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Effects of Δ^4-Cholestenone in Animals and in Man. (23880)

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Feeding of large amounts of Δ^4-cholestenone* will lower the levels of serum cholesterol in rats(1,2), dogs and chickens(3). This effect on serum cholesterol may be related to suppression of endogenous cholesterol biosynthesis by cholestenone, shown first by Tomkins, Sheppard and Chaikoff in acute experiments(4) and confirmed in this laboratory in chronic feeding experiments(1). However, high dose levels (1 g/kg/day) of cholestenone are highly toxic to rats, causing sharp arrest of normal growth rate and accumulation in tissues of dihydrocholesterol, a major product of cholestenone metabolism(5-7). The other outstanding effect of chronic feeding of cholestenone at high dose levels is a remarkable hypertrophy of the adrenal gland to 7 times normal weight(1). This hypertrophy is associated with sharply reduced rate of secretion of corticosteroids—both corticosterone and aldosterone(8). The present studies were carried out to evaluate general toxicity of cholestenone in different species; to study absorption and metabolic fate of the compound, particularly degree of conversion to dihydrocholesterol; and to further explore adrenal changes induced by large doses.

Methods. Male Osborne-Mendel rats were maintained on basic diet of following percentage composition: casein, 34.5; sucrose, 31.0; corn starch, 21.5; cottonseed oil, 6.5; salt mixture(9), 5; cod liver oil, 1; choline, 0.1 and supplements of vitamins. Cholestenone,† dihydrocholesterol and cholesterol were

* Referred to subsequently simply as cholestenone.

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incorporated into basic diet at various levels and the diet fed *ad lib.* Rabbits and guinea pigs were fed standard laboratory chow (B. and B. Small Animal Laboratory Diets). Sterols were added to these diets by blending appropriate amount of ether solution of sterol with weighed amount of ground pellets and then allowing solvent to evaporate. *Lipid analyses.* Published procedures were used for determination of serum cholesterol(10) lipid phosphorus(11) and total lipids(12). Tissue lipids were extracted by homogenizing the tissue in Waring blendor for 3 minutes with 1:1 acetone-ethanol and allowing homogenate to stand overnight at room temperature. Aliquots of extract were analyzed as in the case of serum lipids. Dihydrocholesterol was determined indirectly. Acetone-ethanol extracts of serum were saponified and total cholesterol was determined on one aliquot by Liebermann-Burchard color reaction(13). The digitonin-precipitable sterols were recovered from a second aliquot. The digitonide was washed 3 times with 50% ethanol, once with ether: acetone (2:1), and finally with ether, then dried to constant weight. The contribution of cholesterol, calculated from Liebermann-Burchard color yield, was subtracted and the difference attributed to digitonide of dihydrocholesterol. On several samples, the digitonides were cleaved with pyridine and the recovered sterols were dried to constant weight. The percentage of cholesterol therein as determined by Liebermann-Burchard reaction was in very good agreement with that obtained by the first method. These indirect methods would include any 3-OH sterol yielding little or no color under conditions of Liebermann-Burchard reaction but forming a digitonide with solubility like that of cholesterol. For this reason we have used single quotes throughout to indicate 'dihydrocholesterol' determined by these methods. While these determinations are not specific, the large amount of 'dihydrocholesterol' found in serum and tissues of experimental animals described below is most likely correctly identified as such(14). *Radiochemicals and radioassay.* Cholesterol-4-C^{14} was purchased from Beta Laboratories; cholestenone-4-C^{14} from Tracerlab. Liquid scintillation counting was used for radioassay of lipid fractions (Packard Tri-Carb Scintillation Spectrometer). Samples were taken to dryness in the counting bottle and 15 ml of an 0.6% solution of diphenyloxazole in toluene were added. When necessary, as in counting of highly colored fecal lipid extracts, a correction for quenching was made after adding known amount of palmitic acid-1-C^{14} to each vial and recounting. *Other analyses.* Ascorbic acid was determined by titration of cold 5% trichloroacetic acid (TCA) extracts of tissues with 2, 6-dichloroindophenol(15). In some instances the method of Roe(16) was also applied but in no case was more than 5% of ascorbic acid present in oxidized form. Protein nitrogen was determined by micro-Kjeldahl analysis of TCA-insoluble tissue residues after extraction of nucleic acids with hot TCA and extraction of lipids with alcohol-ether(1:1) and ether.

Results. General toxicity. Fed at a level of 1% by weight in the basic diet, cholestenone is highly toxic to rats, as previously reported(1). Appetite fails and growth curve quickly falls below that of rats on control diet. Since cholestenone was fed as constant percentage in diet, the daily intake of the compound varied with size of rat, ranging from about 1 g/kg in 100 g rats to about 700 mg/kg in 300 g rats that had been on the diet for 5 or 6 weeks. Growth was virtually arrested by the fifth week. Animals became lethargic, the coat became scruffy and there was crusting and discharge about the nose. There was a 20 to 30% mortality in 8 weeks. It was shown in paired feeding experiments that rats receiving control diet in amounts equal to that ingested by rats on cholestenone, had a growth curve not differing significantly from that of the latter (Table I). It was concluded that the principal effect of cholestenone on growth was due to decreased food intake, not to changes in absorption or energy metabolism.

Effects of cholestenone on adrenal gland. The most striking finding in rats given cholestenone at the 1% level was a remarkable hypertrophy of the adrenal glands(1). Histologic examination showed that hypertrophy was limited to the cortex. After 8 weeks on the drug the net weight of combined glands was 5 to 7 times that of glands from control

TABLE I. Adrenal Effects of Δ^4-cholestenone in Paired Feeding Study.*

Rat	Body wt (g)		Combined adrenal wt (mg)	Adrenal ascorbic acid conc. (mg/g)	Adrenal cholesterol conc. (mg/g)	Relative thymus wt (mg/100 g)
	Initial	Final				
Control A	112	185	40.6	5.88	57.4	190
Exp. A	113	185	202.6	.90	5.5	242
Control B	108	219	31.9	5.34	60.6	183
Exp. B	108	215	85.6	1.86		158
Control C	105	189	26.1	6.00	39.5	183
Exp. C	109	189	68.6	2.34	17.4	217

* Experimental rats were fed a 1% Δ^4-cholestenone diet *ad lib* for 4 wk; control rats were fed the basic diet in amounts equal to those consumed by the paired experimental rats.

rats of same age. Little or no increase in gland size occurred during the first 18 days on the diet but after 36 days there was a 3-fold increase. This hypertrophy is readily reversed by removing cholestenone from the diet. After 6 weeks on the drug, protein nitrogen/g wet weight was decreased by only about 20% while total mass had increased more than 3-fold. There was a definite increase in neutral fat, but the most impressive change was the marked fall in cholesterol concentration from normal value about 60 mg g, to less than 10 mg/g. Phospholipid concentration did not change significantly. Another striking finding was an early fall in ascorbic acid concentration. After 18 days on cholestenone, although little or no adrenal hypertrophy had yet developed, there was a 38% fall in ascorbic acid concentration. After 36 days there was 85% decrease in ascorbic acid concentration. Again total quantities and concentrations of cholesterol in glands of treated animals were sharply depressed. Results from paired feeding study (Table I) indicate that this was not due to malnutrition.

Adrenal function in cholestenone-fed rats. As discussed previously(1), the known inhibitory effect of cholestenone on cholesterol biosynthesis suggested that the observed adrenal hypertrophy might be related to a similar block in adrenal steroid biosynthesis or to deficiency of new cholesterol to serve as steroid precursor. Findings of low cholesterol concentration and low ascorbic acid concentration would be compatible with such a postulate. Absence of thymic involution with marked adrenal hypertrophy (Table I) also suggested hypofunction at least/unit mass of gland.

Direct demonstration that adrenocorticosteroid secretion is in fact depressed was reported recently by Fredrickson, Peterson and Steinberg(8). Analyses were carried out on adrenal vein blood of pair fed rats on control diets and on 1% cholestenone diet. Output of corticosterone, the primary adrenal steroid in the rat, was reduced to 15% of control values after 6 weeks on cholestenone. Expressed as output/unit weight of adrenal gland, the rate of corticosterone secretion was only 3% of control values. Attempts to reverse the effects of cholestenone on growth by daily injections of cortisone (0.2 mg daily) were unsuccessful. Even when, in a second study, steroids were started at same time as cholestenone diet and given in larger doses (1 mg daily), growth rate was not improved compared to that of animals receiving no steroid. Corticosterone was no more effective than cortisone. Adrenal hypertrophy on the other hand, was completely or almost completely prevented, thus confirming the importance of pituitary stimulation in development of adrenal hypertrophy. Failure of exogenous steroid to permit normal growth rate suggests that at these high dosages cholestenone has toxic effects over and above those on adrenal glands.

Partial protective effect of dietary cholesterol. Our basic diet was virtually cholesterol-free. It was necessary to consider the possibility that inhibition of endogenous cholesterol synthesis by cholestenone was inducing a relative sterol 'deficiency'. Results of a feeding experiment to determine whether or not simultaneous feeding of cholesterol would protect against the toxic effects of cholestenone are shown in Table II. In terms of growth rate there was a decided protective ef-

TABLE II. Partial Protective Effect of Dietary Cholesterol against Toxic Effects of Δ^4-cholestenone Diet.†

	1% Δ^4-cholestenone alone	1% Δ^4-cholestenone + 1% cholesterol	1% cholesterol alone
Initial body wt, g	113 ± 12	116 ± 14	114 ± 10
Final body wt, g	187 ± 47	296 ± 35	307 ± 56
Serum cholesterol, mg %	43.4 ± 7.3	84.1 ± 13.9	80.2 ± 16.5
Combined adrenal wt, mg	136*	89*	36*

* Mean wt/pair of pooled glands.

† 10 rats in each group.

fect. There was a marked fall in serum cholesterol level of animals receiving cholestenone alone but none in animals receiving cholestenone plus cholesterol. Rats on cholestenone alone showed almost a 4-fold hypertrophy of adrenal glands; those receiving both compounds also had hypertrophied glands, but the degree of enlargement was again somewhat less than that in rats not receiving cholesterol. From these results it appears that simultaneous feeding of cholesterol does indeed partially ameliorate, although it does not completely prevent toxic effects of cholestenone.

Absorption and distribution of Δ^4-cholestenone-4-C^{14}. Two rats weighing approximately 250 g were given by stomach tube cholestenone-4-C^{14} dissolved in Wesson oil. Urine and feces were collected for 24 hours, then the animals were killed. Intestinal contents were pooled with feces and total lipids were extracted into acetone-ethanol (1:1) and counted. As shown in Table III, over 70% of administered dose was absorbed in 24 hours. Simultaneous administration of cholesterol did not interfere with absorption of labeled cholestenone (Table III). Less than 0.01% of administered radioactivity appeared in the urine. To determine organ distribution, a larger dose of cholestenone-4-C^{14} was given by stomach tube to a 210 g rat and the animal killed 20 hours later. Results are summarized in Table IV. Total radioactivity in the non-saponifiable lipid fraction of each organ was determined (cholestenone-C^{14} plus any labeled non-saponifiable products). Highest concentrations of radioactivity were found in liver and intestine. Other tissues contained relatively small amounts of radioactivity with the exception of serum and adrenal glands. In relation to organ weight the concentration in the latter was actually 10 times higher than that in liver. This relative concentration of cholestenone and its metabolites in the adrenal gland is of particular interest in view of the profound effects of the compound on adrenals discussed above.

The feces contained, besides the 5.4 x 10^6 cpm in non-saponifiable lipid, an additional 6 x 10^6 cpm in saponifiable form. This acid fraction most probably represents the modified bile acids shown by Harold, Abraham and Chaikoff to be end-products of cholestenone metabolism in the rat(7). Almost certainly the material in this fraction has been absorbed and subsequently re-excreted into the intestine. If it is included in the "absorbed" fraction in computing a radioactivity balance, 63% of administered dose was absorbed in experiment shown in Table IV. The value of 70% absorption indicated in Table III should, for similar reasons, be considered a minimum value.

Conversion of Δ^4-cholestenone to 'dihydrocholesterol'. That cholestenone is effectively reduced to dihydrocholesterol by rats was clearly shown by isotopic studies of Anker and Bloch(5) and amply confirmed by Stokes, *et al.*(6) and by Harold *et al.*(7). Little or no conversion to cholesterol itself could be demonstrated(5-7). In previous studies on rats receiving 1% cholestenone it was shown that in chronic feeding experiments 'dihydro-

TABLE III. Intestinal Absorption of Δ^4-cholestenone-4-C^{14} in the Rat.

Dose*			
Δ^4-cholestenone-4-C^{14} (mg)	(cpm)	Cholesterol (mg)	Radioactivity absorbed in 24 hr (%)
9.6	4.05 × 10^6	0	72
9.4	3.97 "	0	76
9.4	3.94 "	9.4	73
8.8	3.69 "	8.8	79

* Administered by stomach tube in 1.5 ml Wesson oil to rats weighing between 230 and 280 g.

TABLE IV. Distribution of Radioactivity 20 Hr after Oral Administration of Δ^4-cholestenone-4-C^{14}.*

Tissue	Total radioactivity (cpm)† × 1000
Liver	1,100
Intestine (washed)	505
Serum	280
Adrenals	176
Lungs	68
Spleen	44
Kidney	27
Heart	14
Brain	4
Remaining carcass	1,020‡
Feces§	5,400

* 60 mg (22.5 μc) Δ^4-cholestenone in 4 ml Wesson oil given by stomach tube 20 hr prior to sacrifice.

† Total nonsaponifiable lipid fraction counted in Packard Tri-carb liquid scintillation counter.

‡ 93% of this radioactivity could be precipitated with digitonin.

§ Total 20 hr fecal output pooled with intestinal content at time of sacrifice.

cholesterol' accumulates in liver and other tissues(1). The total non-saponifiable lipid in livers of animals receiving the compound for 8 weeks at 1% level, was more than doubled, while cholesterol content was not appreciably altered. We have now shown that there is an even greater relative accumulation of dihydrocholesterol in the adrenal gland. Here cholesterol concentration falls sharply (Table I) but *total* digitonin-precipitable sterol concentration actually rises somewhat (88 mg g). Over 85% of this material is 'dihydrocholesterol'. The extent to which cholestenone is metabolized to dihydrocholesterol or to related 3-β-hydroxy forms is shown by studies of non-saponifiable carcass lipid after a single dose of cholestenone-4-C^{14}. As indicated in Table IV, 93% of non-saponifiable radioactivity was precipitable with digitonin, indicating that almost all of this material has been converted to a 3-β-hydroxy form.

Accumulation of 'dihydrocholesterol' in serum of rats is shown in Table V. The serum of animals on control diets showed a small amount of 'dihydrocholesterol' (3-6% of total sterol). After 40 weeks on low dose level of cholestenone (0.1%) the average level of 'dihydrocholesterol' was 31 mg%, accounting for 30% of total digitonin-precipitable sterol. Although serum cholesterol level was decreased in these animals, *total* serum sterol remained virtually unchanged. At the high dose level (1%) the 'dihydrocholesterol' concentration after only 7 weeks had reached 62 mg %, accounting for over 70% of total digitonin-precipitable sterol in serum. In this case total sterol level was actually *increased* even though cholesterol level was depressed more than 50%.

Effect of dihydrocholesterol feedings. In view of considerable amounts of dihydrocholesterol in serum and liver of animals receiving large doses of cholestenone it was necessary to consider the possibility that the observed toxic effects, in particular that upon the adrenal, might be due not to cholestenone itself but rather to this metabolic derivative. Dihydrocholesterol was fed to rats at level of 1% in diet. In marked contrast to results obtained with equal dosages of cholestenone there was little or no impairment of growth in an 8-week period. On the other hand, there was an almost 100% increase in adrenal weight and some fall in serum cholesterol (controls 54.4 mg%; dihydrocholesterol-fed, 44.6 mg %).

The fact that dihydrocholesterol was much less toxic in rats than cholestenone does not necessarily rule out the possibility, discussed above, that the former compound may be directly responsible for some of the toxic effects. Cholestenone appears to be better absorbed than dihydrocholesterol and, after conversion, may give rise to higher body concentrations of dihydrocholesterol than are

TABLE V. 'Dihydrocholesterol' in Pooled Sera from Rats on Δ^4-cholestenone Diets.

Diet	Weeks on diet	Serum cholesterol* (mg %)	Serum 'dihydrocholesterol'† (mg %)
Control (9)‡	40	95	6
.1% Δ^4-cholestenone (9)	40	74	31
Control (17)	7	55	2
1.0% Δ^4-cholestenone (37)	7	24	62

* Liebermann-Burchard reactive sterol.

† Sterol precipitated by digitonin but not Liebermann-Burchard reactive.

‡ No. of animals in each group shown in parentheses.

reached by feeding the compound itself.

Results at low dose levels. In contrast to results at high dose levels, drug levels up to 0.1% in the diet (70-100 mg/kg/day) did not impair appetite or limit growth. Rats have been maintained on this level of cholestenone in the diet for over a year without impairment of growth or other gross evidence of toxicity. Furthermore, cholestenone at 0.1% level caused no increase in adrenal weight in 7 weeks. After 40 weeks at this dose level there was approximately 30% increase in adrenal weight, but this was mostly due to lipid accumulation.

Clinical studies. It was of interest to study effects of cholestenone on patients with hypercholesterolemia, since abnormally high initial levels of circulating sterol might lead to a response different from that observed in normocholesterolemic experimental animals. Several patients with essential hypercholesterolemia were given 2.5 to 5.0 g cholestenone daily by mouth. This dosage corresponds to that given to rats receiving 0.1% cholestenone added to basic diet (60-80 mg/kg/day). The drug was well tolerated and there were no clinical signs or symptoms of toxicity. In view of the toxic effects of high dose levels in experimental animals (600-800 mg/kg/day) careful checks were made of hepatic, renal and adrenal function. None of the measured parameters changed with one exception. After several weeks there was a decided elevation in bromsulfalein retention test, up to 26%. None of the other measures of liver function (cephalin flocculation, thymol turbidity, prothrombin time, serum bilirubin and total serum proteins) were affected. The elevation of bromsulfalein retention in these patients was unexpected since at this dosage level in rats there was no accumulation of lipid in the liver and histologically no signs of hepatic damage. Clinical studies were interrupted because of this finding.

The effect on serum sterols in patients was also quite different from that seen in experimental animals on comparable doses. As shown in Table VI, patient C.B., an untreated hypercholesterolemic, had a small but significant amount of 'dihydrocholesterol' in her serum. Patients receiving 2.5 to 5.0 g cholestenone daily developed very high levels of dihydrocholesterol in serum, in one case actually exceeding the level of serum cholesterol. Levels of serum cholesterol did not change significantly. These results are in contrast with results in rats receiving equivalent dosage for 40 weeks, when cholesterol level fell significantly and total sterol level in serum did not rise.

TABLE VI. Serum 'Dihydrocholesterol' Concentrations in Hypercholesterolemic Patients Receiving Δ^4-cholestenone.

Patient	Dosage	Serum cholesterol* (mg %)	Serum 'dihydrocholesterol'† (mg %)
C.B.	None	509	21
F.G.	5 g/day for 46 days	475	549
	After 35 days off drug	365	70
J.K.	2.5 g/day for 32 days and 5 g/day for 70 days	278	212

* Liebermann-Burchard reactive sterol.
† Sterol precipitated by digitonin but not Liebermann-Burchard reactive.

Discussion. The present studies have shown that at low dose levels (70-100 mg/kg/day) Δ^4-cholestenone is well tolerated by rats for a year. Serum cholesterol level is decreased but total serum sterol level is not lowered since there is at the same time an accumulation of dihydrocholesterol. Similar dose levels in man lead to a much more striking accumulation of dihydrocholesterol in serum, in some cases doubling total sterol concentration. The elevation of bromsulfalein retention in patients on Δ^4-cholestenone suggests dihydrocholesterol accumulation in the liver also, an effect observed in rats only at much higher dose levels. Chapman, Nichols and Chaikoff have indicated similar results in birds(17). These findings, particularly in view of the possible atherogenic properties of dihydrocholesterol(18,19), appear to rule out use of Δ^4-cholestenone itself in therapy of hypercholesterolemia. Compounds with related structure but which cannot be converted to dihydrocholesterol deserve study.

At high dose levels (700-1000 mg/kg/day) Δ^4-cholestenone is highly toxic to rats. The unexpected effects on the adrenal gland are of particular interest. There is a sharp depres-

sion of adrenocortical function(8), accompanied by marked hypertrophy. The most likely explanation for this hypertrophy lies in release of pituitary from the inhibitory action of adrenal steroid with consequent over-production of adrenocorticotrophic hormone. Enlargement is greater than any usually obtained with stress. This may be explained, however, since the situation is somewhat unique in that the adrenal gland cannot limit continuing ACTH stimulation as it normally would by steroid production.

The mechanism of effect on adrenal function is not known. It is well established that cholesterol is at least a *potential* precursor for most adrenocortical steroids(20). In view of the demonstrated effectiveness of Δ^4-cholestenone in inhibiting cholesterol biosynthesis the present findings may indicate an *obligatory* role for cholesterol. Δ^4-cholestenone may actually be inducing a relative cholesterol 'deficiency'. The partial protective effect of exogenous cholesterol is compatible with this interpretation. On the other hand, since the biosynthetic pathways for cholesterol and for steroids are closely similar we may be dealing with a simultaneous interference with both and no conclusions regarding the role of cholesterol *per se* as an intermediate should be drawn. Finally, accumulation of dihydrocholesterol in the adrenal glands may be interfering with function mechanically or by adsorption on sites normally reserved for cholesterol.

The results obtained with Δ^4-cholestenone suggest that much valuable information about the role of cholesterol in body economy may be gained through use of this or similar inhibitors of cholesterol biosynthesis.

Summary. 1. Toxic effects of Δ^4-cholestenone fed at high dose levels to rats are described and data on absorption, distribution and fate of 4-C^{14}-Δ^4-cholestenone are presented. 2. A major effect of high dose levels of Δ^4-cholestenone is a marked inhibition of adrenal steroid secretion accompanied by marked hypertrophy of adrenal cortex. Some possible implications with respect to the physiological role of cholesterol in steroid biosynthesis are discussed. 3. Low dose levels are well tolerated, but lead to accumulation of dihydrocholesterol in serum in rats and, more strikingly, in man.

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