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AND LIPOPROTEINS OF RABBIT SERUM

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ABSTRACT

The cholesterol isomer, Δ^7 -cholestenol, has been fed to rabbits as part of a program to compare the effects of this sterol with that of cholesterol on the pathogenesis of atherosclerosis. The results of this research have shown that the serum lipoprotein pattern developed after the ingestion of Δ^7 -cholestenol is very similar to that observed after cholesterol feeding. It has also been observed that the Δ^7 -cholestenol feeding results in a rapid rise in the serum cholesterol level, a rise which is comparable to that which is observed after feeding cholesterol itself.

A method for the quantitative determination of Δ^7 -cholestenol is presented.

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INTRODUCTION

A large number of lipoproteins have been identified in the serum of humans and animals by Gofman et al.⁽¹⁾ through the use of the analytical ultracentrifuge. These lipoproteins account for over 95 percent of all serum lipids and have been divided arbitrarily into two groups: those of high density (> 1.063) and those of low density (< 1.063). In the low density group the lipoproteins are characterized by their varying Svedberg-of-flotation (S_f) values and good correlations have been found between the incidence of atherosclerosis and the quantities of lipoproteins in several of the S_f classes.^(2,3)

The cholesterol-fed rabbit develops huge concentrations of low-density serum lipoproteins and this process is markedly accelerated by the simultaneous feeding of Wesson oil with the cholesterol. Lipoproteins of the

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S_f 5-15 class are the first to increase in concentration with cholesterol feeding, and with time, lipoproteins of successively higher S_f values appear in the serum. After several weeks there are large quantities of all classes of low density lipoproteins in the serum.

The occurrence of Δ^7 -cholestenol as a widespread companion of cholesterol was reported by Fieser,^(4,5) who designated this sterol by the name "lathosterol." Our interest in Δ^7 -cholestenol as a compound of importance in the study of atherosclerosis was initiated by some early Δ^7 -cholestenol analyses which were carried out by Professor Fieser's group and which indicated that there was an unusually low quantity of Δ^7 -cholestenol in human serum which had a high content of S_f 10-20 lipoproteins. It is this particular group of lipoproteins which previously has been shown⁽¹⁻³⁾ to be associated with human atherosclerosis. Although later analyses failed to show anything unusual about the Δ^7 -cholestenol content of the isolated S_f 10-20 lipoproteins, it was decided to determine how this cholesterol analog compared with cholesterol itself in the rates with which the fed sterols cause the appearance of lipoproteins in the various S_f groups. A second object of this research was to determine if Δ^7 -cholestenol appeared to act as a precursor of cholesterol in the biogenesis of the latter sterol. Bloch and Langdon⁽⁶⁻⁸⁾ have reported that, on the basis of Δ^7 -cholestenol's inhibition of the in vivo synthesis of cholesterol from acetate, Δ^7 -cholestenol appears to be a cholesterol precursor.

This paper presents the results observed after feeding rabbits a diet high in Δ^7 -cholestenol and cholesterol (separately and simultaneously), followed by examination of the serum for (a) the appearance of lipoproteins

in various S_f groups, (b) changes in cholesterol concentration, and (c) the appearance of Δ^7 -cholestenol.

EXPERIMENTAL

Synthesis of Δ^7 -Cholestenol

Approximately 510 g. of Δ^7 -cholestenol was synthesized as follows: Cholesteryl benzoate was converted (procedure of Bernstein et al.)⁽⁹⁾ to 7-dehydrocholesterol via N-bromosuccinimide bromination, dehydrobromination with dimethylaniline, and hydrolysis of the 7-dehydrocholesteryl benzoate to the free sterol. The 7-dehydrocholesterol was converted to Δ^7 -cholestenol by dissolving 5 g. of the acetone-recrystallized sterol in 50 cc. of pure dioxane and hydrogenating* overnight in the presence of Raney nickel (prepared according to Adkins)⁽¹⁰⁾ and at about 3 atm. pressure. The completeness of the hydrogenation was followed both by the measured uptake of hydrogen and by the disappearance of the 282 μ absorption maximum of 7-dehydrocholesterol. After the catalyst was removed by filtration, the crude Δ^7 -cholestenol was precipitated on addition of water. The product was then twice recrystallized from acetone and the last traces of solvent were removed by drying under high vacuum.

The 510 g. of Δ^7 -cholestenol were obtained from about 5 kg. of cholesterol -- overall yield from cholesterol was therefore about 10%. The conversions to 7-dehydrocholesterol were carried out on 500 g. quantities of cholesteryl benzoate. The melting points of the recrystallized Δ^7 -choles-

* Conditions for the hydrogenation of 7-dehydrocholesterol to Δ^7 -cholestenol were kindly provided to us by Professor Louis F. Fieser.

tenol product were all in the range of 122-125° and the product was further identified by routine infra-red spectra* which showed the identity of our product with a sample of Δ^7 -cholestenol which was supplied to us by Professor Fieser. Occasionally the infra-red spectra of batches of our product indicated the presence (up to 10%) of cholesterol. These small amounts of cholesterol were not removed.

Rabbit Feeding

Three groups of rabbits were used in this study. There were 6 rabbits (3 males and 3 females) in each group. All rabbits were of the New Zealand white strain and weighed from 2.5 to 4.0 kg. at the start of the experiment. The first group was fed a diet containing 1% cholesterol and Wesson oil. The food was prepared by dissolving 1 pound of cholesterol in 1500 cc. of the oil, using gentle heating. The cholesterol-Wesson oil mixture was added to 100 pounds of Albers "family style" rabbit pellets and thoroughly mixed. The rabbits were given this diet and water ad lib.

The second group was similarly fed except that Δ^7 -cholestenol was substituted for cholesterol. The third group received a diet containing 1% cholesterol and 1% Δ^7 -cholestenol dissolved in the same quantity of Wesson oil as was used for the first and second groups.

Blood specimens were obtained before feeding was begun and at 1-week and 2-week intervals during the feeding. The blood specimens were analyzed for lipoproteins using the method described by Gofman et al. (1-3) and for cholesterol** by the Schoenheimer-Sperry method as modified by Colman. (11)

* We are grateful to Dr. Keith Freeman and Mr. Yook Ng for these spectra.

** The authors are indebted to Mr. David Colman for performing the cholesterol analyses.

The cholesterol analyses were corrected (as described below) to account for the presence of the Δ^7 -cholestenol which would otherwise give high values for the amounts of cholesterol.

Δ^7 -Cholestenol Analyses

The Δ^7 -cholestenol analyses were carried out on 2-5 cc. portions of the serum. The red cells were removed by centrifugation and proteins were precipitated with ten volumes of boiling 1:1 acetone-alcohol. To the supernatant liquid was added 4 or 5 drops of 50% aqueous KOH and the solution was either refluxed for one hour or allowed to stand overnight at room temperature. The solution was concentrated to 5-10 cc., made slightly acid with dilute aqueous HCl, and the sterol digitonides precipitated by the addition of a few cc. of a saturated solution of digitonin in ethanol. The digitonide was recovered by centrifugation, washed with 2:1 ether-acetone solution and then with ether, and dried under vacuum.

Determinations of the amounts of Δ^7 -cholestenol present in the digitonides were made by a modification of the procedure of Moore and Baumann.⁽¹²⁾ This procedure utilizes the great difference in the rates by which the sterols produce color by reacting with the Liebermann-Burchard⁽¹³⁾ reagent (9.5 cc. Ac_2O + 0.5 cc. conc. H_2SO_4). The digitonide to be analyzed was dissolved in glacial acetic acid (1-5 mg./cc.). Exactly 1 cc. of this solution was mixed with 2.0 cc. of the above reagent, the mixture quickly transferred into a cell of a Beckman spectrophotometer (Model DU), and the optical density (D) of the solution at 625 μ determined exactly one minute after mixing of reagent and solution. (The material in the compensating cell of the spectrophotometer was 2 cc. of the Liebermann-Burchard reagent mixed with

1 cc. of glacial acetic acid.) Δ^7 -Cholestenol digitonide reaches its maximum color intensity under these conditions in about one minute; at this same time the color intensity of the cholesterol digitonide is less than 1% of that of the Δ^7 -cholestenol digitonide. The values obtained for the optical densities at one minute for the two digitonides (both on the basis of 1 mg./cc. in the mixture of solution + reagent) are as follows: Δ^7 -cholestenol digitonide (infra-red spectrum demonstrated the absence of cholesterol) showed values for $D_{625m\mu}$ varying from 3.92 to 4.48; cholesterol digitonide (the cholesterol was purified of all Δ^7 -cholestenol by preparation of the dibromide, repeated recrystallization of the dibromide, and regeneration of the cholesterol) showed values for $D_{625m\mu}$ varying from 0.019 to 0.023. The variation in these values resulted from the different temperatures which happened to prevail in the laboratory at the time, and from different sources of solvents. However, each time analysis was made for the percent of Δ^7 -cholestenol in a given digitonide, the above values were determined at the same time and under exactly the same conditions. The recorded optical density for a given digitonide analysis was then converted (using Beer's law) to its value for a 1 mg./cc. solution (i.e., the final mixture of acetic acid solution + Liebermann-Burchard reagent) and, using the optical densities for the pure cholesterol and Δ^7 -cholestenol digitonide in the digitonide mixture was calculated from the relationship (the D values we used are for the digitonides, though they would be just as valid if the free sterols were used):

$$\% \Delta^7\text{-cholestenol} = \left[\frac{D_{\text{recorded}} - D_{\text{cholesterol}}}{D_{\Delta^7\text{-cholestenol}} - D_{\text{cholesterol}}} \right] \times 100$$

From determinations made on known, weighed mixtures of cholesterol and Δ^7 -cholestenol digitonides, it was estimated that percentages of Δ^7 -cholestenol in cholesterol could be obtained with maximum errors in the Δ^7 -cholestenol percentage values of ± 0.4 .

It was further determined that, 30 minutes after mixing the digitonide with the Liebermann-Burchard reagent, Δ^7 -cholestenol gives an optical density which is 2.0 ± 0.3 times that of cholesterol. Therefore cholesterol analyses made without regard to Δ^7 -cholestenol content would be high by a percentage of approximately 2 times the percent of Δ^7 -cholestenol present. Our cholesterol analyses (recorded in the tables below) were therefore corrected to account for the effect of the amounts of Δ^7 -cholestenol which had been determined separately in the manner described above.

RESULTS

The results are presented in Tables I, II, and III.

Table I

RESULTS OF Δ^7 -CHOLESTENOL FEEDING

Rabbit*	Lipoproteins, mg. %				Cholesterol, mg. %		Δ^7 -Cholestenol**** (± 0.4)
	S _f 5-15	S _f 15-30	S _f 30-100	S _f 100-400	Free	Total	
320A**	217	24	0	0		***	0.0
B	1153	374	81	100	333	1190	12.0
C	1193	519	183	164	216	1140	12.1
321A	204	8	0	0			0.0
B	1053	797	293	237	257	1385	8.8
C	786	796	365	211	335	1610	8.3
322A	65	0	0	0			0.0
B	605	122	72	25	94	528	4.8
C	585	257	61	0	108	543	8.4
323A	28	4	4	15			0.0
B	527	193	50	12	—	442	9.3
C	665	314	70	80	109	617	8.9
324A	30	0	0	2			0.0
B	608	144	44	59	112	591	12.5
C	880	220	61	89	167	780	10.5
325A	41	0	0	0			0.0
B	540	449	94	62	229	1145	10.0
C	683	739	178	103	258	1315	10.0

* The first three rabbits are female, the last three are male.

** The letter A denotes data from the rabbit at the start of the feeding program (control), B denotes the data after 1 week and C the data after two weeks of the sterol feeding.

*** The serum cholesterol in all these rabbits at the start of the feeding was in the range of 30-70 mg. %.

**** The Δ^7 -cholestenol percentage is the percentage of the digitonin-precipitable sterol which gives the typical fast-acting behavior of Δ^7 -cholestenol with the Liebermann-Burchard reagent. (See experimental part.)

Table II

RESULTS OF CHOLESTEROL FEEDING

Rabbit*	Lipoproteins, mg. %				Cholesterol, mg. %		Δ^7 -Cholestenol **** ($\pm$ 0.4)
	S _f 5-15	S _f 15-30	S _f 30-100	S _f 100-400	Free	Total	
263A**	15	6	4	4		***	
B	140	281	215	168	250	790	1.1
C	103	580	524	477	275	1305	1.6
264A	17	0	0	0			
B	248	178	42	9	82	303	0.0
C	196	243	150	140	148	1160	0.9
265A	11	0	2	2			
B	384	178	37	0	144	480	0.4
C	168	309	112	0	144	540	0.9
272A	73	0	0	0			
B	842	295	98	131	241	464	0.7
C	458	664	224	9	251	995	0.5
273A	34	0	0	2			
B	482	234	37	0	116	452	0.1
C	514	290	37	0	163	658	1.2
274A	58	6	2	4			
B	281	552	355	505	360	1510	1.7
C	178	617	655	402	360	1610	0.5

* The first three rabbits are male, the last three are female.

** The letter A denotes data from the rabbit at the start of the feeding program (control), B denotes the data after 1 week and C the data after two weeks of the sterol feeding.

*** The serum cholesterol in all these rabbits at the start of the feeding was in the range of 30-70 mg. %.

**** The Δ^7 -cholestenol percentage is the percentage of the digitonin-precipitable sterol which gives the typical fast-acting behavior of Δ^7 -cholestenol with the Liebermann-Burchard reagent. (See experimental part.)

Table III

RESULTS OF THE SIMULTANEOUS FEEDING OF BOTH CHOLESTEROL
AND Δ^7 -CHOLESTENOL

Rabbit*	Lipoproteins, mg. %				Cholesterol, mg. %		Δ^7 -Cholestenol**** (± 0.4)
	S _f 5-15	S _f 15-30	S _f 30-100	S _f 100-400	Free	Total	
266A**	17	0	2	6		***	
B	824	585	159	108	259	1038	6.6
C	337	804	365	56	270	1240	2.2
267A	13	0	0	0			
B	187	271	150	140	208	778	2.9
C	94	477	224	234	252	1157	0.8
268A	49	6	2	6			
B	1216	533	140	140	273	1058	5.7
C	842	767	299	103	286	1122	0.0
278A	40	0	0	0			
B	543	66	19	23	109	386	3.5
C	879	262	37	28	245	1049	1.1
279A	19	0	0	6			
B	458	524	393	309	324	1260	6.4
C	290	673	701	533	391	1760	0.9
281A	13	0	0	2			
B	314	318	154	61	183	1370	1.4
C	187	243	75	19	138	572	0.0

* The first three rabbits are male, the last three are female.

** The letter A denotes data from the rabbit at the start of the feeding program (control), B denotes the data after 1 week and C the data after two weeks of the sterol feeding.

*** The serum cholesterol in all these rabbits at the start of the feeding was in the range of 30-70 mg. %.

**** The Δ^7 -cholestenol percentage is the percentage of the digitonin-precipitable sterol which gives the typical fast-acting behavior of Δ^7 -cholestenol with the Liebermann-Burchard reagent. (See experimental part.)

DISCUSSION

These experiments were undertaken with the hope that Δ^7 -cholestenol might prove to be an antagonist or competitor to cholesterol absorption. The results above indicate that Δ^7 -cholestenol does not act in this way. It produces an increase in serum lipoproteins in the rabbit which is very similar in its overall pattern to the increase produced by cholesterol feeding, the only difference is that the Δ^7 -cholestenol produces a faster rate of increase of the S_f 5-15 lipoproteins. From the lipoprotein standpoint there is no qualitative difference in the way these two sterols are handled by the rabbit.

The observation that the lipoprotein patterns produced by cholesterol and Δ^7 -cholestenol feedings are very similar is doubtless related to, if not entirely explained by, the apparent rapid conversion of Δ^7 -cholestenol into cholesterol. The data of Tables I and II show that the serum cholesterol levels after Δ^7 -cholestenol feeding rise to the same levels as are found after feeding cholesterol itself. This strongly suggests a direct conversion of Δ^7 -cholestenol to cholesterol, in accord with the ideas of Bloch and Langdon.⁽⁶⁻⁸⁾ Further evidence that Δ^7 -cholestenol is a precursor in the biogenesis of cholesterol is presented in the following paper.

The data of Table I, which show that there is a sharp increase in the Δ^7 -cholestenol content of the serum of rabbits fed this sterol, indicate that the Δ^7 -compound is fairly readily absorbed through the intestinal wall.

The data of Table III show that when the two sterols are fed simultaneously there is a rapid rise in the levels of the various classes of lipoproteins

and of the serum cholesterol values. Since this group of rabbits is receiving 2 percent of sterol in the diet, a more rapid rise in the serum lipids is to be expected -- the rate of increase in lipoproteins and of cholesterol in these animals is essentially the same as that which is observed when rabbits are maintained on a 2-percent cholesterol diet alone.

The data of Table III also show that the percentage of Δ^7 -cholestenol rises (after one-week's feeding) to about half that observed when this sterol is fed alone (Table I). These percentages then fall and after two weeks they are comparable to the values obtained for Δ^7 -cholestenol after feeding cholesterol alone for two weeks (Table II). We do not know whether this later decrease represents a failure of absorption, a more rapid conversion to cholesterol, or an increased excretion of the Δ^7 -cholestenol. The 2-percent sterol diet (especially when combined with the Wesson oil) delivers massive amounts of lipids to the rabbit and it is quite possible that in these extreme conditions there is a saturation of one or more enzyme systems.

SUMMARY

1. When Δ^7 -cholestenol is fed to rabbits there is produced a rise in the various classes of low density serum lipoproteins. The lipoprotein "pattern" is qualitatively the same as that observed after feeding cholesterol.
2. Δ^7 -Cholestenol produced a striking rise in serum cholesterol itself. The sterols of the serum after this feeding show a maximum of about 12% of Δ^7 -cholestenol.
3. Δ^7 -Cholestenol and cholesterol produce an additive effect on the levels of serum lipoproteins when fed simultaneously.

4. Five percent of the serum sterols is Δ^7 -cholestenol at the end of one week of feeding both this sterol and cholesterol. At the end of two weeks the Δ^7 -cholestenol falls almost to zero in spite of continued feeding of both sterols.
5. A method is presented for the quantitative analysis of Δ^7 -cholestenol.

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