Benzmalecene: Inhibition of Cholesterol Biosynthesis and Hypocholesteremic Effect in Rats. (25405)

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An agent capable of inhibiting biosynthesis of cholesterol by mammalian systems, would be of possible clinical interest in various hypercholesteremic states. The discovery that 2-C^{14}-mevalonic acid(1) is utilized by rat liver preparations for biosynthesis of cholesterol with an efficiency far greater than any previously tried precursor has made possible the study of a number of compounds which would inhibit this system. This paper describes results obtained with benzmalecene,* a potent inhibitor arising from these investigations.

Methods and materials. In vitro experiments. A cell-free rat liver homogenate system which synthesizes cholesterol was prepared employing the technic described by Bucher(2), as modified by Rabinowitz and Gurin(3). Livers were obtained from male albino rats† received at 75-80 g weight and maintained for one week on Purina Laboratory Chow. The substrate employed was 2-C^{14}-DL-mevalonic acid (2-C^{14}-MVA) having a specific activity of 0.0125 mc/mM as measured by liquid scintillation counting. Each tube‡ contained 0.4 mg of 2-C^{14}-MVA, 1 ml of rat liver homogenate and 0.2 mg each of adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN). The volume was made to 2.2 ml with pH 6.9 buffer which has been previously described(3). The gas phase was 95% O_2 and 5% CO_2. Incubation was carried out at 37° for 4.5 hours with agitation. Two mg of carrier cholesterol were added after incubation and the cholesterol was isolated and counted as the digitonide according to a previously described procedure(3). The benzmalecene was solubilized with an equivalent amount of NaOH in small amount of water and diluted to concentration of 1 mg/ml with the pH 6.9 buffer solution. Aliquots of the solubilized compound were added to the incubation mixture described, at the expense of the buffer solution. *In vivo experiments.* Holtzman male albino rats were obtained at 130-140 g in weight and maintained on Purina Laboratory Chow for one week. The rats were then randomized according to weight into several groups of 20 rats each and placed on Purina Lab Chow diet to which 10% lard had been added. Benzmalecene was administered by incorporation in the diet. After 10 days, 2 ml blood samples were taken by cardiac puncture under light Nembutal anesthesia and placed in tubes containing 0.2 ml of 0.4 M sodium citrate. The plasma was analyzed for total cholesterol by the method of Abell *et al.*(4).

Results. In vitro experiments. Table I shows results typical of those obtained when benzmalecene is added in increasing concentrations to a rat liver homogenate system which converts mevalonic acid to cholesterol. As the concentration of benzmalecene is increased there is a sharp depression in cholesterol synthesis with maximum inhibition being obtained at the 0.15 mg/tube level or about 1.9×10^{-4} M.

To determine if the inhibition by benzmalecene of this multi-enzyme system was competi-

TABLE I. Inhibition of *In Vitro* Incorporation of 2-C^{14}-Mevalonic Acid into Cholesterol by Rat Liver Homogenates.

Compound added*	Level, mg/tube	Recovered cholesterol, epm/mg C	% inhibition
None		1700	
Benzmalecene	.05	975	43
	.075	852	50
	.10	320	81
	.15	9	99
	.20	2	100

* Each tube contained 400 μg of 2-C^{14}-dl-mevalonic acid (specific activity = .0125 mc/mM).

* Generic name for N-(1-methyl-2,3-di-p-chlorophenylpropyl)-maleamic acid (α-isomer). This compound was supplied by Dr. E. M. Schultz of Merck Sharp & Dohme Research Labs., West Point, Pa.

† Sprague-Dawley strain obtained from C & L Breeders, East Greenville, Pa.

‡ Screw cap culture tubes (20 x 125 mm).

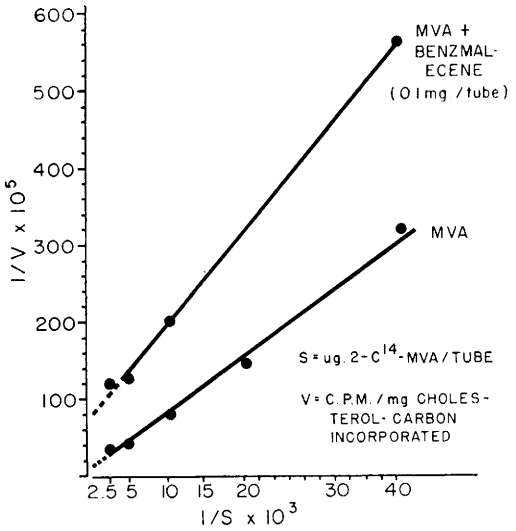

FIG. 1.

tive or non-competitive, the Lineweaver-Burk (5) method of plotting was employed. The incorporation of mevalonic acid into cholesterol over a range of 25 to 400 μg per tube was determined in the absence of inhibitor and then in the presence of a single level of benzmalecene in all tubes. The data, when plotted according to Lineweaver and Burk(5) (Fig. 1), indicate the over-all inhibition to be non-competitive. *In vivo experiments.* Table II illustrates the effect of benzmalecene on plasma cholesterol level of rats when the compound was administered in the diet for 10 days as the free acid and as the sodium and calcium salts. The free acid at levels of 0.1, 0.2 and 0.4% in the diet reduced plasma cholesterol level by 12, 20 and 26% respectively. Little or no effect on body weight gain was apparent. Due to the possibility of poor absorption of the free acid form, the sodium salt was also studied. This salt was more effective, producing decreases in plasma cholesterol of 22, 29 and 39% at levels equivalent to those employed for the free acid. The hygroscopic nature of the sodium salt prompted the testing of the calcium salt. This material, although appearing somewhat less active than the sodium salt at the 2 lower levels, was equal in activity at the 0.4% level. The 2 salts caused some depressing effect on body weight gain.

Discussion. The results of the *in vitro* experiment demonstrate that benzmalecene is a potent metabolic inhibitor of cholesterol synthesis. The exact point at which the metabolic block is exerted in the pathway of mevalonic acid to cholesterol is not known.

When benzmalecene, which blocks the synthesis of cholesterol by rat liver *in vitro,* was incorporated into a chow diet containing lard, a significant reduction in plasma cholesterol level resulted. Benzmalecene has been shown in other studies from these laboratories to produce a prompt depression in blood cholesterol in the dog when given orally at 50 mg/kg day(6). The inhibition of *in vitro* cholesterol synthesis by benzmalecene, coupled with its effect on plasma cholesterol level in *in vivo* experiments, suggests, but does not necessarily prove that the latter effect is a direct result of an *in vivo* reduction of cholesterol synthesis.

A number of other substances have been re-

TABLE II. Effect of Benzmalecene and Its Sodium and Calcium Salts on Plasma Cholesterol Level of Rats.

	Benzmalecene (free acid)		Benzmalecene (sodium salt)		Benzmalecene (calcium salt)	
% compound in diet	Plasma cholesterol, mg %	Wt gain, g	Plasma cholesterol, mg %	Wt gain, g	Plasma cholesterol, mg %	Wt gain, g
0	64.0	53	60.1	53	62.7	46
.1	56.2	50	46.8	44	51.0	47
.2	51.3	52	42.9	37	45.3	39
.4	47.5	51	36.4	31	36.8	
L.S.D.*$_{.05}$	2.9 mg		3.2 mg		3.8 mg	
" *$_{.01}$	3.8		4.3		5.1	

* $\text{L.S.D.} = t \times \gamma \times \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}$.

ported which inhibit hepatic synthesis of cholesterol and lower blood cholesterol; α-phenylbutyric acid(7,8), α-p-bi-phenylbutyric acid (9,10) and vanadium salts(11,12).

It is not known if an increased hepatic synthesis of cholesterol is a contributing factor in the etiology of the various abnormal metabolic states in which cholesterol has been implicated. The clinical study of benzmalecene in these conditions should be useful in obtaining information on their etiology and may offer a practical method of combating hypercholesteremic states.

Summary. Benzmalecene inhibited *in vitro* incorporation of 2-C^{14}-mevalonic acid into cholesterol by rat liver homogenates. The inhibition proved to be non-competitive. Oral administration of this compound to normal rats resulted in a significant reduction in plasma cholesterol.

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Erythropoietin. I. Analysis of Radioiron Assay in Fasted Rats, Using Cobalt as a Reference Standard.* (25406)

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In the past few years research on erythropoietin has expanded rapidly and the number of papers in this field has greatly increased. Unfortunately, however, it has been difficult to compare assay results between laboratories because there has been no reference standard. Gordon(1) documented the need for such a material. We attempted to remedy the situation by choosing cobalt† as a readily available primary reference standard in the radioiron assay developed by Fried *et al.*(2). Using the starved rat as subject, 5 μmoles of cobalt gives a substantial response above the saline blank and therefore has been designated as the one unit response. Further studies have been made in the 1 to 2 unit range, comparing cobalt to various erythropoietin fractions. Finally, a partially purified fraction from anemic sheep plasma has been carefully standardized against cobalt and has been adopted by our laboratory as a day-to-day working standard.

Methods. Normal, 2 month old, male Sprague-Dawley rats, weighing 175 to 200 g were used. The rats were acclimated to the animal room for 4 days, and maintained on Rockland R-4 rat diet. At start of assay all food was withdrawn, but water was supplied *ad lib.* Samples were prepared in 0.05 *M* $NaPO_4$, 0.15 *M* NaCl, pH 6.8 (saline). After 30 hours fasting, the first 2 ml sample was injected intravenously (tail vein) under light

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† Here the simple term "cobalt" is understood to mean "cobaltous ion". Reagent grade cobalt chloride ($CoCl_2 \cdot 6H_2O$) has been used as source of cobaltous ion.