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INDUCTION OF PEROXISOMAL ENZYMES IN RAT LIVER BY THE HYPOLIPIDEMIC AGENT LK-903

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Abstract—The effect of the hypolipidemic agent LK-903 on enzyme activities in hepatic peroxisomes of the rat was investigated. After administration of LK-903 with the chow for 14 days, the activities of hepatic catalase, urate oxidase, D-amino acid oxidase, and the fatty acyl-CoA oxidizing system were increased 2-fold, 2-fold, 2.5-fold and 7-fold, respectively. Subcellular fractionation showed that these peroxisomal enzyme activities were concentrated in the heavy mitochondrial fraction. In contrast, the peroxisomal enzyme activities of control rats were highest in the light mitochondrial fraction. During administration of LK-903, the activities of the particulate catalase, urate oxidase, and fatty acyl-CoA oxidizing system increased gradually, and reached plateau levels in about 12 days. When the rats that had received LK-903 for 14 days were subsequently given the basal diet, the particulate urate oxidase activity decreased gradually, but the activities of catalase and the fatty acyl-CoA oxidizing system remained almost unchanged for 7 days. The enhancement of catalase activity by LK-903 was due to an increase in the synthesis rate of over 2-fold; the half-life and the degradation-rate were unchanged.

Recently, Lazarow and De Duve found that peroxisomes purified from rat liver can oxidize long chain acyl-CoA esters [1]. The fatty acid oxidation capacity of the peroxisomes was strikingly increased by treatment of rats with clofibrate [1, 2], which is well-known to decrease serum triglyceride and cholesterol. Administration of acetylsalicylate or di(2-ethylhexyl)-phthalate (DEHP), a widely used plasticizer, to rats also markedly increases the activity of the peroxisomal fatty acyl-CoA oxidizing system [3, 4]. These two chemicals also have a hypolipidemic effect on rats [3, 4].

Peroxisomes are cytoplasmic organelles present in various tissues of rodents and other vertebrates. The organelles are characterized by their contents of H_2O_2-producing oxidases (urate oxidase, D-amino acid oxidase, L-α-hydroxy acid oxidase, etc.), and an H_2O_2-degrading enzyme, catalase [5]. The hypolipidemic chemicals mentioned above enhance not only the activity of peroxisomal fatty acyl-CoA oxidation, but also the number of peroxisomes and the catalase activity [1–4]. However, such chemicals conversely decrease the levels of other peroxisomal H_2O_2-producing oxidases [6, 8].

LK-903 (α-methyl-*p*-myristyroxycinnamic acid-1-monoglyceride) (Fig. 1) has hypolipidemic activity similar to that of clofibrate, but unlike clofibrate, it does not possess hepatomegalic activity [9–12].

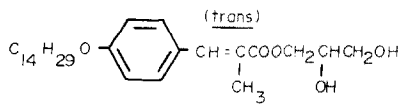

Fig. 1. Structure of LK-903.

In this experiment, the effect of LK-903 on hepatic peroxisomes of the rat was investigated. LK-903 was found to enhance the activities of all the peroxisomal enzymes determined, i.e. catalase, D-amino acid oxidase, urate oxidase, and the fatty acyl-CoA oxidizing system.

MATERIALS AND METHODS

Materials. LK-903 was kindly provided by Dr. Takashima (Tanabe Seiyaku Co., Saitama, Japan). CoA and NAD^+ were purchased from Kyowa Hakko Co. (Tokyo, Japan), and palmitoyl-CoA from Sigma Chemicals Co. (St. Louis, MO).

Administration of LK-903. Wistar male rats weighing about 150 g were fed *ad lib.* on Oriental Laboratory chow (Tokyo, Japan) containing 0.05 per cent (w/w) LK-903, usually for 2 weeks. The content of LK-903 in the chow was that suggested by Dr. Takashima, who provided the agent; it was found to produce a sufficient hypolipidemic effect on the rats. The levels of serum cholesterol (normally 84 mg/dl) and triglyceride (normally 79 mg/dl) were reduced to 25 mg/dl and 43 mg/dl respectively by the administration of LK-903 [9]. The animals were weighed and sacrified by bleeding under light ether anesthesia. The livers were isolated, weighed and homogenized in ice-cold 0.25 M sucrose in a Potter-Elvehjem type Teflon homogenizer.

Subcellular fractionation of the liver. Ten per cent (w/v) liver homogenate was prepared in 0.25 M sucrose and fractionated into nuclear (N) (pellet of 600 *g*, 10 min), heavy mitochondrial (M) (600 *g*,

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10 min-3,300 *g*, 10 min), light mitochondrial (L) (3,300 *g*, 10 min-12,500 *g*, 20 min), microsomal (P) (12,500 *g*, 20 min-100,000 *g*, 60 min), and supernatant (S) (supernatant of 100,000 *g*, 60 min) fractions according to the method of De Duve *et al.* [13]. In the present experiment, Hitachi Ultracentrifuge model 65P, with rotor RP 65 (R_{min} 3.5, R_{max} 7.8 cm) was used.

Sucrose density gradient centrifugation. The large granule fraction (heavy plus light mitochondrial fractions) of LK-903-treated liver was subjected to sucrose density gradient centrifugation. A linear density gradient (36 ml) from density 1.10 to 1.30 was prepared in centrifuged tubes for a Hitachi RPS-27 swinging bucket rotor. Next, 2 ml of the large granule fraction was layered on top of the gradient, and the tubes were centrifuged at 20,000 rpm for 180 min at 4° in a Hitachi centrifuge, model 65P. After centrifugation, the tubes were divided into 10 fractions with an ISCO gradient fractionater (U.S.A.). Each fraction was appropriately diluted with ice-cold water, and washed by centrifugation at 20,000 *g* for 30 min. The precipitate was suspended in 0.25 M sucrose, and the enzyme activities in each fraction were determined.

Determination of the rates of synthesis and degradation of catalase. 3-Amino-1,2,4-triazole (AT, Tokyo Kasei Co., Tokyo, Japan) causes a rapid decrease in hepatic catalase to 5 per cent of the control resulting from the formation of an irreversible complex with the enzyme, however, does not influence the synthesis of the enzyme [14]. Aminotriazole was dissolved in 0.9 per cent saline, and injected intraperitoneally at a dose of 100 mg/100 g body weight into control rats (35 animals) or rats treated with LK-903 for 14 days (35 animals). The administration of LK-903 was continued after the injection of AT. At 2, 8, 10, 12, 24, 36 and 48 hr after the injection, the rats were killed, the livers were isolated, and restored catalase activities in the homogenate, the large granule fraction, and the supernatant were determined. After graphing the semilogarithmic plots of the differences between normal level and the catalase level in AT-treated animals against duration after the treatment, the degradation-rate (K_d) of catalase was obtained from a straight slope of the semilogarithmic plots. The rate of synthesis (K_s) of the activity can be calculated from a theoretical curve according to the following relationship [14] by using experimental data

$$C_t = \frac{K_s}{K_d}(1 - \exp^{-K_d \cdot t})$$

C_t: the activity after t hr of the AT-administration.

Additionally, the half-life time ($T_{1/2}$) of catalase was calculated from the relationship $K_d = \ln 2/T_{1/2}$ [Ref. 14].

Assays of enzyme activities and protein content. Cyanide-insensitive fatty acyl-CoA oxidizing activity was determined by measuring the palmitoyl-CoA-dependent reduction of NAD^+ spectrophotometrically at 340 nm, as described by Lazarow and De Duve [1] with a slight modification. On the basis of the results of Mannaerts *et al.* [15], bovine serum albumin and Triton X-100 were omitted from the determination system of Lazarow and De Duve [1]. The incubation mixture contained, in a total volume of 1.0 ml, the following constituents, 30 mM potassium phosphate buffer (pH 7.4), 0.2 mM NAD^+, 50 μM CoA, 6 mM dithiothreitol, 1 mM KCN, 14 μM palmitoyl-CoA, and various concentrations of enzyme preparation (0.1 ml). Mitochondrial β-oxidation could not be detected under the above conditions because of the presence of KCN [1, 15]. One unit of activity was defined as the amount of enzyme that reduced 1 μM of NAD^+/min.

The activities of catalase and acid phosphatase were also assayed according to our previous report [17]. Cytochrome C oxidase activity was determined by the method of Wharton and Tzagoloff [18] with a slight modification. The incubation mixture (0.9 ml) contained 32 nmol of cytochrome C (reduced by sodium ascorbate) in 7 μmol of potassium phosphate buffer (pH 7.0). The reaction was initiated by the addition of the enzyme preparation (0.1 ml) previously solubilized with 0.4 per cent Triton X-100, and was carried out at 25°. The blank cuvette was oxidized with 1 μmol of potassium fericyanide. Protein was determined according to the method of Lowry *et al.* [19].

RESULTS

Effect of LK-903 *on the activities of liver peroxisomal enzymes*

Table 1 shows the total activities of peroxisomal enzymes of rats which had received LK-903 for 14

Table 1. Changes in various enzyme activities in hepatic peroxisomes of rats given LK-903*

Enzyme	Control	LK-903	$\left(\frac{\text{LK-903}}{\text{Control}}\right)$
Catalase	10,844 ± 644	22,142 ± 2085	(2.04)
Urate oxidase	2.42 ± 0.19	5.07 ± 0.45	(2.10)
D-Amino acid oxidase	1.66 ± 0.30	4.14 ± 0.31	(2.49)
Fatty acid oxidation	23.79 ± 4.14	167.50 ± 34.30	(7.04)
Protein (mg/g liver)	196.9 ± 4.9	192.4 ± 32.0	(0.98)

Rats were given LK-903 for 2 weeks.
Data are represented as units/g liver.
* Values are the means ± S.D. of five rats.

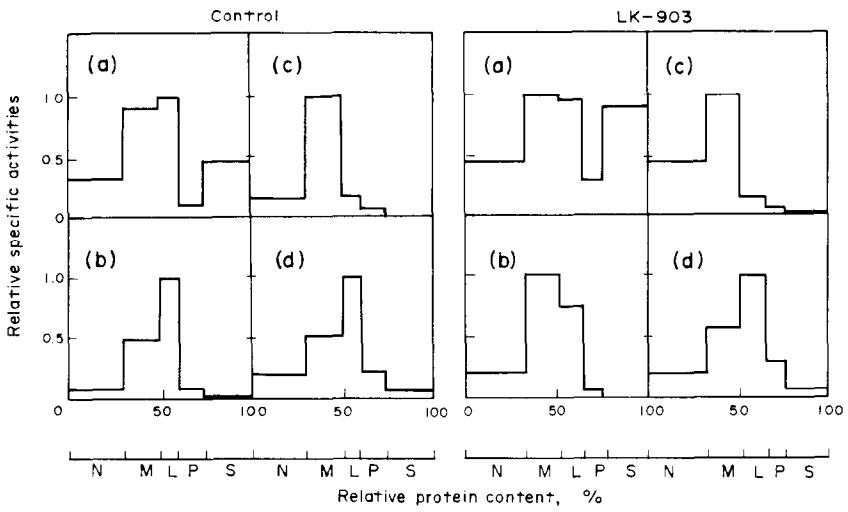

Fig. 2. Subcellular distributions of peroxisomal enzyme activities of the livers from control and LK-903-treated rats. Homogenates were fractionated according to the method of De Duve *et al.* [13]. The ordinate represents relative specific activities to the maximum fraction. The abscissas indicate relative cumulative protein contents; N, M, L, P, and S represent nuclear, heavy mitochondrial, light mitochindrial, microsomal fractions and supernatant, respectively. (a) Catalase, (b) urate oxidase, (c) cytochrome C oxidase, (d) acid phosphatase. Data are means of three experiments.

days. Catalase activity increased 2.04-fold, which is similar to the increases seen upon treatment with clofibrate [6–8], DEHP [4] and acetylsalicylate [3]. D-Amino acid oxidase activity was also enhanced approximately 2.5-fold by LK-903, and this result is quite different from those obtained after the above treatments. Urate oxidase activity of the treated rat liver was 2 times higher than the control. We attempted to determine the activity of the fatty acyl-CoA oxidizing system of peroxisomes in liver homogenate, but it was not possible due to the turbidity. Therefore the activity in Table 1 is only that in the large granule fraction, in which peroxisomes are almost yielded. The activity of the fatty acyl-CoA oxidizing system in liver peroxisomes increased 7-fold, which is similar to the effects of treatment with the above hypolipidemical chemicals. As shown in Table 1, LK-903 enhanced the activities of all the peroxisomal enzymes determined.

Subcellular distribution of peroxisomal enzymes after treatment with LK-903

Figure 2 shows the results of cell fractionation. The highest activities of peroxisomal enzymes were found in the heavy mitochondrial fraction (27.2 per cent for catalase, 49.8 per cent for urate oxidase), in which cytochrome C oxidase activity was also concentrated. The results for peroxisomal enzymes

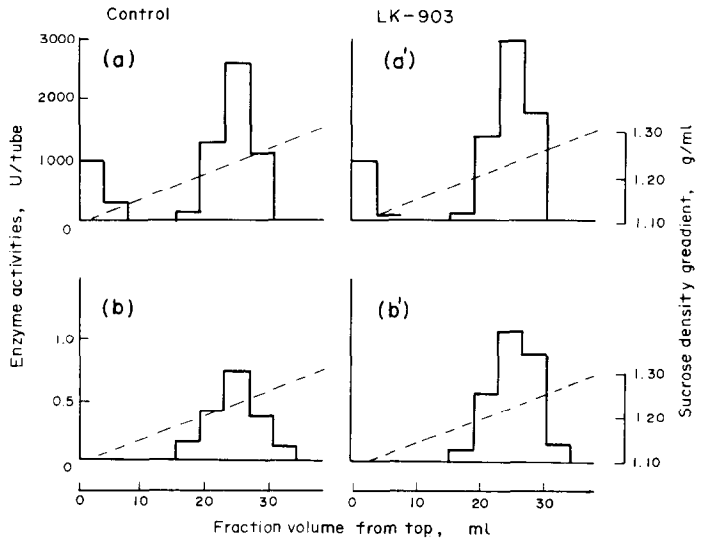

Fig. 3. Sucrose density gradient centrifugation patterns of peroxisomal enzymes. The procedure is described in Materials and Methods. (a) and (a') catalase; (b) and (b') urate oxidase.

of treated liver are very distinct from those for control liver, the highest of which were found in the light mitochondrial fraction. If the density and friction coefficient in sedimentation of the peroxisomes are not greatly altered by the administration of LK-903, the peroxisomes found in the heavy mitochondrial fraction possibly become larger than the normal peroxisomes. Marker enzyme activities other than those of peroxisomes in LK-903-treated rats, and all enzyme activities in control rats appeared in the expected fractions [5, 6, 13], indicating that the present centrifugal procedures were carried out correctly. Significant activities of peroxisomal enzymes were also found in the light mitochondrial fraction (25.6 per cent for catalase, 38.4 per cent for urate oxidase). The supernatant activity of catalase in treated rats was considerably higher than that in control rats.

Subfractionation by sucrose density gradient centrifugation

Figure 3 shows the patterns of catalase and urate oxidase after sucrose density gradient centrifugation. More than 70 per cent of the total activities applied appeared in fractions No. 7 (d = 1.22–1.25) and No. 8 (d = 1.25–1.27). Similar results were obtained for the control rats. Catalase activities in both control and LK-903-treated rats were also found significantly in the first fraction (14.7 and 13.8 per cent, respectively).

Changes in peroxisomal enzyme activities during treatment with LK-903 *and after the cessation of the treatment*

The rats were treated with LK-903 for 2 weeks, then received normal chow without LK-903 for an additional 1 week. Figure 4 shows the changes in the activities of urate oxidase, catalase and the fatty acyl-CoA oxidizing system during the experiment. The data are specific activities in the large granule fraction, supernatant, and the homogenate. The activity of catalase in the large granule fraction gradually increased by the administration up to the 8th day, and rose rapidly during the 8th to 10th days. Even after removing LK-903 at 14th day, the activity of catalase, however, remained almost unchanged for at least 7 days. The supernatant activity of catalase also increased by the treatment, but in a different manner: that is, the activity on the 2nd, 6th and 14th days fell lower than the ones on the respective last days. The activity in supernatant after the cessation of LK-903 fell very rapidly, but then tended to slightly increase again.

Urate oxidase activity in the large granule fraction increased gradually and reached a plateau by the 10th day of the treatment, and no activity was found in the supernatant. The activity in the large granule fraction after the cessation decreased gradually, and approached to the normal value by 5 days of the cessation.

The activity of the fatty acyl-CoA oxidizing system rose suddenly on the 8th day after the administration, then decreased and appeared to reach a plateau by the 14th day. The activity decreased once at 3rd day after removing LK-903, but the activities at 5th and 7th day were almost restored to those at the plateau.

Studies on the kinetics of liver peroxisome proliferation induced by LK-903

In order to determine whether the peroxisome proliferation induced by LK-903 is a result of changes of the degradation rate or the synthesis rate, or both, these changes were evaluated by using catalase activity in the large granule fraction as a marker of peroxisomes. After the administration of aminotriazol, the reappearance of catalase activity is due to *de novo* synthesis of the enzyme. The restored catalase activities were plotted semilogarithmically as the differences between the normal level and the catalase level in the aminotriazole-treated animals (data not shown). The rates of synthesis (K_s) and degradation (K_d) of hepatic catalase were determined by the

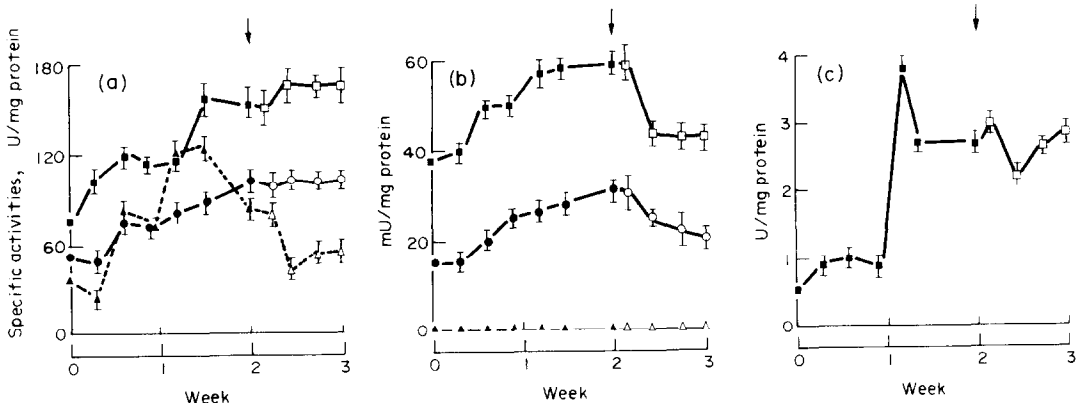

Fig. 4. Changes in peroxisomal enzyme activities during treatment with LK-903 and after cessation of the treatment. Rats were fed *ad lib.* on chow containing 0.05 per cent LK-903 for 2 weeks, and then received normal chow without LK-903. Peroxisomal enzyme activities in liver preparations were determined and are expressed as specific activities. The arrows in the figure mean changeover from LK-903-diet to normal diet. Values are the means of five experiments, with the standard deviations. (a) Catalase, (b) urate oxidase, (c) fatty acyl-CoA oxidizing system. (●)(○) Homogenate, (■)(□) large granule fraction, (▲)(△) supernatant.

Table 2. Turnover parameters for catalase in normal and LK-903-treated rats

	Rate of degradation (K_d)	Rate of synthesis (K_s)	Half-life ($T_{1/2}$)
	Per cent/hr	U/hr	hr
Homogenate			
Control	2.18	158.84	31.79
Treated	1.90	342.90	36.45
Large granule fr.			
Control	2.26	65.02	30.62
Treated	2.39	172.53	29.03
Supernatant			
Control	2.08	37.71	33.30
Treated	1.84	89.05	38.40

3-Amino-1,2,4-triazole was dissolved in 0.9 per cent saline, and injected intraperitoneally at a dose of 100 mg/100 g body weight into control and LK-903-treated rats for 14 days, as described in Materials and Methods. K_s values are expressed as units synthesized per hr, and K_d values as per cent degraded per hr.

method of Price *et al.* [14]. The values for catalase turnover are summarized in Table 2. The K_d of peroxisomes (the large granule fraction) in the treated rats was calculated to be 2.39 per cent/hr, which is almost the same as that of control rats and the value reported by Price *et al.* [14]. The K_d values of the homogenate and the supernatant in control and treated rats were all approximately 2 per cent/hr. The ratio of K_d of the treated rats to that of control rats was in the range of 0.87–1.06. Similarly, the half-life in the treated rats was similar to that in the control rats (about 33 hr). On the other hand, the synthesis rate (K_s) in the treated rats was 2 times or more higher than that in control rats in the supernatant, the large granule fraction and the homogenate. The ratio of the treated rats to control rats in the large granule fraction was especially higher (2.65).

DISCUSSION

Several drugs or chemicals affecting the peroxisomes have been reported. They are clofibrate [6, 7], acetylsalicylate [3], DEHP [4], etofibrate [20], 2-(4-debenzofuranyloxy)-2-methyl propionic acid [21], nafenopin [22, 23], [4-chloro-6-(2,3)-xylidino-2-pyrimidinylythio] acetic acid [24], and 2-chloro-5-(3,5-dimethylpiperidinosulfonyl) benzoic acid [24]. Among them, etofibrate and nafenopin are derivates of clofibrate. The effects of clofibrate on peroxisomes seem to be representative of those of all the above drugs, and are as follows: (1) increase of peroxisomal catalase activity; (2) increase of the activity of the fatty acyl-CoA oxidizing system; (3) increase of the number of peroxisomes; (4) decrease of D-amino acid oxidase activity; and (5) slight decrease or no change of urate oxidase activity. In the present experiment, the effects of LK-903 on the liver peroxisomes were quite different from the above effects. LK-903 enhanced not only the activities of catalase and the fatty acyl-CoA oxidizing system, but also the activities of D-amino acid oxidase and urate oxidase. This is the first report of an agent having such effects. Considered from the results that the altered peroxisomes were sedimented somewhat faster than normal peroxisomes and further its density did not change as shown in Figs. 2 and 3, the size of the peroxisomes after treatment with LK-903 appears to be somewhat larger than normal, or the treatment may produce new characteristics in the peroxisomes, causing them to aggregate more readily than normal peroxisomes, and consequently causing them to appear in the heavy mitochondrial fraction. The former assumption is supported by the results obtained with nafenopin [23], [4-chloro-6-(2,3)-xylidino-2-pyrimidinylthio]acetic acid [24], and 2-chloro-5-(3,5-dimethylpiperidinosulfonyl) benzoic acid [24], while no data are available to support the latter possibility. With the density of the particles, LK-903-administration did not alter the density of peroxisomes (Fig. 3), as well as the densities of lysosomes and mitochondria, using acid phosphatase and cytochrome C oxidase as the respective marker (data not shown). The increases of catalase and urate oxidase activities by LK-903 occurred gradually, and reached plateau levels by the 10th day, while the activity increase of the fatty acyl-CoA oxidizing system occurred abruptly on the 8th day. The activity of a rate-limiting enzyme present in this system may have increased at that time. The enhanced activities of peroxisomal catalase and the fatty acyl-CoA oxidizing system were well maintained after cessation of LK-903 treatment, while with urate oxidase, the activity decreased immediately after the cessation of treatment, and then increased again. On the basis of previous reports by Lazarow and De Duve [25], and Leighton *et al.* [22], which indicate that catalase is present only in the peroxisomes, the supernatant catalase is considered to represent enzyme that has leaked from peroxisomes, and/or intermediates in the synthesis and degradation of peroxisomes. Consequently, the maintenance of high catalase activity in the peroxisomes after cessation of LK-903 treatment may be associated in some way with the supernatant enzymes. The enhancement of peroxisomal catalase activity by LK-903 appears to be dependent on an increase of the synthesis rate; the degradation rate was unaffected. However, the results for peroxisomal catalase in the present experiments may not necessarily reflect those for whole peroxisome particles.

The present results can be summarized as follows. When LK-903 was administered to rats, the activities of all hepatic peroxisomal enzymes increased significantly. Traditional peroxisome-proliferating drugs, such as clofibrate, DEHP, and others enhance the activities of catalase and the fatty acyl-CoA oxidizing system, while they reduce other peroxisomal enzyme activities. However, LK-903 increased the activities of all peroxisomal enzymes determined, so that its use as a peroxisome-proliferating drug should permit more effective studies of the physiological role of peroxisomes.

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