

Plasma cholesterol lowering action of bile acid binding polymers in experimental animals

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SUMMARY

Feeding of bile acid binding polymeric organic bases inhibited cholesterol rise and aortic plaque formation in cholesterol-fed cockerels, lowered plasma cholesterol concentrations in normocholesterolemic cockerels and dogs, and increased fecal bile acid and sterol output in a dog. In experiments lasting as long as one year these substances have not produced visible toxic effects. They are thought to act by binding the bile acids in the intestinal tract.

Siperstein *et al.* (1) have shown that the plasma cholesterol rise in cholesterol-fed cockerels can be inhibited by the feeding of ferric chloride, and have associated this effect with precipitation of the bile acids in the intestinal tract. They have suggested that the binding of bile acids might serve as a means of controlling arteriosclerosis.

Substances that bind bile acids, such as iron salts or various of the alkaloids, generally have toxic or pharmacological properties which preclude their use as blood cholesterol lowering agents. We report here studies of the blood cholesterol lowering action of two bile acid binding polymeric organic bases in cholesterol-fed cockerels and in normocholesterolemic cockerels and dogs. These substances have molecular weight so high that they cannot be absorbed. In experiments lasting as long as one year they have produced no evident toxic effects.

MATERIALS AND METHODS

MK-325 is a water-soluble polymer having quaternary amino groups attached to a polyacrylic skeleton by ester linkages. Its equivalent weight is about 325, and its molecular weight is about two million. The stock material was a clear, viscous 12.5% aqueous solution. MK-135 is a quaternary ammonium anion exchange resin in which the basic groups are attached to a styrene-divinyl benzene copolymer skeleton by carbon-to-carbon bonds. Its equivalent weight is

about 230. It is a lyophobic solid and, as used, contained about 75% water. Except where otherwise noted, both of these materials were given as the chloride salts.

Analyses were conducted with plasma, prepared by mixing 2.0 ml of whole blood with 0.35 ml of ACD solution (22.0 g trisodium citrate dihydrate, 8.0 g citric acid monohydrate, and 22.0 g dextrose per liter). Cholesterol was determined by the procedure of Abell *et al.* (2); and lipid phosphorus by King's procedure (3) after precipitation with trichloroacetic acid, as recommended by Zilversmit and Davis (4). Reported plasma lipid concentrations should be multiplied by 1.3 to approximate values in serum.

RESULTS

Demonstration of Binding Action. The ability of the polymeric bases to bind bile acids is shown in the two following experiments.

A solution containing 100 mg of cholic acid, as sodium cholate, in 40 ml of 15% alcohol was acidified with sulfuric acid until acid to Congo red, and extracted twice with 20 ml portions of chloroform. The solvent extracts were combined and evaporated to dryness. The residue was dissolved in aqueous alcohol and titrated with .05 N sodium hydroxide. Similar extractions were performed with solutions to which MK-325 had been added in various amounts between 5 and 200 mg. In the presence of 0, 5, or 10 mg of MK-325,

recovery of cholate in the extract was 98% or better; with 20 mg, it was 91.5%; with 50 mg, 81.5%; with 100 mg, 60.5%; with 200 mg, 54.5%.

A 1 g sample of MK-135 was stirred with 100 ml of 1% sodium cholate solution for 1 hour. An aliquot of the supernatant was then removed and analyzed for cholic acid by the procedure of Mosbach *et al.* (5). It was found that 91.4% of the cholate had been removed from solution by this procedure.

Short Tests in Cockerels. The polymeric bases were first tested for action on blood cholesterol in 4-day experiments with cholesterol-fed White Leghorn cockerels. Balanced groups of 10 birds, 9 or 11 weeks' old, previously fed basal ration (6), were given a diet containing 2% cholesterol and 5% cottonseed oil, starting on Monday mornings. Treated birds were in addition given polymeric bases as 1% (dry weight) of the diet. On the following Friday mornings blood was drawn for analysis. In tests of this kind plasma cholesterol concentrations normally increase from about 70 mg/100 ml on Monday to about 250 mg/100 ml on Friday. The feeding of polymeric bases inhibited this rise. In four experiments the average plasma cholesterol concentration on Friday of eight control groups was 281 mg/100 ml, and of 4 MK-325-fed groups, 156

mg/100 ml. In another set of four experiments the average for eight control groups was 236 mg/100 ml, and for 4 MK-135-fed groups, 104 mg/100 ml. For each set the observed differences were statistically significant at the 0.001 level.

The effect of polymer feeding was tested in 4-day experiments with normocholesterolemic cockerels fed basal ration containing no added cholesterol. In two such tests the average plasma cholesterol on Friday of four control groups, each containing 10 birds, was 73 mg/100 ml; and of two groups fed MK-325 as 1% of the diet, 58 mg/100 ml. In five tests, the average plasma cholesterol of 11 control groups was 72 mg/100 ml; and of five groups fed MK-135 as 1% of the diet, 57 mg/100 ml. For each set of experiments the observed differences were statistically significant at the 0.001 level.

Longer Tests in Cockerels. The influence of polymer feeding on aortic plaque formation was studied in experiments of 7 or 8 weeks' duration. Birds were set out when 8 or 9 weeks' old, and fed ration containing cholesterol and cottonseed oil until they were 16 weeks' old. Treated birds were also given 1% (dry weight) of polymeric base in the diet. At the end, blood was drawn, the birds were sacrificed, and their aortas

TABLE 1. AVERAGE PLASMA LIPID CONCENTRATIONS AND AORTA SCORES OF COCKERELS GIVEN MK-325 OR MK-135 AS 1% OF THE DIET IN EXPERIMENTS OF SEVEN OR EIGHT WEEKS' DURATION

Treatment	No. of Birds	Total Cholesterol	Lipid Phosphorus	C/PL *	Aorta Score	Incidence of Atheromatosis	Weight Gain
		mg/100 ml	mg/100 ml				g
Experiment I. Cholesterol Feeding, 7 Weeks							
Control	20	489	8.79	2.10	1.75	18/20	872
MK-325	10	370	6.93	1.99	1.1	6/10	841
p †		0.2	0.09	0.7	0.09	0.15	1
Experiment II. Cholesterol Feeding, 8 Weeks							
Control	29	439	8.82	2.02	1.6	25/29	927
MK-325	10	247	5.46	1.77	0.3	3/10	998
MK-135	20	215	5.23	1.62	0.5	7/20	979
p		<.001	<.001	<.01	<.001	<.001	.12
Experiment III. Basal Diet, 7 Weeks							
Control	20	68.7	4.29	.64			807
MK-325	10	53.8	3.85	.57			789
p		0.001	0.12	0.012			1

* Cholesterol/phospholipid ratio.

† p values in each set calculated for treated animals vs. controls.

were removed and graded for degree of atheromatosis by a scale of 0 to 4 (6). The results are presented in Table 1, experiments I and II. The polymer-fed birds had lower incidence and severity of atheromatosis, lower plasma concentrations of cholesterol and lipid phosphorus, and lower cholesterol-to-phospholipid ratios than their controls. Weight gain was not impaired. Plasma cholesterol was also determined approximately every two weeks in experiment I. In the treated birds the average was 55% to 56% of average control for the first 4 weeks, and 71% at 6 weeks.

A similar experiment, of 7 weeks' duration, was conducted with normocholesterolemic birds fed basal diet with no added cholesterol. As shown in experiment III in Table 1, resin feeding lowered plasma cholesterol and lipid phosphorus concentrations, reduced cholesterol-to-phospholipid ratios, but did not impair weight gain. Here again plasma cholesterol levels were determined approximately every 2 weeks during the test. They varied between 74% and 82% of control values.

The influence of the size of the dose of the polymers on their effect on plasma cholesterol concentration was studied in a series of 4-day tests in both cholesterol-fed and normocholesterolemic cockerels. The effect on plasma cholesterol was a function of the amount of polymer fed (Table 2). A concentration of 0.125% in the diet was not large enough to have significant effect.

TABLE 2. AVERAGE PLASMA CHOLESTEROL CONCENTRATIONS OF COCKERELS FED GRADED AMOUNTS OF MK-325 OR MK-135 IN SHORT TESTS

Treatment	Per Cent Polymer in Diet				
	0	.125	.25	.5	1
<i>Plasma Cholesterol, mg/100 ml</i>					
Cholesterol-Fed Birds *					
MK-325	275	243	209	219	144
MK-135	262	242	213	161	120
Normocholesterolemic Birds †					
MK-325	73	73	69	57	54
MK-135	76	75	66	67	60

* Each figure the average for 40 controls or 20 treated birds.

† Each figure the average for 20 controls or 10 treated birds.

MK-135 was prepared as the glycocholate and taurocholate salts. These were fed as 1% of the diet to cholesterol-fed cockerels in 4-day tests. Average plasma cholesterol of 20 control birds was 225 mg/100 ml; of 10 birds fed MK-135 glycocholate, 315 mg/100

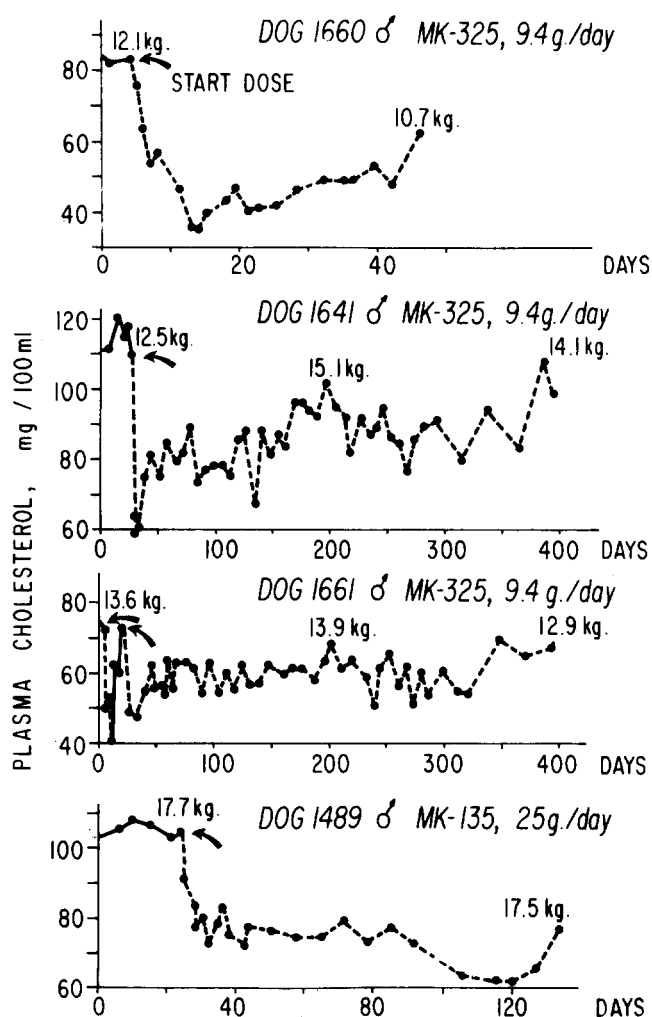


FIG. 1. Plasma cholesterol concentrations of dogs dosed with bile acid binding polymers.

ml; and of 10 birds fed MK-135 taurocholate, 271 mg/100 ml. Thus these forms of the resin did not inhibit cholesterol rise. Resin fed as the stearate salt, however, was effective. Average plasma cholesterol of 30 controls was 225 mg/100 ml; of 10 birds fed MK-135 stearate, 83 mg/100 ml; of 10 birds fed MK-135 chloride, 93 mg/100 ml; and of 10 birds fed stearic acid, 227 mg/100 ml.

Normocholesterolemic Dogs. The polymers were fed for periods of 7 weeks to 1 year to four male beagles maintained on a meat-and-meal diet. The effect on plasma cholesterol and on body weight is shown in Figure 1. The initial plan with the first dog fed MK-325, No. 1660, was to start with a moderate dose and double it each day until signs of toxicity appeared. Twenty-five ml of 12.5% solution added to the diet on the first day, and 50 ml on the second

day were accepted by the dog, but 100 ml on the third day were not retained. A dose of 75 ml (9.4 g dry weight) was successfully maintained thereafter in this dog for 43 days. Dog 1641 was similarly dosed for 1 year. Dog 1661 was given 75 ml per day for 7 days; dosing was then discontinued for 12 days, and resumed for 373 days. Dog 1489 consumed approximately 75 g of MK-135 per day, mixed with the diet, for 113 days.

All dogs behaved normally and were free from toxic effects during the test periods. They varied in weight from time to time, but the changes were neither consistent nor notable. Hematologic examinations, and blood and urine analyses, were made before, at intervals during, and at the end of the tests. Oxalated plasma was analyzed for urea nitrogen, glucose, and fibrinogen. Serum was analyzed for protein, albumin, globulin, A/G ratio, inorganic phosphorus, and alkaline phosphatase. Tests and measurements in urine were for volume, pH, specific gravity, blood, sugar, protein, and creatinine. No deviations from the normal range for dogs were observed in any of these.

Whole blood clotting time and prothrombin time of dogs 1641 and 1661 were determined after they had been on test for 36 and 40 weeks respectively. The results did not depart from expected control values, or from values simultaneously determined in two control dogs. The dogs were then given orally 1 mg per kg per day of Dicumarol (7), for 2 days. This increased both whole blood clotting time and prothrombin time. Recovery from this treatment was as rapid in the MK-325 dosed dogs as in the controls, and thus polymer feeding had neither decreased stores of vitamin K nor impaired its absorption.

Each of the four polymer-fed dogs was sacrificed and autopsied at the end of his period of dosing. The following tissues were then examined microscopically: various parts of the gastrointestinal tract, pancreas, liver, omentum, gall bladder, spleen, adrenal, kidney, urinary bladder, testes, epididymis, seminal vesicles, prostate, lymph nodes, brain, pituitary, lung, trachea, heart, aorta, thyroid, parotid gland, bone marrow, and striated muscle. There were no pathologic changes attributable to the polymeric bases.

When dogs 1641 and 1661 were sacrificed, samples of liver, heart, kidney, adrenal, brain, muscle, and pancreas were digested with KOH and analyzed for cholesterol by the procedure of Abell *et al.* (2). Comparison of the results with those from a simultaneous control showed that polymer feeding had not depleted cholesterol concentrations in these tissues.

The influence of MK-135 feeding on fecal bile acid

and sterol output was studied in an 11 kg male beagle (No. 1987). This dog was fed a meat-and-meal diet, the average daily portion of which was determined to contain about 340 mg of cholesterol. Control fecal collections and plasma cholesterol analyses were made at weekly intervals for 3 weeks. MK-135 was then added to the diet at 25 g, dry weight, per day. Feces were collected and blood was drawn for analysis daily during the feeding period. The feces were extracted with alcoholic ammonium carbonate solution to obtain the sterols and the bile acids, both free and resin bound. Sterols were determined by the Liebermann-Burchard reaction after hydrolysis with alkali and extraction into petroleum ether. The bile acids were separated from interfering substances by an ion-exchange procedure which will be reported separately. Deoxycholic acid was determined by heating with sulfuric acid (5), and cholic acid, by heating with furfural (8). Average fecal bile acid output was increased by resin feeding from 231 mg per day to 609 mg per day, or 2.6-fold (Table 3). Average Liebermann-

TABLE 3. PLASMA CHOLESTEROL CONCENTRATIONS AND FECAL STEROL AND BILE ACID OUTPUT OF A DOG DOSED WITH MK-135, 25 g/DAY

Day	Plasma Cholesterol	Fecal Bile Acid	Fecal Sterol
	<i>mg/100 ml</i>	<i>mg</i>	<i>mg</i>
Control	95	317	285
Control	91	259	301
Control	92	118	116
1	82	949	371
2	80	184	406
3	75	403	405
4	67	838	555
5		No feces	No feces
6		634	515
7	64	331	278
8	67	922	494

Burchard positive sterol output was increased from 234 mg per day to 432 mg per day, or 1.8-fold. Average plasma cholesterol concentration during the control period was 93 mg/100 ml, and during the period of resin feeding, 73 mg/100 ml.

DISCUSSION

It is apparent that feeding of MK-325 and MK-135 to cholesterol-fed cockerels suppressed a rise in plasma cholesterol and lipid phosphorus concentrations and

inhibited aortic plaque formation. In normocholesterolemic cockerels and dogs it lowered plasma cholesterol concentrations. The magnitude of the effect in cockerels was related to the amount of the materials fed.

The results suggest that the effect of these polymeric bases on plasma cholesterol concentration is due to bile acid binding in the intestinal tract. Both polymers bind bile acid *in vitro*. Both have very high molecular weights and thus cannot be absorbed from the intestine. MK-325 contains ester linkages, and is possibly hydrolyzed to some extent in the intestine, but MK-135 contains no hydrolyzable bonds, and, in addition, is a solid. Thus their effect must be exerted in the intestinal lumen. The feeding of MK-135 increased the bile acid output in the feces of a dog. When MK-135 was fed to cholesterol-fed cockerels as the glycocholate or taurocholate salts (forms unable to take up further bile acids), no cholesterol lowering occurred; but feeding of the stearate salt was fully effective.

No toxic effect, either local or systemic, from the feeding of these materials for as long as a year was detected. Treated animals were alert and free from toxic signs. Cholesterol distribution in tissues was not greatly influenced.

No injurious interference with fat absorption was apparent. Animals dosed here with the polymeric bases gained or maintained weight normally, produced normal stools, and had the expected amount and distribution of fatty tissue at autopsy. Dogs 1641 and 1661, after daily dosing with MK-325 for 9 months, had normal prothrombin times, and responded normally to dosage with Dicumarol. Thus vitamin K storage and absorption were unimpaired.

Siperstein *et al.* (1) suggested that the cholesterol lowering effect of bile acid binding is due to impaired cholesterol absorption. The results here suggest that it may also be due to increased cholesterol oxidation, brought about by continued removal of bile acids (9 to 16), which are the oxidation products, from the enterohepatic cycle (17 to 20). In dog 1987 this increased oxidation yielded, on the average, an extra 387 mg of fecal bile acids per day during the period of MK-135 dosing.

It is not unexpected that bile acid binding agents should lower blood cholesterol in cholesterol-fed animals. Hepatic cholesterol synthesis in these should be very slow (21, 22). In animals on basal diet, however, hepatic synthesis should be active, and might be expected to counteract the cholesterol lowering effect. The fact that cholesterol lowering occurred when the polymers were fed to normocholesterolemic animals

indicates that in them loss of steroid nucleus in the feces was more rapid than *de novo* cholesterol synthesis.

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