Alterations in Cholesterol and Fatty Acid Biosynthesis in Rat Liver Homogenates by Aryloxy Acids

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2,4-Dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid inhibited the incorporation of [2-¹⁴C]mevalonate into cholesterol and non-saponifiable lipids. Both compounds inhibited the conversion of [1-¹⁴C]isopentenyl pyrophosphate into cholesterol and the synthesis of cholesterol and fatty acids from [2-¹⁴C]acetate. There was no inhibition of the conversion of [1-¹⁴C]mevalonate into CO₂. At low concentrations (0.5 mM) of the compounds there was a stimulation of acetate incorporation into fatty acids.

The aryloxy acids 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) are widely used as herbicides. Marre & Forti (1958) found that in plants 2,4-dichlorophenoxyacetic acid alters the ATP/ADP ratio by increasing the ATP concentration. They also showed that the GSH/GSSG ratio was increased owing to an increase in the concentration of GSH. Protein and RNA synthesis were observed to be stimulated by 2,4-dichlorophenoxyacetic acid (Baxter & Hanson, 1968). Heene (1969) showed that 2,4-dichlorophenoxyacetic acid inhibited phosphorylase, altered glycogen metabolism and caused myotonia in rats. Kolberg *et al.* (1972) found that 2,4-dichlorophenoxyacetic acid caused the accumulation of triglycerides in mouse fibroblasts. The data suggested that the fatty acids of the accumulated triglycerides were largely derived from the growth medium. To our knowledge, no other report of the effect of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on lipid metabolism has appeared. However, Mann (1968) reported that 2,4-dichlorophenoxyacetic acid increased the incorporation of [2-¹⁴C]malonic acid into lipids in excised hypocotyls. Another aryloxy acid, chlorophenoxyisobutyrate, has marked effects on lipid metabolism (Fallon *et al.*, 1972) and is used clinically. It therefore seemed likely that 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid would exhibit similar effects on lipid metabolism. In the present report we have investigated the effect of the aryloxy acids on cholesterol and fatty acid biosynthesis in rat liver homogenates.

Materials and methods

DL-[2-¹⁴C]Mevalonic acid as the *NN'*-dibenzylethylenediamine salt, [1-¹⁴C]mevalonic acid as the lactone, [1-¹⁴C]isopentenyl pyrophosphate as the

trilithium salt and sodium[2-¹⁴C] acetate were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. ATP, NAD⁺, NADP⁺ and CoA were obtained from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A. Silica-gel Chromagram sheets (type k301R2 without fluorescent indicator) were obtained from Eastman Kodak Co., Rochester, N.Y., U.S.A. 2,4-Dichlorophenoxyacetic acid was purchased from Matheson, Coleman and Bell, Norwood, Ohio, U.S.A. 2,4,5-Trichlorophenoxyacetic acid was obtained from K & K Laboratories, Plainview, N.Y., U.S.A. Both 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid were recrystallized before use and shown to be free of dioxin (less than 1 p.p.m.) by g.l.c. All other chemicals were reagent grade as described previously (Walsh *et al.*, 1969).

Incubation of the homogenates. Incubations were performed essentially as described previously (Walsh *et al.*, 1969). Coenzymes, radioactive substrates, inhibitors and 0.1 M-potassium phosphate buffer were added to each 125 ml Erlenmeyer flask followed by the homogenate in a total volume of 10.5 ml. The flasks were incubated in a Research Specialties oscillation water bath at 37°C for 170 min. In the determination of CO₂ released, 50 ml side-arm Erlenmeyer flasks with fitting scintillation vials were used for the incubations, as described by Saba & DiLuzio (1966).

In the experiments on fatty acid biosynthesis 2 μmol of ATP, 7 μmol of GSH, 0.2 μmol of CoA, 9 μmol of α-D-glucose 1-phosphate, 0.7 μmol of NADP⁺ and 7 μmol of MgCl₂, all dissolved in 1 ml of 0.1 M-potassium phosphate buffer, pH 7.0, [2-¹⁴C]acetate (0.513 μCi), 2 ml of the above phosphate buffer in which the inhibitors were dissolved and 5.0 ml of homogenate were all placed in a 125 ml Erlenmeyer

flask. The reaction mixtures were incubated at 37°C for 3 h.

Control experiments, in which homogenate was omitted, and in which the reaction was stopped at zero time, showed that no radioactivity was extracted when the usual incubation, saponification and isolation procedures were performed.

Isolation of the non-saponifiable lipids. The saponified incubation mixture was extracted four times with 50ml portions of light petroleum (b.p. 30–60°C) by the method of Wright & Cleland (1957). The resulting extracts were pooled.

Determination of radioactivity. Radioactivity was assayed by liquid-scintillation counting in a Packard Tri-Carb liquid-scintillation spectrometer as described previously (Walsh *et al.*, 1969). $^{14}\text{CO}_2$ was counted as bicarbonate on filter paper in 20ml of toluene scintillation fluid [0.4% 2,4-diphenyloxazole–0.01% 1,4-bis-(5-phenoxazol-2-yl)benzene in scintillation-grade toluene], after the paper was dried over CaCl_2 and CaSO_4 overnight, by the method of Saba & DiLuzio (1966).

Determination of cholesterol. Cholesterol was measured by the Liebermann–Burchard reaction (Cook, 1961). The digitonide of cholesterol was prepared by the method of Sperry & Webb (1950). The radioactive digitonide was counted for radioactivity by the method of Avoy *et al.* (1955).

Extraction of fatty acids. After removal of the non-saponifiable fraction, fatty acids were extracted with light petroleum (b.p. 30–60°C) as described previously (Walsh *et al.*, 1969).

T.l.c. and preparative layer chromatography. Silica gel G on plastic plates (Chromagram sheets; Eastman Kodak Co., Rochester, N.Y., U.S.A.) and glass plates (20cm × 20cm, 250 μm thick) were used as the support. The samples were applied as solutions in chloroform. The plates were developed in a glass chamber in diethyl ether–hexane–chloroform–acetic acid (550:400:50:1, by vol.). Compounds were detected by spraying with 10% (w/v) phosphomolybdic acid in ethanol.

Preparative layer plates (20cm × 40cm, 750 μm thick) were prepared by using silica gel H previously purified by the method of Parker & Peterson (1965). Samples were applied as streaks with marker spots and standards were also applied. By using the marker for a guide, the gel was scraped off in bands corresponding to components of the sample. Compounds were extracted from the gel each with 4 × 20ml of chloroform and the chloroform was removed under reduced pressure to yield the sample.

Reverse-phase chromatography of samples was done on silica-gel G plastic plates dipped in 5% mineral oil in light petroleum (b.p. 30–60°C), and developed in 85% (v/v) acetic acid. Bands corresponding to marker spots were cut out and counted for radioactivity.

Results

Effects of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on incorporation of [2- ^{14}C]mevalonate into non-saponifiable fraction and cholesterol. The incorporation of [2- ^{14}C]mevalonate into non-saponifiable lipids ranged from 22.6 to 30.6% (nine experiments). Both 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid inhibited this incorporation, the maximum inhibition being 76.7% with 9.0mm-2,4-dichlorophenoxyacetic acid and 64.9% with 4.5mm-2,4,5-trichlorophenoxyacetic acid (Table 1).

The non-saponifiable lipids from the control and 2,4-dichlorophenoxyacetic acid- and 2,4,5-trichlorophenoxyacetic acid-treated homogenates were subjected to t.l.c. on silica-gel G precoated plastic plates in ether–hexane–chloroform–acetic acid (550:400:50:1, by vol.). Compounds with R_F values corresponding to those of cholesterol (R_F 0.68), farnesol (R_F 0.79) and squalene (R_F 0.91) were removed and their radioactivities determined. Similarly, samples were subjected to preparative layer chromatography on silica gel H in the same solvent system and the com

Table 1. *Effects of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on incorporation of [2- ^{14}C]mevalonate into non-saponifiable lipids*

Rat liver homogenates were incubated with [2- ^{14}C]mevalonate (0.05 Ci) at 37°C for 170 min, in the presence and absence of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid. Non-saponifiable lipid was isolated and the radioactivity determined as described under 'Materials and methods'. Details of the procedure have been reported previously (Walsh *et al.*, 1969). Comparisons are made between values obtained with pooled homogenates from the same group of animals. Data are reported as % $\pm$ S.E.M.

No. of experiments	2,4-Dichlorophenoxyacetic acid (mm)	2,4,5-Tri-chlorophenoxyacetic acid (mm)	Inhibition (%)
2	—	—	0.00 (control)
2	4.5	—	50.8 $\pm$ 0.45
2	9.0	—	76.7 $\pm$ 0.17
6	—	—	0.00 (control)
6	4.5	—	27.9 $\pm$ 1.49
6	9.0	—	67.2 $\pm$ 0.92
3	—	—	0.00 (control)
2	1.0	—	12.3 $\pm$ 2.23
3	9.0	—	69.2 $\pm$ 0.98
3	—	4.0	64.9 $\pm$ 1.14
2	—	1.0	18.2 $\pm$ 4.10
3	—	0.5	5.6 $\pm$ 0.48

pounds isolated by extraction with chloroform. Identification of cholesterol was confirmed by digitonin precipitation. It was shown that 93.5% of the radioactivity was in the digitonin-precipitable compound. Identification of cholesterol was confirmed by reverse-phase chromatography and by g.l.c. by using a 7.8 m glass column (0.62 cm inside diameter), 6% diethylene glycol succinate on Diatapore-S and an F & M model 402 gas chromatograph. No significant radioactivity was found in compounds whose R_f values in both reverse-phase t.l.c. and in ether-hexane-chloroform-acetic acid (550:400:50:1 by vol.) corresponded to desmosterol, 7-dehydrocholesterol, dihydrocholesterol or lanosterol. In the control, 89.4% of the total radioactivity was in cholesterol, 2% in squalene and 4.4% in farnesol. The distribution of radioactivity changed with increasing concentration of inhibitor. Distribution of radioactivity in the presence of 2,4-dichlorophenoxyacetic acid was cholesterol (92.4%), squalene (2.8%) and farnesol (1.5%) at 1.0 mM and cholesterol (36.6%), squalene (35.3%) and farnesol (20.8%) at 9.0 mM. At 0.5 mM-, 1.0 mM- and 4.5 mM-2,4,5-trichlorophenoxyacetic acid the distributions were cholesterol (83.7, 90 and 4.8% respectively), squalene (1.6, 2.5 and 54% respectively) and farnesol (1.3, 4.2 and 21.6% respectively).

Effect of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on the conversion of [1- 14 C]mevalonate into CO_2 by rat liver homogenates. No decrease in $^{14}\text{CO}_2$ production was observed when concentrations as high as 9.0 mM-2,4-dichlorophenoxyacetic or 4.5 mM-2,4,5-trichlorophenoxyacetic acid were used.

Effect of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on the incorporation of [1- 14 C]isopentenyl pyrophosphate into non-saponifiable fraction and cholesterol by rat liver homogenates. Inhibition of [1- 14 C]isopentenyl pyrophosphate incorporation into cholesterol and non-saponifiable lipids was comparable with that observed with [2- 14 C]mevalonate. Cholesterol accounted for 83.6% of the total radioactivity in the non-saponifiable lipids.

Effects of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on the incorporation of [2- 14 C]acetate into non-saponifiable lipids and cholesterol. Analysis of the non-saponifiable lipids was performed as described above in the experiments in which [2- 14 C]mevalonate was the substrate. Maximum inhibition was obtained with both 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid at 4.5 mM. 2,4-Dichlorophenoxyacetic acid at 9.0 mM gave $99.6\% \pm 0.21$ [S.E.M. (3)] inhibition, which was not significantly different from that observed with 4.5 mM [$96.2\% \pm 0.62$ S.E.M. (3)]. Cholesterol contained 76.7% of the total radioactivity. At the lower concentration (0.5 mM),

2,4,5-trichlorophenoxyacetic acid [$73.1\% \pm 9.6$ S.E.M. (3)] was a more effective inhibitor than 2,4-dichlorophenoxyacetic acid [$5.6\% \pm 1.1$ S.E.M. (3)].

Effects of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid on the incorporation of [2- 14 C]acetate into fatty acids. 2,4-Dichlorophenoxyacetic acid (0.5 mM and 1.0 mM) and 2,4,5-trichlorophenoxyacetic acid (0.5 mM and 1.0 mM) caused stimulation [2,4-dichlorophenoxyacetic acid, $6.3\% \pm 1.1$ S.E.M. (3) and $64\% \pm 10$ S.E.M. (3) respectively; 2,4,5-trichlorophenoxyacetic acid, $59\% \pm 11$ S.E.M. (3) and $66.7\% \pm$ S.E.M. (3)] of [2- 14 C]acetate incorporation into fatty acids. At 4.5 mM 2,4-dichlorophenoxyacetic acid increased fatty acid biosynthesis by $56.7\% \pm 5.0$ S.E.M. (3), whereas 2,4,5-trichlorophenoxyacetic acid at the same concentration decreased it by $37.8\% \pm 5.2$ S.E.M. (3). Inhibition [$63.3\% \pm 0.3$ S.E.M. (3)] by 2,4-dichlorophenoxyacetic acid was obtained at 9.0 mM concentration.

Discussion

The data clearly demonstrate that 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid inhibit the incorporation of [2- 14 C]mevalonate into cholesterol and non-saponifiable fractions of rat liver homogenates. 2,4,5-Trichlorophenoxyacetic acid was a more effective inhibitor of the latter processes, giving essentially the same inhibition at half the concentration of 2,4-dichlorophenoxyacetic acid. The concentrations used were in the range in which cholesterol biosynthesis is inhibited by chlorophenoxyisobutyrate. At the higher concentrations there was a change in the distribution pattern of radioactivity in the non-saponifiable fraction, more radioactivity appearing in squalene and other non-sterol products. The latter finding suggests the possibility of inhibition at a site between squalene and cholesterol, but does not exclude the possibility of inhibition at earlier sites in the pathway. Similar results were obtained when [1- 14 C]isopentenyl pyrophosphate was the substrate. There was no apparent inhibition, by either of the aryloxy acids, of the conversion of [1- 14 C]mevalonate into CO_2 . The latter result indicates that 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid do not inhibit pyrophosphomevalonate decarboxylase activity. Conversion of acetate into the non-saponifiable lipids and cholesterol was inhibited by both of the aryloxy acids, as was observed with mevalonate. At the low concentrations there was a stimulation of fatty acid synthesis from acetate, whereas non-saponifiable lipid formation was inhibited. At the higher concentrations, fatty acid synthesis was inhibited. It is not possible to deduce the mechanism of action of the aryloxy acids from the results of the present experiments. The results indicate that the compounds have effects on lipid biosynthesis similar to those of chlorophenoxyisobutyrate, a drug

whose mechanism of action has not been completely described. The compounds in question are compounds known to have profound metabolic effects in plants. It is therefore of interest to demonstrate alterations in lipid metabolism in an animal system by the aryloxy acids.

It should be understood that no implications are intended with respect to potential or actual toxicity or lack thereof of such compounds. Hansen *et al.* (1971) found no significant deleterious effects of 2,4-dichlorophenoxyacetic acid in rats and dogs with doses as high as 1250 p.p.m., which is considerably higher than the calculated 0.3 p.p.m. expected from maximum quantities allowed on crops. 2,4-Dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid apparently do not accumulate in most tissues, the epithelium of the yolk sac being an exception (Lindquist & Ullberg, 1971). The concentrations in the present study are higher than those used in the latter but are in the range used for administration of chlorophenoxyisobutyrate, the hypolipidaemic drug.

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