treatment gives the location of pH optimum at 7·25. The observed value of pH_{opt} is lower than the calculated one by 0·25 unit. The small differences between observed and calculated values of pH_{opt} for oxime reactivation of organophosphate inhibited acetylcholinesterase were observed also by other authors.[7,8]

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Effect of 3-methylcholanthrene induction on the carbon tetrachloride-induced changes in rat hepatic microsomal enzyme system

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THE WELL known impairing effects of carbon tetrachloride (CCl_4) on oxidative enzymes in hepatic microsomes[1–3] are probably due to a decrease in microsomal heme components.[4–6] It is now believed that these effects are mediated by an active metabolite of CCl_4.[7–10] In accord with this view, treatment of rats with phenobarbital (PB) enhances the impairing effects of CCl_4 on ethylmorphine (EM) *N*-demethylation, the destruction of cytochrome P-450[4] and the metabolism of CCl_4[11] and decreases the LD_{50} of the toxicant.[11]

While treatment of rats with PB increases the metabolism of a wide variety of drugs presumably by increasing the amount of the type of cytochrome P-450 already present in hepatic microsomes,[12] treatment with 3-methylcholanthrene (3-MC) enhances the metabolism of relatively few compounds,[13] presumably by inducing the formation of a different kind of cytochrome P-450 called cytochrome P-448.[12] It therefore seemed important to study the effects of CCl_4 in 3-MC treated rats.

In these studies, 3-MC dissolved in corn oil was administered, i.p., in a dose of 20 mg/kg three times at 12-hr intervals to 180-g male Sprague–Dawley rats from Hormone Assay (Chicago, Ill.). Control rats received the same volume of corn oil. Forty-eight hr after the last injection the animals, which had been fasted overnight, received 2·5 ml of CCl_4/kg orally, while controls received the same volume of saline. At different time intervals thereafter the rats were decapitated, and hepatic microsomes were isolated as previously described.[6] Protein concentrations were determined by the biuret method.[14] The amounts of cytochrome P-450, cytochrome P-420 and cytochrome b_5 were determined spectrophotometrically as described by Omura and Sato.[15] The amount of total heme was estimated as described by Falk.[16] An Aminco-Chance dual wavelength spectrophotometer was used for these determinations. EM metabolism was determined in incubation mixtures, consisting of 5 mM $MgCl_2$, 12 mM glucose 6-phosphate, 1·00 U of glucose 6-phosphate dehydrogenase per 3 ml, 0·33 mM nicotinamide adenine dinucleotide phosphate (NADP), 50 mM tris buffer (pH 7·4), 5 mM EM and

6 mg of microsomal protein per 3 ml. Metabolism of 3,4-benzpyrene was carried out in incubation mixtures consisting of 250 μg microsomal protein, 30 mg bovine serum albumin, 0·064 mM benzpyrene and the above-mentioned cofactors. The mixtures were incubated in a Dubnoff metabolic shaker for 10 min at 37°. The formaldehyde formed from the *N*-demethylation of EM was estimated by the method of Nash.[17] The 3,4-benzpyrene metabolites were assayed according to the method of Wattenberg *et al.*[18] as modified by Kuntzman *et al.*[19] Students' *t*-test was used to evaluate the significance of differences between treated animals and the corresponding controls.

As shown previously[6] in unpretreated rats receiving CCl_4, the decline in EM *N*-demethylase activity with time approximately parallels the decline in cytochrome P-450 content and the microsomal heme content that is not associated with cytochrome P-420 and cytochrome b_5 (Table 1). The decline in 3,4-benzpyrene hydroxylase activity, however, was initially faster than that of EM *N*-demethylase.

Pretreatment of the animals with 3-MC caused the expected increases in 3,4-benzpyrene hydroxylase activity, in the amount of cytochrome P-448 and the total heme content but no change in EM *N*-demethylase activity. In the 3-MC-treated animals receiving CCl_4, the EM *N*-demethylase and the 3,4-benzpyrene hydroxylase activities were not significantly changed during the first 3 hr, but were decreased at 14 and 24 hr. The cytochrome P-448 content, however, and the heme content not associated with cytochrome P-420 and cytochrome b_5 were significantly decreased at 3 hr after CCl_4 administration and continued to decrease thereafter.

The metabolic activities and the amounts of the heme components, however, were always significantly higher in the 3-MC-pretreated rats receiving CCl_4 than in the animals receiving CCl_4 alone, and the per cent decrease in these parameters was less in the rats induced with 3-MC. Furthermore, cytochrome P-420, which normally begins to appear 14 hr after CCl_4 administration to control rats, was not found when CCl_4 was given to 3-MC-pretreated animals.

Several interpretations of these results are possible. For example, the cytochrome P-448 induced by 3-MC may be less susceptible to destruction by CCl_4 or its intermediates than is cytochrome P-450. But this explanation would not account for the ameliorating effects of 3-MC on EM *N*-demethylation since this reaction is presumably not catalyzed by cytochrome P-448. It seems more likely, therefore, that 3-MC induction alters the pathways of CCl_4 metabolism causing a decrease in formation of toxic intermediates. Studies on the metabolism of CCl_4 in control and 3-MC-treated rats are needed to clarify the protective effects of 3-MC on CCl_4-induced destruction of microsomal enzymes.

The results are in agreement with the findings of Carlson *et al.*,[20] who noticed that exposure of rats to CCl_4 vapor caused a smaller decrease in hepatic cytochrome P-450 when the rats had been induced with 3-MC than when they were induced with PB. But whether these changes in cytochrome P-450 and cytochrome P-448 are related to tissue damage is debatable because Pitchumoni *et al.*[21] found a more extensive hepatic necrosis after i.p. injection of CCl_4 when the rats were induced with benzpyrene, and Reuber[22] observed an increased incidence of hyperplastic lesions and cirrhosis of the liver when Buffalo rats were given 3-MC and CCl_4 simultaneously over a period of several weeks.

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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.

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