Comparison of *in vitro* Bile Acid Binding Capacity and *in vivo* Hypocholesteremic Activity of Cholestyramine. (30723)

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Cholestyramine, an anion exchange resin, lowers plasma cholesterol levels in experimental animals(1) and in man(2). This resin binds and decreases resorption of bile acids from the intestine, thereby stimulating the rate of oxidation of cholesterol in order to replenish the bile acid supply(3). With the use of cholestyramine for therapeutic purposes(4,5), the need has arisen for a method for measuring and assuring uniform bile acid binding capacity. In this paper, a rapid *in vitro* method for bile acid binding capacity is described and the findings compared with *in vivo* measurement of hypocholesteremic activity in chicks.

Methods. A stock supply of cholestyramine (99%<100 mesh) was used for development of *in vitro* and *in vivo* assays, and as a standard in assays of other preparations of cholestyramine, as well as other resins.

In vitro *assay.* Preliminary studies were carried out on the effects of pH, buffer concentration, reaction time and temperature on cholate binding. Weighed quantities of cholestyramine resin, 20 ml phosphate buffer, and 1 ml aqueous solutions containing 20 or 40 mg sodium cholate were added to 50 ml Erlenmeyer flasks. These were capped with aluminum foil and shaken mechanically in a water bath for specified times. The contents were filtered through Whatman No. 42 filter paper, and an aliquot was diluted and assayed for cholate by the method of Kier(6) or in later experiments by the Pettenkoffer reaction as described by Gordon *et al*(7).

In vivo *assay.* Groups of one-day-old White Leghorn cockerels were fed a hypercholesteremic diet containing 36.2% ground yellow corn, 40% soybean oil meal (50% protein), 10% coconut oil, 5% corn starch, 3.5% steamed bone meal, 3% dehydrated alfalfa meal (17% protein), 1% cholesterol, 0.4% iodized salt, 0.35% methionine-hydroxy-analogue,† 0.02% manganese sulfate, and a complete vitamin mixture. In some studies the test resins were included in this diet from the start and blood was obtained for cholesterol analysis after one week. In other studies, the hypercholesteremic diet alone was fed for one week and the resins were added to the diet during the second week, after which plasma cholesterol was determined.

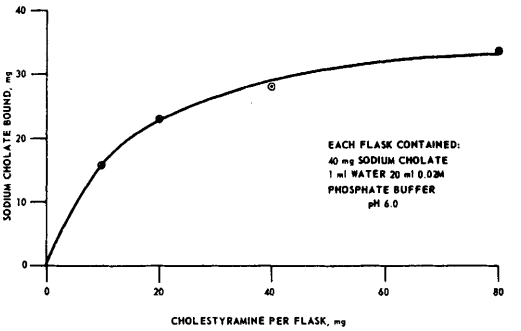

FIG. 1. Binding of sodium cholate by cholestyramine *in vitro.*

Blood was collected by cardiac puncture in heparinized tubes and centrifuged. Plasma cholesterol levels were determined by a modification of the method of Zlatkis *et al*(8), using the Technicon AutoAnalyzer(9).

Results. In vitro *assay.* Cholate was found to be rapidly bound by cholestyramine. Most of the binding occurred within one minute, and after 20 minutes, binding of cholate was not further increased. Increasing the temperature from 25°C to 37°C had no effect on binding after 10 minutes or more of shaking. In subsequent studies the flasks were shaken 30 minutes at 25°C.

The binding of cholate by graded amounts of cholestyramine in 0.02 M phosphate buffer at pH 6.0, is shown in Fig. 1. The amount of

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† Calcium DL - 2 - hydroxy-4-methylthiobutyrate. This was generously supplied by Monsanto Chemical Co., St. Louis, Mo.

TABLE I. Effect of pH and Phosphate Concentration on Cholate Binding by Cholestyramine.

Molarity of phosphate buffer	pH	Cholate bound (mg)	Cholate bound (%)
.02	5.0	25.2	63
	6.0	23.4	59
	7.0	18.4	46
	8.0	15.8	39
.15	6.0	15.6	39
	7.0	16.4	41
	8.0	16.4	41
	8.8	16.2	41
.3	6.0	16.8	42
	7.0	17.0	42
	8.0	16.8	42
	8.8	16.0	40

Each flask contained 40 mg sodium cholate and 20 mg cholestyramine.

cholate bound was increased as the resin was increased from 10 to 80 mg.

With 0.02 M phosphate buffer, cholestyramine bound progressively less cholate as the pH was increased from 5.0 to 8.0 (Table I). At the higher phosphate concentrations, changes in pH did not influence binding. At pH 6.0, cholate binding was significantly less with 0.15 M or 0.3 M phosphate than with 0.02 M phosphate; at pH 8.0, phosphate concentration did not affect binding.

The standard conditions chosen for subsequent assays included the use of 20 ml of 0.3 M phosphate buffer at pH 6.0, 20 or 40 mg resin, and 40 mg sodium cholate. Flasks were shaken for 30 minutes at 25°C, the mixture filtered, and the filtrate assayed for cholate (7).

In vivo *assay*. Addition to the diet of 0.3, 0.6, and 1.0% cholestyramine resulted in stepwise lowering of plasma cholesterol levels of the chicks (Table II). Plasma cholesterol levels of duplicate groups were similar with 0, 0.6 or 1.0% cholestyramine, but there were often marked variations with 0.3% cholestyramine. In repeating the first week assay at different times, considerable differences in degree of hypercholesteremia were found with the diet alone and with the resin. This may have been related to the date of hatching and initial body weights of the chicks(10). In the second week assay, plasma cholesterol levels of the chicks not receiving resin were higher and more consistent than those of the control chicks in the 1-week assay (Table II). The resin also had a greater hypocholesteremic effect in the 2-week assay, providing a better assay than the 1-week test.

An experiment for which data are not reported herein showed that substitution of other fats for the dietary coconut oil or measurement of tissue cholesterol levels in the chick did not provide a better assay. Use of olive or peanut oil in the diet did not improve the reproducibility of the plasma cholesterol levels. Cholesterol levels of aorta and liver were reduced by cholestyramine, but little or

TABLE II. Effect of Cholestyramine on Plasma Cholesterol Levels of Chicks During First and Second Weeks on Hypercholesteremic Diet.

Chicks per group	Cholestyramine in diet: 0	.3%	.6%	1.0%
		Plasma cholesterol, mg/100 ml*		
1st week assays				
20	552 ± 175	413 ± 95	310 ± 106	163 ± 30
10	489 ± 240	227 ± 94	160 ± 53	144 ± 28
10	420 ± 167	189 ± 75	149 ± 26	116 ± 19
10	501 ± 164	319 ± 116	159 ± 27	—
10	310 ± 130	178 ± 33	106 ± 20	121 ± 12
10	234 ± 62	176 ± 78	—	—
10	282 ± 147	267 ± 142	173 ± 107	—
2nd week assays				
20	730 ± 225	593 ± 213	314 ± 134	182 ± 21
10	709 ± 132	632 ± 242	220 ± 46	—
10	793 ± 230	471 ± 172	—	—
10	902 ± 260	719 ± 315	—	—
10	714 ± 194	527 ± 224	—	—
20	760 ± 167	—	250 ± 97	168 ± 25

* Values given with standard deviations.

TABLE III. Hypocholesteremic Activity and Cholate Binding Capacity of Cholestyramine Preparations and Other Anion Exchange Resins.

	1st week chick assay			2nd week chick assay			*In vitro* assay		
	Plasma cholesterol* (mg/100 ml)			Plasma cholesterol† (mg/100 ml)			mg cholate‡ bound by		
	Resin in diet		Activity, % of cholestyramine Standard	Resin in diet		Activity, % of cholestyramine Standard	20 mg resin	40 mg resin	Binding capacity, % of cholestyramine Standard
	.6%	1.0%		.6%	1.0%				
Exp 1									
Cholestyramine—Std <100 mesh	160	144	100	250	168	100	12.7	18.2	100
" —Coarse—not ground	309	223	55– 77	443	359	62– 68	5.8	10.4	46– 57
" —Fine—200 mesh	163	132	99–103	249	170	81–100	12.0	18.2	95–100
" —Ball milled	169	134	97– 97	—	—	—	12.4	17.6	98– 97
" —Standard (starch coated)	171	150	97– 98	—	—	—	13.2	19.4	103–106
Exp 2									
Cholestyramine—Std	155	127	100	—	—	—	12.8	18.1	100
Dowex 2 ×1	218	164	84– 91	404	219	70– 91	9.0	13.2	71– 72
Exp 3									
Cholestyramine—Std				—	—	—	13.0	18.4	100
Product 5865-7D§, Batch 193				259	157	98–102	11.6	16.3	89– 89
" " Batch 147				257	169	99–100	11.4	18.4	88–100
" " Batch 159				203	180	109– 98	11.5	17.5	89– 95
Exp 4									
							30 mg resin		
Cholestyramine—Std	—	121	100	—	—	—	14.3		100
Amberlite XE- 58 (fine)	—	213	51	—	—	—	1.0		7
" XE- 67 (fine)	—	212	52	755	653	1– 16	4.3		30
" XE-220 (fine)	—	139	90	516	384	48– 64	1.9		13

* Plasma cholesterol levels of control groups (no resin) in the 1-week assays were 489, 525 and 310 mg/100 ml for Exp 1, 2 and 4, respectively.
† All values shown were obtained in the same assay.
‡ 40 mg sodium cholate per flask.
§ Questran®, brand of cholestyramine, Mead Johnson & Company.

no effect on heart cholesterol levels was found. The tissue cholesterol changes were less sensitive than those in plasma and were not as useful in determining the relative activities of cholestyramine preparations.

Comparison of in vitro *and* in vivo *assays.* Cholestyramine was milled and screened to provide materials of varying particle size. Some of these were mixed with a variety of inert ingredients in the development of palatable forms for clinical studies. These were assayed on the basis of cholestyramine content. The various preparations and other anion exchange resins were assayed for cholate-binding capacity *in vitro* and for hypocholesteremic activity *in vivo* in 1-week and 2-week chick assays (Table III). Activities are expressed as per cent of that found with the standard cholestyramine preparation. In the chick assay these were calculated from the extent to which the resin decreased plasma cholesterol levels from that found with no resin in the diet.

The activities of the cholestyramine preparations in the chick assays and in the *in vitro* assay agreed quite well (Table III). The coarse beadlets had 46 to 57% of the standard activity in the *in vitro* assay, as compared to 55 to 77 and 62 to 68% in the chick assays. The other cholestyramine preparations shown in Experiment 1 had close to 100% activity by both methods.

Three batches of Product 5865-7D,‡ the form of cholestyramine under clinical study, showed no diminution in activity due to excipients, in either the *in vivo* binding assay or the chick assay. Other anion exchange resins, such as Dowex 2 × 1§ and three Amberlite resins‖ had much lower activity than cholestyramine in both the *in vitro* binding and the 2-week chick assays.

Summary. Methods for *in vitro* assay of cholate binding capacity and for *in vivo* assay of hypocholesteremic activity of preparations of cholestyramine, an anion exchange resin, were developed and compared. In the *in vitro* assay, 20 ml of 0.3 M phosphate buffer at pH 6.0, 20 or 40 mg of the resin and 40 mg of sodium cholate were mixed and shaken for 30 minutes; the filtrates were assayed for cholate spectrophotometrically, and the cholate bound by the resin determined by difference.

In the *in vivo* method, a hypercholesteremic diet was fed to day-old chicks for 2 weeks; graded levels of the anion exchange resin were added to the diet during the second week. Plasma cholesterol levels were then determined as an indicator of the activity of the resin.

The relative activities of the various preparations of cholestyramine and other anion exchange resins compared very well when tested by the two methods of assay. Addition of excipients in preparations of cholestyramine for clinical use had no effect on cholate binding or hypocholesteremic activity.

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‡ Questran®, brand of cholestyramine, Mead Johnson & Company.

§ Dowex 2 x 1 was generously supplied by Dow Chemical Co., Midland, Mich.

‖ Amberlite resins were generously supplied by Rohm and Haas, Inc., Philadelphia, Pa.

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