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Studies on New Nicotinic Acid Ester Derivatives. VII.[1] Effect of 2,2,6,6-Tetrakis(nicotinoyloxymethyl)cyclohexanol (K-31) on Excretion and Biosynthesis of Cholesterol in Rats

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K-31, a proved hypocholesterolemic agent, was studied about its effect on the metabolism as well as biosynthesis of cholesterol in rats using ^{14}C-labeled cholesterol and acetate. K-31 increased the recoveries of ^{14}C in the 2nd day's bile and feces in rats injected [4-^{14}C] cholesterol. Addition of K-31 as hydrochloric acid salt solution to the liver homogenates from normal rats did not affect the incorporation of [1-^{14}C] acetate into cholesterol. However, increased cholesterogenesis was observed in the liver homogenates from rats fed 0.5% K-31 in diet for 21 days.

K-31 significantly enhanced hepatic cholesterogenesis in intact rats. Increased hepatic-cholesterogenesis restored to control levels by the withdrawal of the feeding of K-31. Thus, the hypocholesterolemic action of K-31 was considered to depend on the inhibition of the intestinal cholesterol absorption with increasing excretion of cholestrol into bile and feces.

Among various nicotinic acid ester derivatives, it was found that 2,2,6,6-tetrakis(nicotinoyloxymethyl)cyclohexanol (K-31) had a potent hypocholesterolemic action in experimental hypercholesterolemic mice.[3] In the previous paper,[1] it was shown that K-31 markedly suppressed the elevation of serum cholesterol and other lipids in cholesterol fed rabbits and significantly reduced the absorption of [4-^{14}C]cholesterol into lymph in thoracic duct fistula rats. The present paper describes the influences of K-31 on the excretion and the biosynthesis of cholesterol in rats.

Material and Method

Animals——Male Wistar strain rats were used for all the experiments and were fed a commercial rat chow (CE-2; Nippon Clea Co., Ltd.).

Materials——K-31 was synthesized in Kyorin Chemical Laboratory and its structure is shown in Fig. 1.

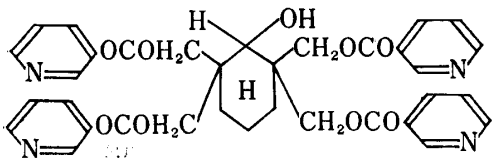

Fig. 1. Structure of K-31

Physical and chemical properties of K-31 were described in the previous paper.[3] [4-^{14}C] Cholesterol and [1-^{14}C] acetate were obtained from Daiichi Pure Chemical Co., Ltd., PPO (2,5-diphenyloxazole) and dimethyl-POPOP (1,4-bis-2-(4-methyl-5-phenyloxazole)benzene) from Packard Instrument Company, Inc., and HCO-120 from Nikko Chemical Co., Ltd. Digitonin was obtained from E. Merck, A.G., and NAD was obtained from C.F. Boehringer and Soehne GmbH.

Measurement of Biliary Excretion of [4-^{14}C] Cholesterol in Bile Duct Fistula Rats——Rats weighing about 160 g were used. Normal control group was fed CE-2 for 14 days, while K-31 treated group was fed 0.5% K-31 in CE-2 for the same days. On the 14th day, [4-^{14}C]cholesterol in 1 ml of 3% HCO-120 was injected into each tail vein of the rat 1 hr after the establishment of bile fistula cannulation under light ether anesthesia.[4] Just before the injection of labeled cholestrol, the K-31 treated group was orally administrated

1) Part VI: Y. Aso, Y. Abe, K. Higo, T. Naruke, and T. Irikura, *J. Atheroscler. Res.*, **10**, 391 (1969).
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3) T. Irikura, Y. Abe, and K. Higo, *Pharmacometrics (Japan)*, **2**, 237 (1968).
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300mg/kg of K-31 suspended in water. Collected bile was extracted with 5 volumes of boiling ethanol and washed 3 times with hot ethanol, and the filtrates were combined. After the evaporation under reduced pressure, residual product was hydrolyzed in 5 ml of 5N NaOH at 120° for 7 hr. The hydrolysates were extracted 3 times with 20 ml of petroleum ether (cholesterol fraction). The aqueous layer, after acidification to pH 2 by adding conc. HCl, was subjected to the successive extraction with petroleum ether (fatty acid fraction), with diethyl ether (bile acid fraction) and with *n*-butanol (acidic *n*-butanol fraction), respectively. The radioactivity of ^{14}C in each fraction was determined with a liquid scintillation counter (Beckman Model LS-100) by adding toluene[1] or dioxane scintillator to the solution. The composition of dioxane scintillator was: dioxane 1000 ml, 5 g of PPO and 100 g of naphthalene. Counting efficiency for each sample was determined by the external standard quench correction method. The dose of K-31 presented as mean mg/kg/day by calculating from consumed diet. Statistical comparisons were carried out with Student's *t* test.

Measurement of Fecal Excretion of [4-^{14}C] Cholestrol in Intact Rats——Rats weighing about 180 g were used. Normal control group was fed CE-2 for 14 days, while K-31 treated group was fed CE-2 containing 0.5% K-31 for the same days. On the 14th day, [4-^{14}C] cholesterol was injected intravenously to each of the rats. Just before the injection, K-31 treated group was orally given 300 mg/kg of K-31 suspended in water. Feces were daily collected for 4 days, extracted and separated to the fractions by the aforesaid method. Further, on the 4th day after the injection of [4-^{14}C] cholesterol, rats were killed and the liver was excised and minced. Minced liver was hydrolyzed by boiling with alcoholic KOH (10% KOH in 90% ethanol) for 3 hr under reflux. After the evaporation, hydrolysates were dissolved in 50% ethanol and extracted by the aforesaid method to separate into the fractions. The radioactivity of ^{14}C in each fraction was determined as previously shown.

Measurement of Incorporation of [1-^{14}C] Acetate into Cholresterol in Liver Homogenate——Liver homogenates were prepared from livers of normal rats or rats fed 0.5% K-31 for 21 days, both weighing about 250 g, according to Frantz[5] and Ozawa.[6] Excised livers were washed with 1.15% KCl and then minced. Minced livers were ground in a very loose-fitting chilled glass homogenizer containing 2.5 volumes of suspending medium. The suspending medium was composed of potassium phosphate buffer pH 7.4, 0.1M; nicotinamide, 37.5 mM; $MgCl_2 \cdot 6H_2O$, 6 mM in final concentration. The incubation mixture contained 8 ml of the supernatant of the homogenate, 1 ml of NAD (0.8 mM in final concentration) and 1 ml of sodium [1-^{14}C]acetate (13 mM in final concentration). An aliquot of the supernatant of the homogenate was measured by the Folin's method. K-31 aqueous solution as HCl salt was added to the incubation mixture prepared from normal rat liver. Incubation was carried out under aeration of 100% O_2 at 37° for 90 min. After the incubation, 50 ml of ethanol and 6 g of solid KOH were added to each incubation mixture. The nonsaponifiable substances were extracted with petroleum ether after alkaline hydrolysis under reflux for 3 hr and precipitated as the digitonide.[7] The digitonide was dissolved in pyridine.[8] The petroleum ether soluble material extracted from the hydrolysate after acidification constituted the fatty acid fraction.

Measurement of Incorporation of [1-^{14}C]Acetate into Liver or Intestinal Cholesterol in Intact Rats——Rats weighing 120—130 g were fed CE-2 for 12 days. K-31 treated group was fed 0.5% K-31 in CE-2 for the same days. On the 21st day, [1-^{14}C]acetate (20 μCi/20 μmole in 1 ml saline) was injected into each tail vein. The K-31 treated group was given oral K-31 (300 mg/kg) suspended in water 5 hr before the injection. Each injected rat was placed in a metabolic chamber. Rats were all sacrificed 1 hr after the injection, and the liver and the intestine were immediately excised and subjected to the analysis. Further, 92 rats weighing 140—150 g were used to examine the effects of K-31 on the hepatic cholesterolgenesis. K-31 treated group was fed 0.5% K-31 in CE-2 for 36 days. A part of K-31 treated group were withdrawn the CE-2 containing 0.5% K-31 at the 21st day of feeding and fed only with CE-2 for a further 28 days.

Result

Effect on the Biliary Excretion of [4-^{14}C]Cholesterol in Bile Duct Fistula Rats

As shown in Table I, there was no significant difference between the K-31 treated group and the normal control group in the volume of bile, the total activity and distribution pattern of ^{14}C in the bile samples collected for the first 24 hr. In the 2nd day's bile samples, however, volume of bile and the total activity of the K-31 treated group was significantly higher than those of the normal control group. Also the % distribution in fatty acid fraction was significantly higher in the rats treated with K-31.

5) I.D. Frantz, Jr. and N.L.R. Bucher, *J. Biol. Chem.*, **206**, 471 (1954).
6) H. Ozawa and H. Ichikawa, *Yakugaku Zasshi*, **90**, 143 (1970).
7) T. Gerson, *Biochem. J.*, **77**, 446 (1960).
8) J.M. Dietschy and M.D. Siperstein, *J. Lipid. Res.*, **8**, 97 (1967).

Table I. Effect of K-31 on Biliary Metabolism of Labeled Cholesterol injected intravenously to Bile Duct Fistula Rats

Group	No. of animals	Final[a] body weight (g)	Bile collection time (hr)	Bile volume (ml)	Total recovery in bile (%)
Control	5	172±3.1	0—24	8.6±0.86	12.2±1.32
			24—48	5.6±0.21	8.8±0.79
K-31	4	179±6.5	0—24	9.2±1.20	13.7±0.88
			24—48	7.4±0.23[b]	12.7±1.29[c]

% Distribution of ^{14}C in each fraction of bile			
Cholesterol fraction	Fatty acid fraction	Bile acid fraction	*n*-Butanol fraction
13.6±2.21	2.3±0.57	58.8±1.01	25.2±1.47
6.1±0.26	3.4±0.45	62.7±5.69	27.9±5.14
12.8±1.62	1.5±0.26	64.9±3.30	20.9±2.50
5.3±0.92	1.2±0.41[c]	76.9±5.13	16.5±4.17

K-31 treated group was fed 0.5% K-31 in CE-2 for 14 days (491 mg/kg/day), and received 300 mg/kg of K-31 orally before the injection of labeled cholesterol. Values represent mean±standard error. *a*) body weight after 14 days of feeding *b*) significantly different from control ($p < 0.01$) *c*) significantly different from control ($p < 0.05$)

Effect on the Fecal Excretion of [4-^{14}C]Cholesterol in Intact Rats

As shown in Table II, there was no significant difference between the K-31 group and the normal control group in the total activity and the distribution pattern in the feces collected for the first day. Significant difference, however, was observed in the 2nd day's feces. Weight of the feces and the total activity of the K-31 group was significantly higher than those of

Table II. Effect of K-31 on Fecal Excretion of Labeled Cholesterol injected to Intact Rats

Group	No. of animals	Final[a] body weight (g)	Feces collection time (day)	Dried feces (g/rat/day)	Total recovery in feces (%)
Control	9	230±5.9	1st	2.09±0.24	3.1±0.19
			2nd	3.19±0.57	6.6±0.61
			3rd	3.95±0.45	7.7±1.00
			4th	3.93±0.20	6.2±0.57
K-31	9	230±4.5	1st	2.10±0.37	3.1±0.38
			2nd	4.95±0.28[b]	8.7±0.49[b]
			3rd	4.49±0.69	6.4±0.70
			4th	4.66±0.33	6.9±0.51

	% Distribution of ^{14}C in each fraction of feces			
	Cholesterol fraction	Fatty acid fraction	Bile acid fraction	*n*-Butanol fraction
(1st)	10.9±1.25	10.7±1.29	72.0±0.93	6.4±0.87
(2nd)	20.2±2.85	16.9±2.29	52.9±2.98	9.9±1.79
(1st)	11.0±2.21	12.6±0.72	67.9±2.66	8.4±0.31
(2nd)	21.2±2.90	14.3±1.34	54.9±1.75	9.7±0.95

K-31 treated group was fed 0.5% K-31 in CE-2 for 14 days (467 mg/kg/day). Values represent mean ± standard error.
a) body weight after 14 days of feeding *b*) significantly different from control ($p < 0.05$)

Table III. Distribution of Labeled Compounds in the Liver 4 Days after Intravenous Injection of [4-^{14}C] Cholesterol

Group	% distribution of ^{14}C in each fraction			
	Cholesterol fraction	Fatty acid fraction	Bile acid fraction	*n*-Butanol fraction
Control	87.5±1.31	3.9±0.75	1.3±0.31	7.5±0.48
K-31	85.7±1.22	4.5±0.79	1.1±0.19	8.7±0.58

K-31 group was fed 0.5% K-31 in CE-2 for 14 days. Values represent mean ± standard error.

the normal control group. In Table III, the distribution pattern of the activity in the liver was shown for each group, but there was no difference between both groups.

Effect on the Incorporation of [1-^{14}C]Acetate into Liver Cholesterol *in Vitro*

As shown in Table IV, the addition of K-31 HCl salts (5×10^{-4}M) to normal rat liver homogenate did not affect the cholesterol synthesis from [1-^{14}C]acetate. However, liver homogenates from rats fed 0.5% K-31 for 21 days synthesized significantly more cholesterol from acetate than did homogenates from the normal control rats (Table V).

Table IV. Effect of K-31 on Cholesterol Synthesis from [1-^{14}C]Acetate in Liver Homogenate prepared from Control Rats

Concentration of K-31 ($\times10^{-4}$M)	Total counts of cholesterol ($\times10^4$ dpm/100mg protein)
0	1.43±0.006
0.2	1.42±0.021
1	1.41±0.043
5	1.51±0.027

K-31 was added to the medium as HCl salt solution. values represent mean ± standard error.

Table V. Incorporation of [1-^{14}C]Acetate into Cholesterol by Liver Homogenate prepared from K-31 Treated Rats

	No. of animals	Final[a] body weight (g)	Wet liver weight (g)	Total counts of cholesterol ($\times10^3$ dpm/100 mg protein)
Control	8	266±5.8	11.78±0.39	3.16±0.59
K-31	7	264±5.0	11.59±0.45	6.46±0.57[b]

K-31 treated rats were fed 0.5% K-31 in CE-2 for 21 days (460 mg/kg/day) before application to the liver homogenates preparation.
a) body weight after 21 days of feeding *b*) significantly different from control ($p<0.01$)

Effect on the Incorporation of [1-^{14}C]Acetate into Cholesterol in Intact Rats

K-31 had no significant effect on the synthesis of cholesterol and fatty acids in intestine. Also, there was no significant difference in the incorporation of acetate into hepatic fatty acid between control and K-31 treated group. K-31, however, significantly increased hepatic cholesterogenesis than control (Table VI). The effect of K-31 on the hepatic cholesterogenesis was shown in Fig. 2. K-31 increased the hepatic cholesterogenesis on the 7th day of continuous feeding of this compound, and significant difference was noted on the 14th and also 36th day of the feeding. Increased hepatic cholesterogenesis in rats by continuous feeding of K-31

for 21 days restored to an almost control level after the administration of K-31 was withdrawn. In these experiments, however, there was a wide variation in the hepatic cholesterogenesis in the normal control group.

Table VI. Effect of K-31 on the Incorporation of [1-^{14}C]Acetate into Cholesterol in Rat Liver and Intestine

Group	No. of animals	Final[a] body weight (g)	Tissue	Wet weight (g)	Total counts ($\times 10^{3}$) dpm)	
					Cholesterol	Fatty acid
Control	6	207±9.9	liver	7.85±0.46	1.53±0.27	7.05±0.95
			intestine	12.35±0.48	3.89±0.38	18.3±1.35
K-31	5	190±8.9	liver	7.32±0.55	2.60±0.36[b]	10.6±2.18
			intestine	12.86±0.93	4.21±0.55	20.6±1.93

K-31 treated group was fed 0.5% K-31 in CE-2 for 21 days (496 mg/kg/day), and received 300 mg/kg of K-31 orally before the injection of labeled acetate. Values represent mean ± standard error
a) body weight after 21 days of feeding *b*) significantly different from control ($p<0.05$)

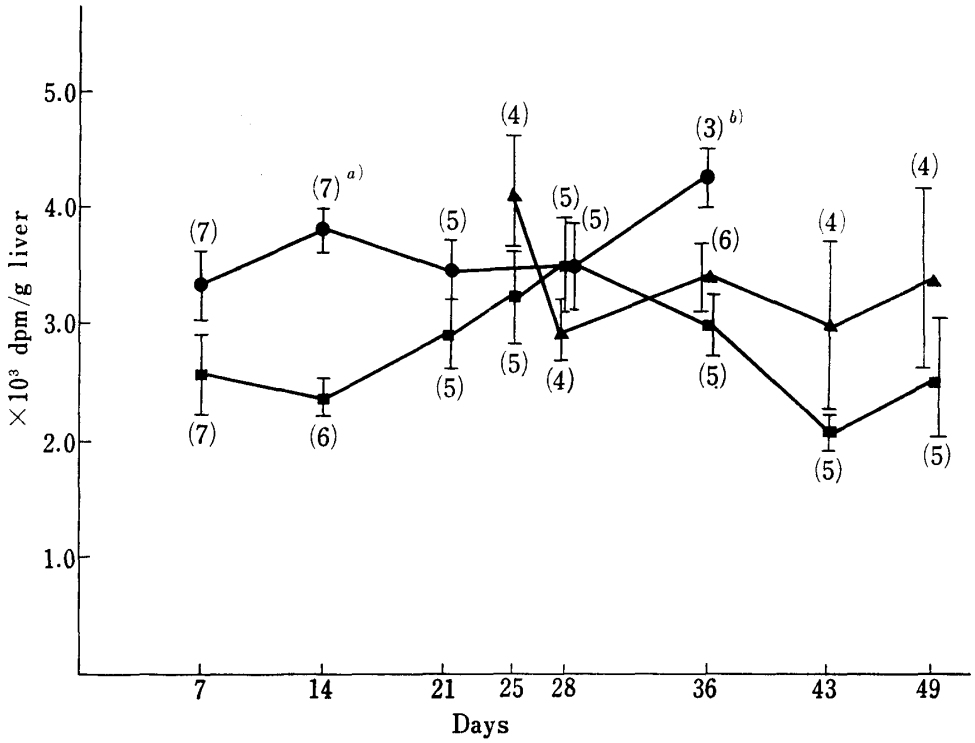

Fig. 2. Influence of K-31 on Hepatic Cholesterogenesis in Intact Rats
Numbers in parentheses indicate number of rats, total number of rats is 92.
Each bar represents mean ± standard error.
■—■, control; ●—●, continuous feeding of K-31; ▲—▲, 21 days of K-31 feeding and then ceased
a),*b*) significantly different from control ($p<0.05$).

Discussion and Conclusion

In the first paper of this series,[3] 2,2,6,6-tetrakis(nicotinoyloxymethyl)cyclohexanol (K-31) was shown to have significant lowering effects on serum total cholesterol in experimental hyperlipemic mice. Recently, it was found that K-31 had a suppressing effect on the elevation of serum total cholesterol, triglycerides and phospholipid levels as well as lowering

effect on the hepatic total cholesterol in the regressive study, and also was shown that K-31 had a prophylactic action on experimental hypercholesterolemia in rabbits in the progressive study.[1)]

Serum hypocholesterolemic agents may act by one or more of the following mechanisms: inhibition of intestinal absorption or reabsorption of cholesterol, depression of cholesterol biosynthesis, increased cholesterol degradation and excretion, and redistribution of cholesterol from blood to tissues. The hypocholesterolemic action of K-31 was considered to depend mostly on the inhibition of intestinal cholesterol absorption.[1)] In order to confirm the mechanism of the action of K-31, this study was undertaken.

Most of the activity of intravenously injected [4-^{14}C]cholesterol was recovered in bile[9)] and about 60% of ^{14}C was present in the form of bile acids.[10)] The distribution of ^{14}C in various lipid fractions in the feces resembled that observed for bile, but somewhat more activity was recovered in the nonsaponificable fraction in feces, which represented the contribution of ^{14}C from the intestinal wall.[11)]

In this paper, the total recovered activity in the 2nd day's, bile was significantly higher in the rats fed K-31 than in the control animals, and higher activity in the 2nd day's feces was also observed. Thyroxine and its analogues were reported to enhance the excretion of bile acid resulting from increased chenodeoxycholic acid.[12)] Anabolic steroids increased the excretion of bile acid and sterols without influencing on the volume of excreted bile.[13)] Unsaturated fatty acids were reported to increased cholesterol degradation and excretion.[14)] The increased total radioactivities in bile and feces of the K-31 treated group seem to be resulted from the increased volume of the bile without modifying the excretion pattern of each fraction.

Most of animals are capable of maintaining the cholesterol in plasma relatively at a constant level in spite of wide variations in dietary cholesterol. Hepatic cholesterogenesis is affected by cholesterol feeding or fasting. Synthesis of cholesterol from acetate is thought to be inhibited by dietary cholesterol not at the conversion of mevalonic acid to cholesterol but at the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid.[8,15)] Intestinal cholesterogenesis, however, depends on the concentration of bile acid.[16)]

Addition of K-31 HCl salt to normal rat liver homogenates did not affect the incorporation of [1-^{14}C]acetate into cholesterol. On the other hand, cholesterol synthesis was stimulated in the liver homogenates from the rats fed K-31 for a long time. Also in the intact animals, hepatic cholesterogenesis was increased, but the acetate incorporation into hepatic fatty acids was not influenced by the feeding of K-31. K-31 had not significant effect on the intestinal cholesterogenesis. In the long term feeding of K-31, increased hepatic cholesterogenesis was observed, too. Withdrawal of the feeding of K-31 lowered the increased hepatic cholesterogenesis to the levels of control rats. In the present study, however, wide variations of hepatic cholesterogenesis in control rats was noted. Hepatic cholesterogenesis in normal rats might change with their ages on the day of the determination.

Some serum hypocholesterolemic agents were reported to have an accompanying stimul-

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ative effect on the hepatic cholesterogenesis.[17] In the previous experiments,[1] K-31 significantly depressed the absorption of [4-^{14}C]cholesterol into lymph. Stimulated hepatic cholesterogenesis in K-31 fed rats might be related to its inhibitory action on the absorption of cholesterol. The hypocholesterolemic action of K-31 in experimentally hypercholesterolemic animals would depend on the inhibition of the intestinal cholesterol absorption with increasing excretion of cholesterol into bile and feces.

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