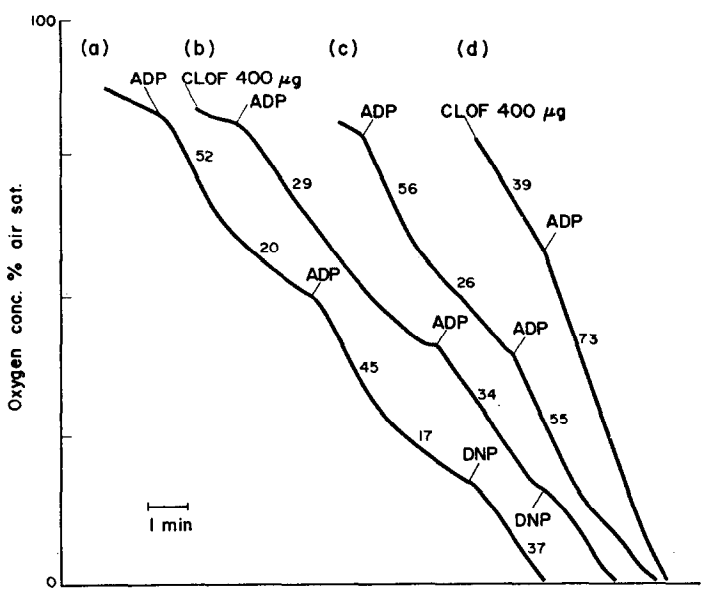

FIG. 2. Effect of clofibrate (Clof.) on respiration of mouse liver mitochondria. A and B are with 8·3 mM glutamate; C and D, with 8·3 mM succinate. Other details are given in the legend for Fig. 1.

Figure 2 shows the effect of clofibrate on mitochondrial respiration. The addition of 400 μg clofibrate into the incubation medium with glutamate as substrate resulted in an inhibitory effect on the state 3 respiration rate (curve B). A similar inhibitory effect on state 3 respiration was observed when alpha-ketoglutarate plus malonate was used instead of glutamate. On the other hand, state 4 respiration was not significantly altered when glutamate or alpha-ketoglutarate was used. When added to a succinate-utilizing system, 400 μg clofibrate brought about an increase in the respiration rate (state 4), whereas the state 3 respiration rate in the presence of drug was not altered.

TABLE 1. EFFECT OF DIFFERENT CONCENTRATIONS OF AY 9944 ON MITOCHONDRIAL ATPASE ACTIVITY*

Additions	AY 9944 (μg added/mg of mitochondrial protein)			
	0	25	50	100
None	0·73	2·82	3·91	5·00
5 mM $MgCl_2$	2·91	3·91	4·09	4·36
10 μM DNP	2·36	4·63	6·18	7·82
10 μM DNP + 5 mM $MgCl_2$	4·64	5·82	6·18	7·00

* The ATPase activity is expressed as micromoles of phosphorus liberated per milligram of protein per hour. The reaction system contained 6 mM ATP (pH 7·4), 75 mM KCl, 10 mM tris–HCl buffer, pH 7·4, and 2 mg mitochondrial protein in 0·3 ml of 0·25 M sucrose. Total volume: 1 ml; incubated for 15 min at 30°.

TABLE 2. EFFECT OF DIFFERENT CONCENTRATIONS OF CLOFIBRATE ON MITOCHONDRIAL ATPASE ACTIVITY*

Additions	Clofibrate (μg added/mg of mitochondrial protein)			
	0	100	200	400
None	0·46	0·82	0·82	2·18
5 mM $MgCl_2$	3·82	3·72	4·36	6·91
10 μM DNP	3·09	3·27	2·91	2·91
10 μM DNP + 5 mM $MgCl_2$	6·27	5·72	6·45	7·63

* The ATPase activity is expressed as micromoles of phosphorus liberated per milligram of protein per hour. The reaction system was the same as that described in the footnote to Table 1.

TABLE 3. EFFECT OF CLOFIBRATE AND AY 9944 ON MITOCHONDRIAL ATPASE ACTIVITY IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF DNP*

Additions	Concentration of DNP (mM)				
	0	0·01	0·025	0·05	0·1
None	2·63	3·54	4·18	4·91	5·36
AY 9944 (200 μg)	3·91	5·45	5·82	6·63	
Clofibrate (800 μg)	5·18	5·18	5·27	5·27	5·45

* The ATPase activity is expressed as micromoles of phosphorus liberated per milligram of protein per hour. The reaction system was the same as that described in the footnote to Table 1, except that 5 mM $MgCl_2$ was also included.

The effect of different concentrations of AY 9944 on mitochondrial ATPase activity is presented in Table 1. AY 9944 stimulates ATPase activity with and without DNP at all the concentrations of AY 9944 used in the study. Addition of $MgCl_2$ stimulated ATPase at lower concentrations (i.e. 25 and 50 μg AY 9944/mg of mitochondrial protein); however, at higher concentrations (100 μg AY 9944/mg of protein), it brought about a decrease in the ATPase activity as compared to that without Mg^{2+}. The action of different concentrations of clofibrate on mitochondrial ATPase is shown in Table 2. Clofibrate at a concentration of 400 μg/mg of mitochondrial protein brought about an increase in the ATPase activity. The effects of DNP plus clofibrate and of $MgCl_2$ plus clofibrate appear to be additive.

The results of clofibrate and AY 9944 on mitochondrial ATPase activity in the presence of varying concentrations of DNP are given in Table 3. The addition of 400 μg clofibrate/mg of mitochondrial protein in the presence of varying concentrations of DNP did not bring about any change in the ATPase activity. The value was 5·18 at zero concentration of DNP is against 5·45 at 0·1 mM concentration. A different picture emerged when 200 μg AY 9944/mg of mitochondrial protein was added to increasing concentrations of dinitrophenol, i.e. an increase in the stimulation of ATPase activity over that brought about by different concentrations of DNP. These studies reflect the presence of different mechanisms involved in the stimulation of ATPase activity for clofibrate and AY 9944.

The recent work with microorganisms has pointed out that cholesterol inhibitors may not only affect sterol synthesis but also growth and respiration of microorganisms.[13] Two such compounds, Benzmalecine and Triparanol, were reported to uncouple oxidative phosphorylation in rat liver mitochondria.[7,14] Diazacholesterol acted as a respiratory inhibitor in heart submitochondrial particles.[8] Vanadyl salts are inhibitors of oxidative phosphorylation and also inhibited cholesterol biosynthesis.[15] The present investigation also revealed an uncoupling action of two hypocholesterolemic drugs, clofibrate and AY 9944. These agents stimulate ATPase activity with and without DNP and inhibit the oxidation of DPNH-linked substrates. The oxidation of DPNH-linked substrates, such as glutamic acid, could also be affected by transaminase activity. Levels of transaminase, however, are known to be increased in liver and serum of animals treated with clofibrate.[16] Consequently, the inhibition of glutamate oxidation could not be due to an effect on transaminase activity.

Our results are not sufficient to allow the conclusion that the physiological levels of the drug would also bring about changes in mitochondrial oxidative phosphorylation, thus inhibiting the synthesis of ATP. However, there is evidence of cytological changes occurring as a result of clofibrate feeding, especially those in mitochondria and lysosomes.[17] Oxygen consumption and respiration quotients of rats treated with clofibrate are decreased by 5–10 per cent.[16] On the other hand, feeding of clofibrate at a level of 0·25 per cent in diets to rats for 14 days did not bring about any change in ATP content of liver.[18] But this study does not rule out the possibility of the appearance of a defect in ATP synthesis by feeding the drug for a longer time so as to bring about changes in mitochondria. Maragoudakis and Hankin[19,20] reported that clofibrate, when added *in vitro* at concentrations ranging from $4{\cdot}2 \times 10^{-5}$ M to 180×10^{-5} M inhibited the activity of acetyl CoA carboxylase by 3·5–98·7 per cent. However, no effect was found on the activities of a number of other enzymes tested in the presence of the same concentrations of the drug. In the present investigation, concentrations of the drug showing an effect on mitochondrial respiration also fall within this same molar range. All these observations lead to the conclusion that hypocholesterolemic drugs have some influence on ATP levels. Indeed, this inhibitory effect on ATP synthesis may have bearing on the overall synthesis of lipids in liver, especially that of cholesterol, which requires ATP at least at four different intermediary steps between acetate and cholesterol. It remains to be proven whether the action of these drugs *in vitro* can explain their effects *in vivo*.

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Dissociation of morphine tolerance and dependence from brain serotonin synthesis rate in mice

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ALTHOUGH acute and chronic administration of morphine, as well as morphine withdrawal, results in little or no change in brain serotonin (5-HT) levels in animals,[1–5] there is indirect evidence of a role for this brain amine in the action of morphine. Thus, administration of the 5-HT precursor, 5-hydroxytryptophan, has been reported to potentiate effects of morphine,[6,7] while *p*-chlorophenylalanine (pCPA), an inhibitor of 5-HT synthesis at the tryptophan hydroxylase step,[8] is said to antagonize morphine analgesia, tolerance and physical dependence.[9,10] Way *et al.*[10,11] have related morphine tolerance in mice to an increased rate of brain 5-HT turnover. In the present studies, we have measured the activity of tryptophan hydroxylase in whole brains of morphine-tolerant and control mice, and also measured whole brain 5-HT levels in mice treated similarly to those in the studies of Way *et al.*[10,11]

Three groups of male mice (18–30 g) were prepared that were tolerant to and physically dependent on morphine. Two of these groups were N.I.H. random-bred mice implanted for 72 hr with 75-mg morphine pellets of our manufacture (prepared according to the published formulation[10]), or implanted for 72 hr with 75-mg morphine pellets kindly supplied by Dr. Way. The third group consisted of CF-1 mice (Carworth Farms) implanted with morphine pellets from Dr. Way's supply. Control mice were implanted with placebo pellets.

Tolerance to morphine was assured by demonstrating at least a 3-fold increase in the dose of morphine necessary to prolong the reaction time on a hot plate at 55°[12] to 30 sec. or longer in treated animals, when compared to controls. Pellets were removed 72 hr after implantation; 6 hr after pellet removal, mice were given subcutaneous doses of morphine and tested. With 9 mg/kg of morphine, all controls responded by lifting and licking the forepaws in less than 30 sec. No morphine-implanted mouse given up to 30 mg/kg responded within 30 sec. Physical dependence was established by the jumping behavior precipitated by naloxone.[13] Seventy-two hr after morphine pellet implantation, the ED_{50} of naloxone required to elicit jumping within 15 min was 0·035 mg/kg subcutaneously. Placebo-implanted mice did not jump even after administration of 20 mg/kg.

Tryptophan hydroxylase activity was determined by a tritium release assay using 5-tritio-tryptophan as the substrate material.[14] Brains were removed 72 hr after implantation of the morphine or placebo pellet and duplicate samples were assayed from each mouse brain.

The rise of brain serotonin levels after intraperitoneal injection of pargyline hydrochloride, 75 mg/kg, was used as an index of the rate of serotonin synthesis.[15] At 0, 30, 60 and 120 min after pargyline, the mice were sacrificed by decapitation and the brains removed for assay. Whole brain 5-HT was measured by the method of Bogdanski *et al.*[16] with one modification; the final acid extract was reacted with orthopthalaldehyde[17] to increase the sensitivity so that triplicate samples from single mouse brain homogenates could be assayed. Periodic comparisons of assays with and without this modification yielded comparable results.