Bioassay of Hypocholesterolemic Steroids

By R. M. TOMARELLI, T. M. DOUGHERTY, and F. W. BERNHART

The hypocholesterolemic activity of a number of natural and synthetic steroids was determined in a 3-day oral assay using male rats with hypercholesterolemia of dietary origin. The reduction in serum cholesterol was linearly related to the log dose of the administered natural or modified estrogen; the effect was evident 6 hr. after oral administration and had reached a maximum in 24 to 30 hr. Active steroids lowered the elevated serum phospholipids, cholesterol/phospholipid ratios, triglycerides, α and β lipoproteins, and β/α lipoprotein ratios, but not the elevated serum globulins. The effect of the steroids on body weight permitted a semiquantitative estimation of feminizing activity.

THE LOWER incidence of atherosclerotic heart disease in premenopausal women than in men and the demonstration that estrogen administration will lower serum lipids in man and animals have prompted a search for antilipemic steroids that have little or no feminizing activity (1,2). The present report describes the development of a 3-day bioassay for the screening of orally active hypocholesterolemic compounds and presents the results obtained with a number of natural and synthetic steroids.

EXPERIMENTAL

Analytical Procedures.—The following methods were used for determining serum constituents: cholesterol, Zlatkis *et al.* (3) and Technicon Auto-Analyzer procedure (4); triglyceride, van Handel and Zilversmit (5); phospholipid, Whitley and Alburn procedure (6); protein, Lowry *et al.* (7).

Serum lipoproteins were estimated from the cholesterol content of serum protein fractions separated by paper electrophoresis. The relative proportions and locations of the protein fractions were determined on strips stained with azocarmine G; cholesterol associated with these areas was eluted from duplicate unstained strips and determined colorimetrically. The designation of protein fractions as α or β lipoproteins was according to Searcy *et al.* (8).

Assay Procedure.—The assay animals were male rats with serum cholesterol levels elevated 7 to 10 times normal by the feeding of a hypercholesterolemic diet. The diet used was essentially that of Nath *et al.* (9); it consisted of 18% casein,[1] 25% hydrogenated coconut oil,[2] 4% salts, (U.S.P. XIV) 1% cholesterol, 0.5% cholic acid, 0.2% choline chloride, an adequate amount of all required vitamins (10), and sucrose to make 100%. Groups of 70 to 80 young male Sprague-Dawley rats, 100–120 Gm. body weight, were prepared for assay by feeding this diet *ad libitum* for 3 weeks. Average weight gain during this period was 70–100 Gm. Average serum cholesterol levels determined on 0.01 ml. of serum from tail blood varied from 600–1000 mg.%.

The rats were divided into groups of six with nearly equal average body weights and serum cholesterol levels. Steroids were given orally for 3 days in 0.2 ml. cottonseed oil solution; the control group received oil vehicle alone. Three groups received graded doses of an appropriate standard steroid; test compounds were administered at a single 1-mg. dose level. On the morning of the fourth day the rats were weighed and serum cholesterol was determined. The average serum cholesterol values of the three standard groups, plotted against the log dose, gave a linear relationship from which the hypocholesterolemic activity of the test compounds were evaluated. Activity was expressed relative to the standard.

The effect of the test steroid on body weight was also evaluated by comparison with that of the standard. Since estrogen administration to a male rat will inhibit food consumption and retard body weight gain in a young animal or cause weight loss in an adult (11), a lack of effect of the steroid on body weight was accepted as an indication of a low degree of feminizing activity. Loss of weight, being nonspecific, could be considered as only suggestive evidence of feminization. The effect of the estrogenic steroids on body weight and food intake was roughly proportional to the log dose, particularly in young animals (Fig. 1). For semiquantitative evaluation the effects were graded 0 to +3, with 0 equal to the control value and +1 to that of the standard steroid. Pair-feeding experiments showed that the reduction in the intake of food (and cholesterol), comparable to that found in rats treated with estrogen for 3 days, had no appreciable effect on serum cholesterol concentration.

Fifteen days after the completion of a test, serum cholesterol was again determined and the rats regrouped for the next test which started 4 days later. Each group of hypercholesterolemic rats were routinely used for 4 to 6 assays.

The 3-day assay period followed by the 19-day rest period was selected after time studies revealed that serum cholesterol levels (*a*) decrease measurably about 5 hr. after oral administration, (*b*) drop to a constant value within 24–28 hr., and (*c*) return to pretest levels 5 days after the last dose of the steroid is given (Fig. 2). In earlier assays in which the steroids were administered for 3 weeks, no significant differences were found in the serum cholesterol levels determined at weekly intervals (Fig. 3).

The steroid used as a standard in all the tests reported in the present study was *dl*-13β-ethyl-3-methoxy-8α-gona-1,3,5(10)-trien-17β-ol (Wy-3359). This recently synthesized compound (12) has an antilipemic activity by subcutaneous injection 2.6

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[1] High nitrogen, Sheffield.
[2] Hydrol-100, Durkee.

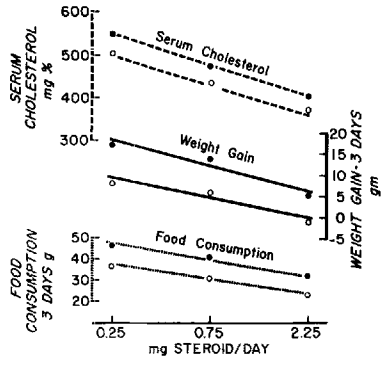

Fig. 1.—The effect of β-estradiol (○) and Wy-3359 (●) on serum cholesterol, weight gain, and food consumption of hypercholesterolemic rats.

times that of *d*-estrone but only 1% of its estrogenic activity (13). Oral administration of Wy-3359, 0.25 to 2.25 mg./day, resulted in a reasonably reproducible drop in serum cholesterol, 197 ± 10 mg./100 ml. (21 three-dose assays with six rats per group). This response, when expressed as per cent of the control, did not appear to be influenced either by the body weight in the 200–350-Gm. range, the initial serum cholesterol level, the season of the year, or the use of the animal in previous assays.

The confidence limits of the assay value could be estimated by any conventional procedure for a 4-point assay (three standards and one unknown) such as that of Gaddum (14). In practice, a convenient estimate of confidence limits was devised from the observation that the standard error was proportional to the average serum cholesterol level (9.2% ± 1.7). This value was calculated from the data obtained from 32 groups, six rats per group, which included controls and groups treated with a variety of steroids of different hypocholesterolemic activity. The 95% confidence limits were as follows: for compounds having activity near 25%, 7–75%; near 100%, 68–160%; and near 200%, 145–280%.

Serum Constituents.—Serum samples taken under routine assay conditions, *i.e.*, from unfasted rats after 3 days of oral steroid administration, were analyzed for a number of constituents in addition to cholesterol. Typical results are presented in Table I and Fig. 4. Compared with Purina chow-fed controls, the hypercholesterolemic rats had elevated serum proteins, phospholipids, cholesterol-phospholipid ratios, α and β lipoproteins, and β-to-α lipoprotein ratios. Serum triglycerides were slightly elevated with considerable individual variation presumably because of the unfasted state of the animal. The increase in the serum proteins was in the globulins, particularly in the β fraction (Fig. 4).

The oral administration of β-estradiol or Wy-3359 reduced all the lipid constituents (Table I); the decrease in cholesterol was relatively greater than that of phospholipids resulting in a reduced cholesterol–phospholipid ratio. Both α and β lipoproteins were reduced, the β considerably more than the α (Fig. 4). With increasing doses of Wy-3359, the β/α lipoprotein ratio of the hypercholesterolemic rat dropped from 1.08 to 0.59, approaching that of the chow-fed controls, 0.41. However, steroid administration had no effect on the elevated globulin fractions (Fig. 4); *i.e.*, only the cholesterol moiety of the globulin fractions was decreased.

Comparative Hypocholesterolemic Potencies.—Table II presents the hypocholesterolemic activity, as determined by the 3-day oral assay, of a number of natural and synthetic steroids. Many of these compounds have been studied previously by other investigators for antilipemic activity in the rat (see references in table). The most active compound by far was ethinyl estradiol, followed by estriol, β-estradiol, estrone, equilin, and equilenin, all with approximately equal activity. This order of activity is similar to that reported for oral estrogenicity (26). 17α-Estradiol was about half as active as the β isomer. The 3-methyl ethers of estrone and β-estradiol were slightly more active than the parent compounds. The Δ^4 steroids, methyltestosterone, progesterone, and norbolethone were inactive. The ineffectiveness of methyltestosterone corroborates the results obtained by Abel and Mosbach (20), who found that while the steroid had hypocholesterolemic activity in rats with normal or slightly elevated serum cholesterol, it was inactive in hypercholesterolemic animals.

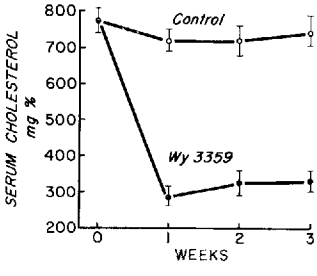

Fig. 3.—Effect of Wy-3359, 1 mg./day, 6 days a week for 3 weeks, on serum cholesterol.

Compared with the standard, Wy-3359, all of the natural steroid estrogens and diethylstilbestrol had a relatively greater adverse effect on body weight than on serum cholesterol reduction. The favorable separation of antilipemic and estrogenic activity found in the synthetic steroids, Wy-3271, Wy-3714, and Wy-6012, has previously been reported (21–23).

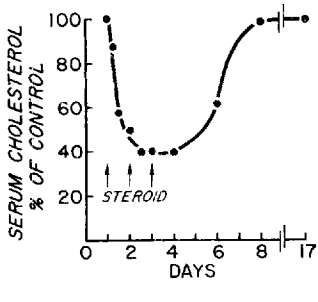

Fig. 2.—Time study of the effect of Wy-3359, 2.25 mg./day for 3 days, on serum cholesterol.

DISCUSSION

The hypocholesterolemic activity of a number of steroids, determined by this 3-day procedure with hypercholesterolemic rats, was in general agreement with values obtained by other procedures, indicating that a 3-day assay period is of adequate duration.

TABLE I.—EFFECT OF HYPOCHOLESTEROLEMIC STEROIDS ON SERUM CONSTITUENTS UNDER ASSAY CONDITIONS

Dietary Group[a]	Steroid[b]	Serum Constituent/100 ml. ± S. E. Protein, Gm.	Cholesterol,[c] mg.	Phospholipid, mg.	Chol./Phosp. Ratio	Triglyceride, mg.
Hypercholesterolemic	None	8.9 ± 0.3	643 ± 33	342 ± 4	1.87 ± 0.06	181 ± 36
Hypercholesterolemic	Wy-3359 0.75 mg.	8.8 ± 0.2	251 ± 16	235 ± 7	0.98 ± 0.06	70 ± 19
Hypercholesterolemic	β-Estradiol 0.75 mg.	8.9 ± 0.2	247 ± 37	267 ± 33	0.91 ± 0.05	39 ± 14
Chow	None	6.8 ± 0.1	73 ± 4	163 ± 10	0.46 ± 0.05	84 ± 21

[a] Six rats per group; average body weight 320 Gm. [b] Daily oral dose for 3 days. [c] Total cholesterol; the free cholesterol proportion of 20% was not affected by steroid administration.

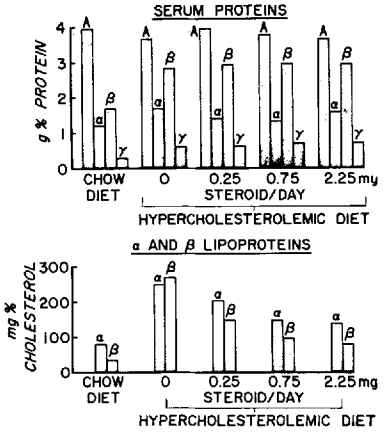

Fig. 4.—Effect of Wy-3359 on serum proteins and α and β lipoproteins. The β/α ratio was 0.41 for chow-fed rats and 1.08 for the hypercholesterolemic rats, but decreased in the latter to 0.87, 0.67, and 0.59 with the increasing doses of the steroid.

In addition to saving in time and material, the procedure has several other advantages over assays of longer duration with rats fed diets containing no added cholesterol. The small amount of hypercholesterolemic blood required for chemical analysis does not necessitate sacrifice of the animals; they can be used for a series of assays, and determinations can be run on the same animal before and after steroid administration, permitting a more precise measurement of effect. The decrease in food consumption, resulting from estrogenic steroid administration, did not lower serum cholesterol, presumably because of the high content of cholesterol in the diet and the brevity of the treatment.

While the rats varied in their response to the hypercholesterolemic diet, with serum values ranging from 400 to 1300 mg./100 ml., daily variations were small and the response to an antilipemic steroid was not related to the degree of existing hypercholesterolemia. The seasonal fluctuations in serum cholesterol in male rats fed diets without added cholesterol, noted by Thorp and Waring (24) and Edgren (27), has not been noted in our animals

TABLE II.—HYPOCHOLESTEROLEMIC ACTIVITY OF NATURAL AND SYNTHETIC STEROIDS

Common Name	Chemical Name	Hypocholesterolemic Activity	Body Wt. Effect	Ref.
Wy-3359 (std.)	*dl*-13β-Ethyl-3-methoxy-8α-gona-1,3,5(10)-trien-17β-ol	1.0	1	(12)
d-Estrone	*d*-3-Hydroxyestra-1,3,5(10)-trien-17-one	2.0	3	(15, 16)
β-Estradiol	*d*-Estra-1,3,5(10)-trien-3,17β-diol	1.8	>3	(17–19)
17α-Estradiol	*d*-Estra-1,3,5(10)-trien-3,17α-diol	1.0	2	...
Estriol	*d*-Estra-1,3,5(10)-trien-3,16α,17β-triol	3.5	>3	(2, 17)
Equilin	*d*-3-Hydroxyestra-1,3,5(10),7-tetraen-17-one	2.3	>3	...
Equilenin	*d*-3-Hydroxyestra-1,3,5(10),6,8-pentaen-17-one	2.3	>3	...
d-Estrone-3-methyl ether	*d*-3-Methoxyestra-1,3,5(10)-trien-17-one	2.5	3	...
β-Estradiol-3-methyl ether	*d*-3-Methoxyestra-1,3,5(10)-trien-17β-ol	3.8	>3	...
17α-Estradiol-3-methyl ether	*d*-3-Methoxyestra-1,3,5(10)-trien-17α-ol	0.8	1	...
Ethinyl β-estradiol	*d*-17α-Ethynylestra-1,3,5(10)-trien-3,17β-diol	65–90	>3	(19)
Methyltestosterone	*d*-17α-Methylandrost-4-en-17β-ol-3-one	0	0	(20)
Progesterone	*d*-Pregn-4-en-3,20-dione	0	0	(17)
Norbolethone	*dl*-13β,17α-Diethyl-17β-hydroxygon-4-en-3-one	0	0	...
Wy-3271	*dl*-13-Ethyl-3-methoxygona-1,3,5(10)-8,14-pentaen-17-one, cyclic ethylene acetal	1.2	1	(21)
Wy-3714	*dl*-13-Ethylgona-1,3,5(10)-triene-3,17β-diol	0.3	0.3	(22)
Wy-6012	*dl*-3-Methoxy-6-oxa-8α-estra-1,3,5(10)-trien-17β-ol	0.9	0.9	(23)
Diethylstilbestrol	4-4′-Dihydroxy-α,β-diethylstilbene	2.9	>3	(17, 19)
	Conjugated equine estrogens[a]	2.5	>3	...
	Ethyl(*p*-chlorophenoxy)isobutyrate[b]	0.007	0	(24)
	1-[*p*-(β-Diethylaminoethoxy)phenyl]-1(*p*-tolyl)-2-(*p*-chlorophenyl)ethanol[c]	Trace	2	(25)

[a] Marketed as Premarin by Ayerst. Activity relative to estrone sulfate content. [b] CPIB. Imperial Chemical Industries. Daily dose, 20–50 mg. [c] Trademarked as Triparanol by Merrell. Daily dose, 25 mg.

presumably because these changes are inconsequential in relation to the high concentration of serum cholesterol.

It should be noted that the above assay procedure is unsuitable for the screening of compounds that lower serum cholesterol by interfering with cholesterol biosynthesis, since this process is markedly reduced in rats fed high cholesterol diets (28, 29).

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Interaction of Substituted Benzoic Acids with Cationic Surfactants

By LUCY S. C. WAN

The interaction of benzoic acid and a series of hydroxy, amino, chloro, and nitrobenzoic acids with surfactants of the quaternary ammonium type was studied by means of viscosity measurements. Only salicylic acid was found to interact with the surfactants leading to an increase in viscosity which reached a maximum at approximately the concentration at which the surfactant solution became saturated with salicylic acid. Apparently the interaction is specific and is limited to the *ortho* hydroxy substitution of benzoic acid, as no viscosity effect was observed with the *ortho, meta,* and *para* isomers of amino, chloro, and nitro substituted benzoic acids. In addition, sodium lauryl sulfate and cetomacrogol 1000 did not exhibit viscosity changes in the presence of salicylic acid. The viscosity effect was not related to pH.

BENZOIC ACID and its substituted isomers have been found to interact with a varied number of pharmaceutical compounds. Salicylic acid, *meta*, and *para* hydroxybenzoic acids have been shown to complex with polyethylene glycols (1) and with polyvinylpyrrolidone (2). In addition, the hydroxybenzoic acids have been found to interact with caffeine (3) and with theophylline and theobromine (4). Goodhart and Martin (5) reported that the solubilities of benzoic acid and its substituted isomers were greatest in the least hydrophilic of the polyethylene stearates. The solubilities of benzoic acid, salicylic acid, and *p*-hydroxybenzoic acid have also been found to increase in the presence of their sodium salts and the addition of a hydroxy group to the acid led to the formation of complexes (6).

Chelation of ferric and aluminum ions with salicylic acid and its amino, chloro, and nitro derivatives have been demonstrated by Foye and Turcotte (7). Results from the investigation of the action of Schardinger dextrins on benzoic acid and hydroxybenzoic acids did not seem to indicate a clear cut mechanism of the complexation involved. Apparently hydrogen bonding is an important factor in the cyclodextrin–hydroxybenzoic acid interaction (8). Schlenk and Sand (9) showed that the molecular ratio of cyclodextrin to certain benzoic acid derivatives was a function of the physical state of the complex. Monohalogenated benzoic acids and

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