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Hypocholesterolemic Activity of *N*-(Ferrocenylmethyl)piperidine (33743)

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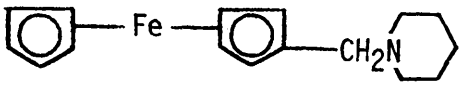
The search for new hypocholesterolemic agents has resulted in the discovery of many active compounds in the last few years. It is highly probable that the majority of these affect cholesterol biosynthesis in some way. In particular, several have been reported which inhibit one of the later reactions in the sequence (1–3).

The purpose of the present paper is to report the investigation of a member of a series of new hypocholesterolemic agents having ferrocene as a component of their structure. The results of this study show that this compound, *N*-(ferrocenylmethyl)piperidine,



causes abnormal quantities of a compound resembling 7-dehydrocholesterol to acumulate in serum and liver, and probably acts by inhibiting 7-dehydrocholesterol reductase. Very little work has been reported on the pharmacological activity of ferrocene compounds, although it has been suggested that ferrocene or ferrocene derivatives could be used as hematinic agents (4, 5).

Experimental Methods. Animal studies. The chemical was adsorbed on silica gel from an acetone solution and mixed with ground rodent mash on a roller mill to give a concentration of 0.007%. Groups of six male weanling rats (Harlan Wistar strain, Harlan Industries, Cumberland, In.) were maintained on the experimental diet for 1 week, then killed and blood and liver samples were collected.

Analytical. A modification (6) of the method of Sperry and Webb (7) was used for the determination of "fast-acting" sterols and cholesterol. This method takes advantage of the fact that the so-called "fast-acting" sterols give full color development in 12 min at 0°, whereas cholesterol does not react at all in this time. After standing at 25° for an additional 30 min, the absorbancy is again determined. After a suitable correction for the contribution of "fast-acting" sterols to the latter reading, the amount of cholesterol can be determined. Materials absorbing at 281.5 mμ were determined spectrophotometrically by measuring the absorbancy in ethanol of nonsaponifiable material from serum and liver. Correction for extraneous absorption was made using a three-point procedure (8) with selected wavelengths of 276.5, 281.5, and 284.5 mμ. The reference material for "fast-acting" sterols and the 281.5 mμ determination was 7-dehydrocholesterol.

A spectrophotometric assay (9) was used to determine inhibition of the enzymatic reduction of 7-dehydrocholesterol *in vitro*. Each flask contained 13,000*g* rat liver supernate equivalent to 0.25 g of liver, 0.75 μmoles of 7-dehydrocholesterol, 1.7 μmoles of NADP, 30 μmoles of nicotinamide, 15 μmoles of glucose-6-phosphate, and 70 μmoles of Tris–maleate buffer, pH 7.2. The total volume was 1.0 ml and the incubations were carried out in duplicate at 37° for 2 hr under nitrogen.

TABLE I. Chromatographic Behavior of Isolated Sterol.

	Thin-layer chromatography R_f			Gas chromatography; relative retention time (cholestane = 1.00)
	I	II	III	
Cholesterol	.37			2.95
7-Dehydrocholesterol	.14	.38[a]	.51[a]	3.42
Isolated sterol	.15	.39[a]	.53[a]	3.40

[a] Small amount of fluorescent material which was not visible after spraying with Rhodamine 6G.

Isolation of 7-dehydrocholesterol. Digitonin-precipitable sterols were isolated from about 30 g of liver [from animals treated for 2 weeks with a diet containing 0.007% *N*-(ferrocenylmethyl)piperidine], and the digitonide was decomposed with pyridine (10). The resulting sterol mixture was chromatographed on a 1 mm thin-layer plate (Adsorbosil-1, Applied Science Labs.) impregnated with 20% silver nitrate and developed in 5:1 (v/v) benzene–ethyl acetate (11). Two major zones were discovered, cholesterol and a slower-moving zone. The latter was eluted from the adsorbent, reprecipitated with digitonin, and regenerated with pyridine. After recrystallization from methanol, a white crystalline product was obtained, mp 144–146° (uncorrected). The unknown compound was identified as 7-dehydrocholesterol by comparison of its infrared and ultraviolet spectra, melting point, and behavior in chromatographic systems with that of freshly recrystallized authentic material. Ultraviolet (absolute ethanol) and infrared (KBr pellet) spectra were determined on a Bausch and Lomb 505 recording spectrophotometer and a Perkin-Elmer 337 grating infrared spectrophotometer, respectively. Gas chromatographic analyses were performed on a Micro-Tek 2000R instrument equipped with a flame ionization detector and a 6 ft × ¼ in. glass column containing 1% QF-1 on 100-140 mesh Gas-Chrom P. Helium flow was 45 ml/min, column temperature was 195°, and the elution time for cholestane was 4.83 min.

Radiochemical studies. Incorporation of radioactive substrates was studied *in vivo* in male rats by giving an intravenous injection via a tail vein of 1.0 μCi of potassium mevalonate-2-^{14}C (1.58 μmoles/μCi; Nuclear-Chicago) in 0.5 ml of normal saline. After 2 hr the animals were stunned by a blow to the neck and rapidly exsanquinated. The liver was immediately homogenized in 25 vol of 1:1 (v/v) acetone–ethanol and suitable aliquots of the supernate were taken for determination of radioactivity in nonsaponifiable material, digitonin-precipitable material, and cholesterol. All radioactivity determinations were carried out in a Packard Tri-Carb liquid scintillation spectrometer using a toluene system containing PPO (5 g/liter) and dimethyl POPOP (0.1 g/liter). The nonsaponifiable extract was prepared by adding 2 vol of 5% (w/v) ethanolic KOH to an aliquot of the acetone–ethanol extract, heating at 60° for 1 hr, and extracting with petroleum ether after diluting the saponified mixture with an equal volume of water. The digitonide was prepared as described previously (7) and dissolved in 1.0 ml of NCS reagent (Nuclear-Chicago) after moistening with water. Total sterols were isolated from the digitonide by treatment with pyridine and extraction with ether. Cholesterol was isolated via the dibromide derivative (12).

Results and Discussion. Soon after activity was discovered in this new series of compounds, it became evident that significant amounts of a "fast-acting" sterol were present in the treated animals. A few milligrams of the unknown sterol were isolated by preparative thin-layer chromatography. The chromatographic behavior of this material was very similar to 7-dehydrocholesterol (Table I). 7-Dehydrocholesterol has an ultraviolet spectrum with absorption maxima at 271, 282, and 293 mμ which is typical of $\Delta^{5,7}$

TABLE II. Incorporation of Potassium Mevalonate-2-^{14}C into Liver Lipids in Rats.

Group	× 10^{3}[a]		
	Nonsaponifiable material (dpm/g of liver)	Digitonin-precipitable material (dpm/mg)	Cholesterol (dpm/mg)
Control	13.6 ± 1.8	5.23 ± 0.66	2.61 ± 0.55
Experimental	15.4 ± 2.7	5.08 ± 0.88	0.74 ± 0.59[b]

[a] Mean ± SD.

[b] Significantly lower than control $p < 0.01$.

unsaturation (13). The absorption spectrum of the unknown in the 250–300 mμ range was identical to that of 7-dehydrocholesterol. In addition, the infrared spectrum of the unknown was identical to that of 7-dehydrocholesterol. The results of studies of the incorporation of potassium mevalonate-2-^{14}C into liver lipids supported the suggestion that the latter stages of cholesterol biosynthesis were inhibited. Six male rats were treated with *N*-(ferrocenylmethyl)piperidine (0.007% in the diet) for 1 week and then given 1.0 μCi of potassium mevalonate intravenously. A group of control animals was similarly treated. The data in Table II clearly show that the treated animals incorporated large amounts of radioactivity into nonsaponifiable material, and digitonin-precipitable material, but very little into cholesterol. While the conclusions one can draw from radiochemical experiments are somewhat limited, it seems fairly certain that the block in cholesterol synthesis must come after the C_{27} sterol nucleus is formed.

The serum and liver of these animals were analyzed for cholesterol, "fast-acting" sterols, and materials absorbing at 281.5 mμ. These and other data from the experiment are compiled in Table III. It is apparent that the "fast-acting" sterol which is present is accounted for entirely by the material absorbing at 281.5 mμ. The discrepancy in the case of serum in which the 281.5 mμ material accounts for more than the analyzed amount of "fast-acting" sterol is probably due to difficulty in determining very small quantities of the latter. These results are consistent with the suggestion that the unknown material which results from treatment with the ferrocene derivative is 7-dehydrocholesterol.

The ability of *N*-(ferrocenylmethyl)piperidine to inhibit the reduction of 7-dehydrocholesterol is evident from the data in Table IV.

TABLE III. Effect of *N*-(Ferrocenylmethyl)piperidine on Serum and Liver Cholesterol in Rats.

	Group[a]	
	Control	Experimental
Body wt. (g) initial	94 ± 7	90 ± 9
final	142 ± 12	130 ± 14
Liver wt. (g/100 g of body wt.)	4.68 ± 0.34	4.52 ± 0.15
Food consumption (g)	635	585
Serum		
Cholesterol (mg/100 ml)	61 ± 6	7 ± 5[b]
281.5 mμ absorbing material (mg/100 ml)	0	18 ± 3
"Fast-acting" sterol (mg/100 ml)	0	13 ± 2
Liver		
Cholesterol (mg/g)	2.56 ± 0.17	0.67 ± 0.16[b]
281.5 mμ absorbing material (mg/g)	0	2.26 ± 0.25
"Fast-acting" sterol (mg/g)	0	2.22 ± 0.17

[a] Mean ± SD.

[b] Significantly lower $p < 0.01$.

TABLE IV. Inhibition of the Reduction of 7-Dehydrocholesterol *in Vitro* by *N*-(Ferrocenylmethyl)piperidine.

Inhibitor conc (moles/liter)	7-Dehydrocholesterol reduced (μmoles)	Inhibition (%)
0	0.232	0
1×10^{-7}	0.227	2.2
5×10^{-7}	0.144	37.9
1×10^{-6}	0.074	68.1
5×10^{-6}	0.007	97.0

Essentially complete inhibition is achieved at a very low molar concentration of inhibitor. With the information presently available, the only physiological function of this compound that is apparent is the ability to inhibit the reduction of 7-dehydrocholesterol and cause abnormal quantities of this sterol to appear in serum and liver. This is probably the mechanism by which total serum sterol is lowered. However, this is by no means obvious. If the rate of production of 7-dehydrocholesterol is not lowered, it would not be unreasonable to expect this sterol to compensate for the loss of cholesterol in the serum. However, this is not the case. It is possible that the animal is capable of eliminating 7-dehydrocholesterol rapidly by excretory or catabolic routes other than via cholesterol.

Summary. A ferrocene derivative, *N*-(ferrocenylmethyl)piperidine possesses marked hypocholesterolemic activity. This activity appears to be due to the ability of this agent to alter the metabolism of 7-dehydrocholesterol. Abnormal quantities of this sterol were found in serum and liver of animals treated with this chemical. In addition, the ferrocene derivative is an effective inhibitor of the reduction of 7-dehydrocholesterol *in vitro*.

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