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EFFECTS OF 2,4-DICHLOROPHENOXYACETIC ACID AND 2,4,5-TRICHLOROPHENOXYACETIC ACID ON PEROXISOMAL ENZYMES IN RAT LIVER

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Abstract—The effects of feeding 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) on the level of peroxisomal enzymes in rat liver were studied. The concentration of triglyceride in serum was decreased and the activity of cyanide-insensitive palmitoyl-CoA oxidation, catalase and carnitine acetyltransferase increased. However, the extent of the increase in the activity of these enzymes by treatment with 2,4-D was less pronounced than that by 2,4,5-T treatment. The administration of 2,4-D or 2,4,5-T increased the concentration of polypeptide with a mol. wt of 80,000 in the light mitochondrial fractions of the liver from the rats.

Several hypolipidemic agents are known to cause proliferation of peroxisomes in rat liver [1–6]. Ethyl 4-chlorophenoxyisobutyrate (clofibrate) is one of the most common inducers of peroxisomes and is used clinically as a drug. Lazarow and de Duve [7] presented evidence suggesting that hypolipidemic action of clofibrate is attributable to the induction of cyanide-insensitive fatty acyl-CoA oxidizing activity and that the activity is localized in the peroxisomes. Recent studies on several peroxisome proliferators including clofibrate have shown that these compounds are carcinogenic in rats and mice [8–14]. It is important therefore to study the effects of other aryloxy acids on peroxisomal enzymes. 2,4-D and 2,4,5-T are well known as herbicides. In spite of their structural similarity to clofibrate, little information is available about their effects on lipid metabolism, particularly their effects on peroxisomal enzymes. The present study was undertaken to investigate the effects of 2,4-D and 2,4,5-T on the induction of peroxisomal enzymes involved in lipid metabolism.

MATERIALS AND METHODS

Chemicals. Palmitoyl-CoA, acetyl-CoA, L-carnitine, clofibric acid and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO); NAD and CoA from Oriental Yeast Co. (Tokyo, Japan); and 2,4-D and 2,4,5-T from Tokyo Chemical Industry Co. (Tokyo, Japan). All other chemicals were of analytical grade.

Animals. Male rats of Wistar strain, weighing 150–200 g, were used. Rats were fed *ad lib.* a commercial diet or a ground diet containing 0.25% (w/w) 2,4-D, 2,4,5-T or clofibric acid for 3, 7 or 14 days.

Subcellular fractionations. The rats were decapitated and the livers isolated. The livers were perfused with ice-cold 0.9% NaCl and rinsed in cold 0.25 M sucrose. Part of the liver was homogenized with cold 0.25 M sucrose and fractionated into nuclear, heavy mitochondrial, light mitochondrial, microsomal and cytosolic fractions by the method of de Duve *et al.* [15]. The other part of the liver was frozen at −30° until the peroxisomal enzymes were assayed. The frozen livers were used within 1 month as described previously [16].

Enzyme assays. Cyanide-insensitive palmitoyl-CoA oxidation was assayed by the method of Lazarow and de Duve [7] with minor modification [16]. One unit of activity was defined as the amount of enzyme required to reduce 1 μmole of NAD per min. Catalase activity was determined by the method of Aebi [17]. Carnitine acetyltransferase was assayed according to the method of Kahonen [18]. Urate oxidase and glutamate dehydrogenase were assayed according to Leighton *et al.* [19].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed essentially according to the method reported by Reddy and Kumar [20], except for the use of slab gel of 2 mm thickness. Light mitochondrial protein (20 μg) was loaded onto a stacking gel. Electrophoresis was carried out at 25 mA for *ca* 5 hr until the marker dye reached the bottom of the gel. The proteins were fixed in the gel with 20% trichloroacetic acid for 30 min and then stained with 0.25% Coomassie brilliant blue in methanol–acetic acid–water (5:1:5, v/v) for 2 hr. Destaining was done by repeated washing in 20% methanol containing 7% acetic acid.

Other analytical procedures. The concentration of cholesterol in the serum was measured by the method of Zurkowski [21]. To measure the concentration of triglyceride in the serum, lipid was extracted from serum by the method of Bligh and Dyer [22] and triglyceride was isolated by TLC on silica gel G using

Table 1. Effects of 2,4-D and 2,4,5-T on the concentration of triglyceride and cholesterol in rat serum

Conditions		Triglyceride (μmoles/ml serum)	Cholesterol (μmoles/ml serum)
Control	(3)	1.15 ± 0.14	2.50 ± 0.25
2,4-D	(3)	0.37 ± 0.03	1.65 ± 0.11
2,4,5-T	(3)	0.36 ± 0.10	1.85 ± 0.54

Each value represents mean ± S.D. The amount of triglyceride and cholesterol was determined as described in the text. Numbers in parentheses are the numbers of animals used. The rats were fed a control diet or a diet containing 2,4-D or 2,4,5-T for 7 days.

petroleum ether–diethyl ether–acetic acid (80:30:1, v/v) as developing solvent. After adding pentadecanoic acid as internal standard, triglyceride was recovered from the silica gel by extraction with chloroform–methanol–water (2:2:0.5, v/v) according to Stern and Pullmann [23]. The amount of triglyceride was determined by measuring the amount of fatty acid in trigylceride by GLC. The conditions of gas chromatographic analysis have been reported previously [24]. The concentration of protein in the enzyme preparations was determined by the method of Lowry *et al.* [25], with bovine serum albumin as standard.

RESULTS

Table 1 shows the effects of 2,4-D and 2,4,5-T on the concentration of triglyceride and cholesterol in the serum. On administration of 2,4-D or 2,4,5-T for 7 days, the concentration of serum triglyceride decreased to approximately one-third of the control. The concentration of cholesterol was also decreased by this treatment, although the extent of the decrease was less considerable. The effects of 2,4-D, 2,4,5-T and clofibric acid on relative liver weight are compared in Fig. 1. Marked hepatomegaly was produced following the administration of clofibric acid to rats, in good agreement with earlier reports [16, 26]. The relative liver weight increased 1.6 times compared to that of control rats after treatment with clofibric acid for 14 days. Significant hepatomegaly was produced by the administration of 2,4,5-T, although the effect was less marked than that observed with clofibric acid. 2,4-D did not cause hepatomegaly.

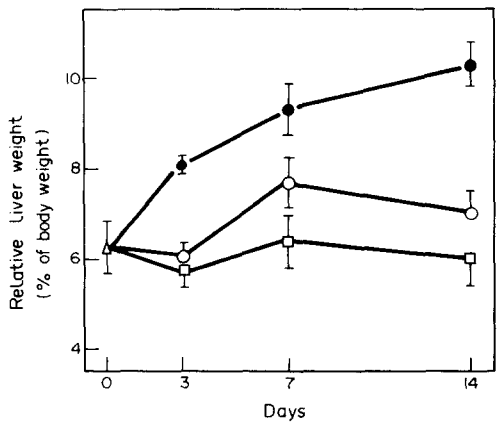

Fig. 1. Effects of 2,4-D and 2,4,5-T on relative liver weight. 2,4-D (□), 2,4,5-T (○) and clofibric acid (●). Each value was obtained from three to ten animals. Vertical bars represent the S.D.

Figure 2 shows the time-course of changes in activity of cyanide-insensitive palmitoyl-CoA oxidation, catalase and carnitine acetyltransferase when rats were fed a diet containing 2,4-D, 2,4,5-T or clofibric acid. The activity of cyanide-insensitive palmitoyl-CoA oxidation was increased by the administration of 2,4,5-T and reached its maximum value 7 days after the initiation of the administration. The maximum activity was approximately 10 times that of the control and approximately 60% of that induced by clofibric acid, when compared on a g liver basis. However, the total activity of cyanide-insensitive palmitoyl-CoA oxidation in the whole liver was 34% of that induced by clofibric acid feeding owing to the less hepatomegalic effect of 2,4,5-T compared with that of clofibric acid. The administration of 2,4-D also increased the activity of cyanide-insensitive palmitoyl-CoA oxidation, although the extent of the increase was less pronounced than that obtained by treatment with 2,4,5-T. The activity of catalase per g liver was slightly increased by treatment with either 2,4,5-T or clofibric acid, whereas 2,4,5-T did not change total activity of catalase in the whole liver. 2,4-D did not affect catalase activity. The activity of carnitine acetyltransferase, as well as cyanide-insensitive palmitoyl-CoA oxidation, was induced by treatment with either 2,4,5-T or clofibric acid. Treatment with 2,4-D caused a slight, but significant, increase in the activity of carnitine acetyltransferase.

Figure 3 shows the data on subcellular distribution of cyanide-insensitive palmitoyl-CoA oxidizing activity and marker enzymes for both mitochondria and peroxisomes. The highest activity of cyanide-insensitive palmitoyl-CoA oxidation was localized in the light mitochondrial fraction. The distribution pattern of the activity is similar to that of urate oxidase, but not to that of glutamate dehydrogenase.

Figure 4 shows the electrophoretic profiles of protein from the light mitochondrial fractions from livers of rats which had been fed 2,4-D, 2,4,5-T or clofibric acid. In accordance with the findings reported by Reddy and Kumar [20], the concentration of polypeptide with a mol. wt of approximately 80,000 in light mitochondria was increased by feeding with clofibric acid. 2,4-D and 2,4,5-T also increased effec-

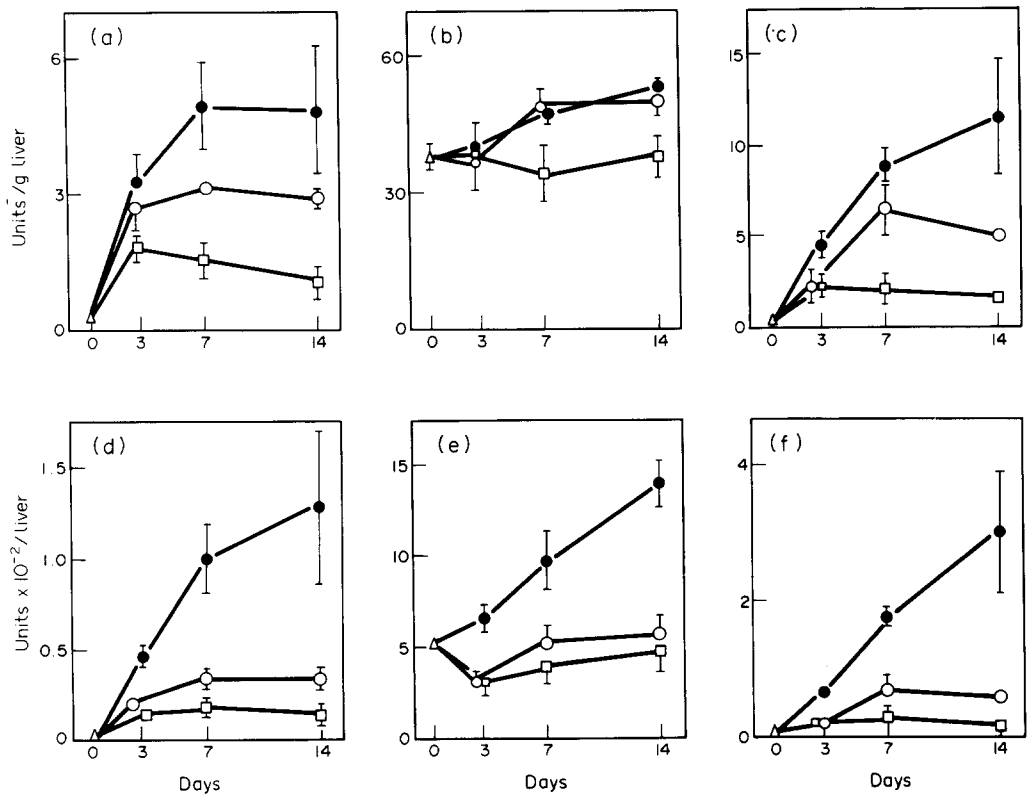

Fig. 2. Effects of 2,4-D and 2,4,5-T on peroxisomal enzymes. Rats were fed a diet containing 0.25% (w/w) 2,4-D, 2,4,5-T or clofibric acid. Enzyme activity in the post-nuclear supernatant of the livers was assayed as described in the text. Values represent the mean ± S.D. from three or four experiments. A and D, cyanide-insensitive palmitoyl-CoA oxidation; B and E, catalase; C and F, carnitine acetyltransferase. 2,4-D (□), 2,4,5-T (○) and clofibric acid (●).

tively, but somewhat less markedly than clofibric acid, the concentration of the polypeptide in the light mitochondrial fractions.

DISCUSSION

Clofibrate is a hypolipidemic drug and a potent inducer of peroxisomal β-oxidation [7]. Since many other peroxisome proliferators increase the activity of peroxisomal β-oxidation [27–29] and decrease the serum lipid level [4, 30], it is considered that the hypolipidemic action may be due to the increased activity of peroxisomal β-oxidation. The peroxisomal β-oxidation system is distinct from the mitochondrial one in its response to KCN [7].

In the present study, we showed that the administration of either 2,4-D or 2,4,5-T causes a decrease in the serum triglyceride concentration and an increase in the cyanide-insensitive palmitoyl-CoA oxidizing activity. Not only the oxidation activity, but also catalase and carnitine acetyltransferase were induced by 2,4,5-T. Catalase is a peroxisomal enzyme and, in rats, is induced slightly by treatment with clofibrate [7, 16]. Carnitine acetyltransferase has been reported to be localized in both mitochondria and peroxisomes [18] and to be greatly induced by treatment with peroxisome proliferators [6, 18]. Our subcellular fractionation studies showed that the distribution of cyanide-insensitive palmitoyl-CoA oxidizing activity induced by 2,4,5-T is consistent with that of urate oxidase, a marker enzyme for peroxisomes. These results suggest that the increase in the activity of palmitoyl-CoA oxidation in the liver is due to the increase in peroxisome activity. In spite of the fact that catalase is a peroxisomal enzyme, a large amount of catalase was distributed in the cytosolic fraction. This may result from leakage of the enzyme from the peroxisomes into cytosol, because it has been suggested that peroxisomes grow and then burst [31]. Similar leakage of catalase into cytosol was observed in the livers of rats which had been treated with other peroxisome proliferators [29, 30].

The present study showed that the concentration of polypeptide with a mol. wt of approximately 80,000 in the light mitochondrial fractions was effectively increased by either 2,4-D or 2,4,5-T. The polypeptide has been reported to be increased markedly in peroxisomes of livers from rats treated with peroxisome proliferators [20]. Osumi and Hashimoto [32] showed that a peroxisomal protein having activities of both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase corresponds to the peroxisome-specific polypeptide with a mol. wt of 80,000. Accordingly, our results show that both

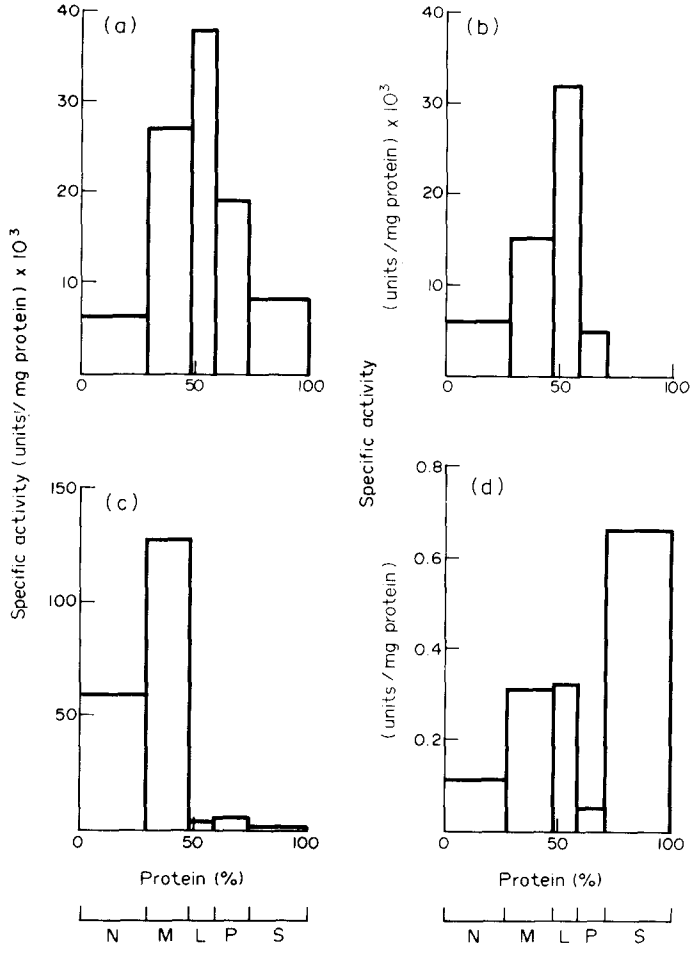

Fig. 3. Subcellular distribution of cyanide-insensitive palmitoyl-CoA oxidizing activity induced by 2,4,5-T. The livers were homogenized and fractionated into nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P) and cytosolic (S) fractions as described in the text. Enzyme activity was determined as described in the text. Each value represents the mean of three separate experiments. Rats were fed a diet containing 0.25% (w/w) 2,4,5-T for 7 days. A, Cyanide-insensitive palmitoyl-CoA oxidation; B, urate oxidase; C, glutamate dehydrogenase; D, catalase.

2,4-D and 2,4,5-T, like clofibric acid, are inducers of peroxisomal β-oxidation enzymes.

The ability of 2,4,5-T to induce peroxisomal β-oxidation was less pronounced than that of clofibric acid, whereas the effect of 2,4,5-T on peroxisomal enzymes was greater than that of 2,4-D. Most of the peroxisome proliferators are known to cause hepatomegaly and induction of catalase as well as of peroxisomal β-oxidation and carnitine acetyltransferase. In fact, 2,4,5-T produced hepatomegaly and induced catalase, peroxisomal β-oxidation and carnitine acetyltransferase. However, 2,4-D differed from 2,4,5-T in not causing hepatomegaly or catalase induction. Lazarow *et al.* [33] pointed out that an increase in activity of peroxisomal β-oxidation is not always accompanied by an increase in catalase activity or liver size. Our previous study also showed that the increase in peroxisomal β-oxidation activity by clofibric acid is not always correlated to the production of hepatomagaly and an increase in catalase activity in rats of a hormonally altered state [16]. The potency of 2,4-D and 2,4,5-T to induce peroxisomal enzymes may be due to their structural similarity to clofibric acid. However, it should be noted that phenoxyacetic acid, 2-chlorophenoxyacetic acid and 4-chlorophenoxyacetic acid hardly caused hepatomegaly or an increase in the activity of peroxisomal β-oxidation, catalase and carnitine acetyltransferase (data not shown).

Several inducers of peroxisome, including clofibrate and its derivatives [8–14], have been found to produce tumours when administered to rats for prolonged periods and a relatively low dose of clofibrate promotes hepatocarcinogenesis induced by dimethylnitrosamine [34]. A case-control study showed that exposure to 2,4-D and 2,4,5-T causes a 6 times increase in the risk of soft-tissue sarcomas [35]. It may therefore be necessary to take the potency of these herbicides to proliferate peroxisomes into consideration in the study of tumourigenesis of these aryloxy acids.

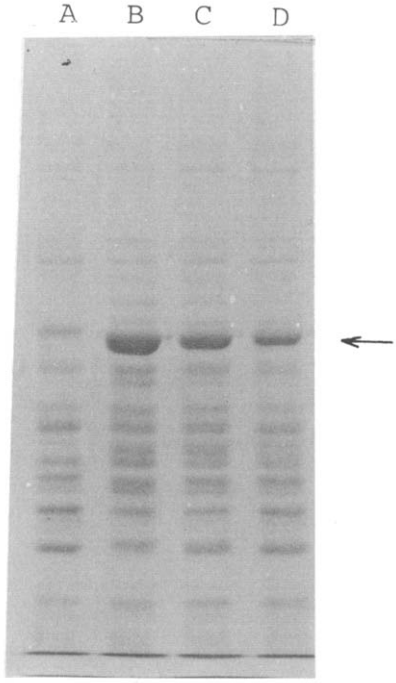

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the light mitochondrial fractions of the livers. Rats were fed a diet containing 0.25% (w/w) clofibric acid, 2,4-D or 2,4,5-T for 7 days. Approximately 20 μg of light mitochondrial protein was analyzed by electrophoresis as described in the text. A, Control; B, clofibric acid fed rats; C, 2,4,5-T fed rats; D, 2,4-D fed rats. The arrow indicates the position of the polypeptide with a mol. wt of 80,000.

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