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Effect of *p*-chlorophenoxyisobutyric acid, ethyl *p*-chlorophenoxyisobutyrate, and Atromid on the synthesis of nonsaponifiable material and cholesterol in the bovine aorta*

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ATROMID, a mixture of *p*-chlorophenoxyisobutyric acid ethyl ester and 3-α-hydroxy-17-androstanone (androsterone) lowers the serum and liver cholesterol in rats.[1] *p*-Chlorophenoxyisobutyric acid ethyl ester (chlorophenisate), alone, lowers the serum and liver cholesterol levels of rats.[1] Although parenterally administered androsterone lowers serum cholesterol,[2, 3] oral administration of the compound produces no effect on serum cholesterol.[4] However, orally administered androsterone does lower serum cholesterol when chlorophenisate is given simultaneously.[1] These findings have led to the suggestion that perhaps ethyl *p*-chlorophenoxyisobutyrate exerted its action by influencing normal hormonal mechanisms;[5] that is, chlorophenisate in some way releases endogenous androsterone from plasma proteins. More recently Azarnoff and Tucker[6] reported the inhibition of cholesterol synthesis in crude homogenates of rat liver by ethyl *p*-chlorophenozyisobutyrate; their results indicated that possibly there are other modes of action of chlorophenisate.

The present study was undertaken to determine whether *p*-chlorophenoxyisobutyric acid and related compounds have an effect on the synthesis of nonsaponifiable material (NSF) and cholesterol in vascular tissue, *in vitro*. Ethyl *p*-chlorophenoxyisobutrate, *p*-chlorophenoxyisobutyric acid, and Atromid each inhibited the incorporation of mevalonic acid-2-^{14}C into nonsaponifiable material, *p*-chlorophenoxyisobutyric acid being the most effective. A similar decrease in cholesterol synthesi from mevalonic acid-2-^{14}C was observed with *p*-chlorophenoxyisobutyric acid.

EXPERIMENTAL

Materials. Bovine aortas (about 12–14 inches in length) were obtained immediately after sacrifice of the cattle, courtesy of the D. E. Nebergall Meat Co., Albany, Ore. Atromid and *p*-chlorophenoxyisobutyric acid ethyl ester were a gift of Ayerst Laboratories, Inc., Rouses Point, N.Y. All other chemicals were reagent grade commercial products.

Preparation of cell-free system. Excess fat and adventitia of the aortas were removed with scissors and the aortas ground in a meat grinder. The preparation was next homogenized in a Waring Blendor for 30 sec with an equal weight of 0·1 M phosphate buffer (containing 0·03 M nicotinamide and 0·006 M $MgCl_2$), pH 7·0. The resulting homogenate was squeezed through cheesecloth. This crude preparation was centrifuged at 60,000 *g* in a model L Spinco centrifuge for exactly 120 min to give a clear cell-free extract; the preparation can be stored frozen for about two weeks without loss of activity.

Incubation. Into a 125-ml Erlenmeyer flask were placed 1·0 ml ATP (2 mg/ml), 2·5 ml NAD (20 mg/ml), 2·5 ml 0·1 M phosphate buffer (pH 7·0), 1·0 ml mevalonic acid-2-^{14}C (0·05 μc/ml, 0·5 mg mevalonic acid/ml added as carrier), and 5·0 ml bovine aorta cell-free preparation. Compounds being tested for inhibition were substituted for the buffer; other conditions were identical. Atromid and ethyl *p*-chlorophenoxyisobutyrate were dissolved in a small amount of ethanol and the solution brought to the appropriate volume with water. Controls were run with the same amount of ethanol without the inhibitor; *p*-chlorophenoxyisobutyric acid was dissolved in 0·1 M phosphate buffer (pH 7·0). Each flask was oxygenated (95% oxygen, 5% CO_2) for about 15 sec by swirling while a stream of the gas entered the flask. After sealing the flask tightly with rubber stoppers, they were incubated for 3 hr in a constant-temperature, shaking water bath at 37°; after removing the flasks from the water bath, the reactions were stopped by the addition of 10 ml alcoholic KOH (10%) to each flask.

Determination of radioactivity of nonsaponifiable material and cholesterol. After the saponification, the reaction mixtures were extracted with 30–60° petroleum ether according to the method of

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Wright and Cleland.[7] The radioactivity in the resulting extracts (NSF) was determined by means of a Packard Tri-Carb liquid scintillation spectrometer. The cholesterol in the NSF was separated by column chromatography on silicic acid, according to the method of Wycoff and Parsons.[8] For the quantitative estimation of cholesterol the Sperry and Webb method[9] was employed. The radioactivity of the cholesterol was determined by scintillation counting in the manner described for NSF.

RESULTS AND DISCUSSION

The effect of Atromid, ethyl *p*-chlorophenoxyisobutyrate, and *p*-chlorophenoxyisobutric acid on the incorporation of mevalonic acid-2-^{14}C into nonsaponifiable material is shown in Table 1. *p*-Chlorophenoxyisobutyric acid elicited the greatest inhibition; Atromid appears to be the least effective. The results in Table 2 indicate that *p*-chlorophenoxyisobutyric acid decreased the incorporation of mevalonic acid into cholesterol.

TABLE 1. EFFECT OF ATROMID, ETHYL *p*-CHLOROPHENOXYISOBUTYRATE, AND *p*-CHLOROPHENOXYISOBUTYRIC ACID ON THE SYNTHESIS ON NONSAPONIFIABLE MATERIAL BY CELL-FREE EXTRACTS OF THE AORTA

Exp.	Atromid* (M × 10^{-3})	Ethyl *p*-chlorophenoxyisobutyrate (M × 10^{-3})	*p*-Chlorophenoxyisobutyric acid (M × 10^{-3})	Counts per minute	
				Average†	Range
1				1,997	1,794–2,089
2	9·6			997	995–999
3	4·8			1,254	1,214–1,295
4	0·96			1,930	1,850–2,009
5				2,109	1,968–2,251
6		9·6		365	220–510
7		4·8		912	892–931
8		0·96		1,540	1,286–1,794
9				2,127	2,030–2,255
10			9·6	24	10–37
11			7·6	76	61–91
12			5·7	234	191–272
13			3·8	707	656–758
14			1·0	1,424	1,185–1,663

* Concentration of Atromid based on the *p*-chlorophenoxyisobutyrate content of the drug.
† Each value is the average of 2 to 4 determinations.

TABLE 2. EFFECT OF *p*-CHLOROPHENOXYISOBUTYRIC ACID ON THE SYNTHESIS OF CHOLESTEROL BY CELL-FREE EXTRACTS OF THE AORTA

Experiment	*p*-Chlorophenoxyisobutyric acid (M × 10^{-3})	Counts per min per mg cholesterol
1		20,725
2	3·8	6,240
3	7·6	316
4	9·6	272

In vivo experiments with Atromid have indicated that the simultaneous use of androsterone with ethyl *p*-chlorophenoxyisobutyrate brings about a greater decrease in serum levels.[1, 5] In the present *in vitro* experiments such was not the case. When equivalent amounts of *p*-chlorophenoxyisobutyric acid were given as Atromid and as the free acid, inhibition elicited by the latter (Table 1) was much greater. The present experiments were performed in a system from the aorta, whereas the former experiments involved studies on rats, monkeys, and man. Thus no real comparison can be made. It is possible that the lower inhibition observed with ethyl *p*-chlorophenoxyisobutrate and Atromid in the cell-free system from the bovine aorta is due to a lack of an esterase to cleave the ethyl ester to the free acid.

Azarnoff and Tucker[6] found in rat liver homogenates that ethyl *p*-chlorophenoxyisobutyrate at certain concentrations had minimal inhibition of the incorporation of mevalonic acid into squalene and maximal inhibition into lanosterol and cholesterol. In the present system *p*-chlorophenoxyisobutyric acid appeared to have the same effect on cholesterol and nonsaponifiable material at various levels of the compound; the data seem to indicate a similar inhibition at some point before squalene and between squalene and cholesterol in the reaction sequence.

The results reported in the present paper suggest that in the cell-free system from the bovine aorta there is a direct inhibition of the syntheses of NSF and cholesterol by *p*-chlorophenoxyisobutyric acid.

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Effect of chronic sympathetic denervation on subcellular distribution of some sympathomimetic amines*

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CONVERSION of tyramine to octopamine has been demonstrated in tissue slices,[1] perfused organs,[2–4] and in intact animals.[5–7] This transformation is impaired by chronic sympathetic denervation,[5, 6] and presumably occurs in the sympathetic nerves. Tyramine and α-methyltyramine (p-hydroxyamphetamine) rapidly enter the norepinephrine storage granules, where their β-hydroxylated derivatives are formed,[8] selectively retained,[8] and released by sympathetic nerve stimulation.[4] These storage granules have been separated from the heart and salivary glands.[9]

Although binding is markedly impaired, some administered norepinephrine[10] or tyramine[5, 6] is taken up into chronically denervated salivary glands, probably at extraneuronal binding sites. Norepinephrine formation from dopamine-^{14}C, however, cannot be demonstrated after chronic denervation.[11] Absence of demonstrable norepinephrine-^{14}C may be due to inability of the denervated salivary gland to take up dopamine, to form norepinephrine, or to retain norepinephrine which is formed. Dopamine may not be present because it can be destroyed by monoamine oxidase or catechol-O-methyl transferase. These enzymes do not destroy α-methyltyramine, and destruction of tyramine can be prevented by pretreatment with a monoamine oxidase inhibitor. These amines, therefore, have been used to examine the effects of chronic sympathetic denervation on the subcellular distribution and β-hydroxylation of amines in the salivary gland.

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