The Administration to Rats of Cholesta-3:5-dien-7-one

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The fact that cholesta-3:5-dien-7-one occurs in arteriosclerotic aorta and some other tissues, in small amounts (Hardegger, Ruzicka & Tagmann, 1943; Prelog, Ruzicka & Stein, 1943), and that it is not necessarily an artifact (Kantiengar & Morton, 1955a) makes it desirable to study the effect of administering it to animals. In the present work it has been given to rats orally at the rate of 25 mg./rat. Absorption is slow and partial, but a little unchanged material reaches the liver; conversion into a similar compound (probably cholesta-4:6-dien-3-one) occurs in the gut to an appreciable extent, but absorption of this substance is very poor.

It has been shown (Kantiengar & Morton, 1955b) that by feeding cholesterol (2% of diet) the resulting fatty livers contain small, but significant, amounts of the 3:5-dien-7-one. One result of the present study is the finding that on an otherwise sterol-free diet providing 83.3 mg. dienone/rat/day, the part which reaches the liver is stored to only a minute extent and, although the digitonin-precipitable sterol becomes higher than in the sterol-free controls, it is quite comparable with that of normal rat liver. This may mean that the appearance of cholesta-3:5-dien-7-one in tissues is a result of excess cholesterol production or deposition.

EXPERIMENTAL

Animals. Healthy adult male albino rats were used. They were given 12 g. rat cubes on each of 4 days before dosing.

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After fasting for 12 hr. the rats were then given cholesta-3:5-dien-7-one (25 mg.) in arachis oil (0.25 ml.) by mouth from a syringe. Water, but not food, was then provided until the animals were killed. After an appropriate time interval (see below) the rats were anaesthetized with CHCl_3 and the abdominal wall was laid open. The liver and the small intestine were removed. The intestine was washed out with normal saline (using a syringe fitted with a hypodermic needle) until the liquid emerging from the open end was clear. The intestinal washings (400–500 ml.) were repeatedly extracted with ether. The residual tissue was dried between sheets of filter paper and weighed.

The liver was roughly dried similarly and the 'wet weight' was recorded. The tissues were separately subjected to alkali digestion (in the presence of ethanol) and the unsaponifiable fractions were extracted and examined for ultraviolet absorption. The fractions were then chromatographed on alumina (P. Spence and Co., grade 0; weakened by stirring in water, 5%, w/w) and the various products were then examined in the spectrophotometer.

Cholesta-3:5-dien-7-one. This compound was prepared via 7-oxocholesteryl acetate following Daniel, Lederer & Velluz (1945). The pure dienone (m.p. 112°) shows λ_{max} 268 m μ , $E_{1\%}^{1\text{cm}}$ 693 in light petroleum or cyclohexane and λ_{max} 278 m μ (with no change in intensity of absorption) in ethanol or CHCl_3 . In conc. H_2SO_4 a single absorption band λ_{max} 356 m μ , $E_{1\%}^{1\text{cm}}$ 800 was shown. The infrared spectrum agreed very closely with that given by Jones & Dobriner (1949). $[\alpha]_D^{25} - 304 \pm 5^\circ$ in CHCl_3 (Daniel *et al.* 1945, give $[\alpha]_D - 306^\circ$ in benzene). (Found C, 84.7, H, 10.6; calc. for $\text{C}_{27}\text{H}_{48}\text{O}$, C, 84.7; H, 11.1%.)

Experiments 1–4. Table 1 summarizes four experiments (1–4) in each of which two rats were given 25 mg. each of cholesta-3:5-dien-7-one. The amounts of dienone recovered are calculated from the weights and intensities of absorption of the appropriate chromatographic fractions. In Expts. 2–4 a substance distinct from the cholesta-3:5-dien-7-one but derived from it was found in the intestinal washings.

Table 1. Absorption of cholesta-3:5-dien-7-one by male rats given 25 mg. in arachis oil and killed 4–18 hr. later

Means for two rats in each case.				
Expt.	1	2	3	4
Dose (mg./rat)	25	25	12.5 + 12.5 after 2 hr.	25
Animals killed (hours after dose)	4	6	8 (after first dose)	18
Dienone recovered/rat (mg.)				
(a) From intestinal washings:				
(i) Unchanged	20.8	8	0.62	0.4
(ii) 280 m μ . substance*	—	0.27	0.85	1.8
(b) From washed intestinal tissue	0.57	1.6	0.65	1.0
(c) From liver	0.2	0.38	0.85	0.83

* Probably cholesta-4:6-dien-3-one.

The weights of this substance given in the table are calculated on the basis of $E_{1\text{cm}}^{1\%}$, 280 $\mu\mu$. = 700 for the intensity of absorption at the maximum. The faeces from the rats in Expt. 4 were collected and the unsaponifiable matter was obtained; the weight (19 mg./rat) was higher than that (9 mg./rat) obtained from two similarly treated undosed rats.

Experiment 5. Six male albino rats were maintained on a 'sterol-free' diet for 9 days (casein 520 g., rice starch 1000 g., glucose 300 g., arachis oil, 100 g., Marmite 120 g., salt mixture (Hawk & Oser, 1931) 80 g.). The control group of three rats remained on this diet until the end of the experiment (12 g./rat/day for 21 days). The experimental group of three rats had the same diet supplemented by 250 mg. of dienone for 12 days (i.e. 3 g. spread over 12 days for three rats). The results are summarized in Table 2.

Experiment 6. Two rats (202, 220 g.) were given 12 g./rat/day of the 'sterol-free' diet and the faeces were collected

daily and dried at 60° for 3 hr. At the end of 3 days the dried faeces were weighed and the ether-soluble material was extracted. The ether extract was dissolved in ethanol and the titratable acidity was determined on a known volume of the solution (0.05 N ethanolic KOH and phenolphthalein). The remainder was saponified and the unsaponifiable fraction obtained. Its absorption spectrum was determined and the total sterol precipitable by digitonin was determined gravimetrically.

Similar tests were carried out every 3 days until the sterol excretion had reached a steady low value. Thereafter the diet was changed to the extent that each rat was given 100 mg. of dienone/day in 12 g. of the 'sterol-free' diet. After 12 days (during which the faeces were collected and tested as already described) the rats were returned to the original diet.

The results of this experiment are summarized in Table 3.

Table 2. *Effects of feeding cholesta-3:5-dien-7-one to three rats over 12 days on a 'sterol-free' diet (83.3 mg./rat/day)*

	Experimental group	Control group
	Livers	
Wt. (3 livers)	30 g.	28 g.
Unsaponifiable matter	140.5 mg.	99.5 mg.
Digitonin-precipitable sterol	75 mg.	37.5 mg.
2% Ether-light petroleum eluate	2.2 mg.	1.2 mg.
	$\lambda_{\text{max.}}$ { 268 $\mu\mu$. (petrol) 278 $\mu\mu$. (ethanol)	Inflexion 278 $\mu\mu$. (ethanol)
5% Ether-light petroleum eluate	1.4 mg.	Negligible
7% Ether-light petroleum eluate	12.6 mg.	Negligible
	$\lambda_{\text{max.}}$ { 280 $\mu\mu$. (petrol) 285 $\mu\mu$. (ethanol)	
15% Ether-light petroleum eluate (vitamin A fraction)	{ 53.5 mg. $\lambda_{\text{max.}}$ 325 $\mu\mu$.	48 mg. $\lambda_{\text{max.}}$ 325 $\mu\mu$.
	Intestines	
Wt.	30 g.	28 g.
Unsaponifiable matter	{ 65 mg. $\lambda_{\text{max.}}$ 278 $\mu\mu$. (definite band)	76 mg. $\lambda_{\text{max.}}$ 278 $\mu\mu$. (small band)

Table 3. *Faecal lipids of two rats on a 'sterol-free' diet with and without addition of cholesta-3:5-dien-7-one (100 mg./rat/day)*

Wts. of rats 216–222 g. and 199–204 g. throughout the experiment. 12 g. food/day throughout.

Day	Diet	Wt. of faeces (dried at 60°) (g.)	Faecal lipid (mg.)	Acidity (ml. 0.1N)	Unsaponi- fiable matter (mg.)	'Dienone' (mg.)	Unsap. minus dienone (mg.)	Sterol pptd. by digitonin (mg.)
-2, -1, 0	Stock	12.6	448	6.9	146	None	146	80
1, 2, 3		9.6	337	4.7	102	None	102	70
4, 5, 6	Sterol-free	4.6	250	3.4	141	None	141	61
7, 8, 9		3.9	138	2.5	57	None	57	24
10, 11, 12		3.6	465	3.0	340	203	137	53
13, 14, 15		4.4	786	4.2	463	249	214	97
16, 17, 18	Sterol-free	4.5	805	7.4	516	181	335	94
19, 20, 21	plus dienone	4.2	795	7.2	493	277	216	* { 112 103
22, 23, 24		3.2	310	2.7	166	31	135	40
25, 26, 27	Sterol-free	4.1	202	2.1	90	None	90	31
28, 29, 30		3.5	121	2.5	55	None	55	28

* Two determinations.

Experiment 7. Six adult male rats were maintained on the 'sterol-free' diet for 9 days and three of them were then given a supplement of cholesta-3:5-dien-7-one (250 mg./36 g. diet) for 12 days, the other three animals still on the sterol-free diet serving as controls. The faeces of both groups were collected daily, dried at 60° and worked up as in Expt. 5. The main findings are recorded in Tables 4 and 5.

In all some 1.3 g. of cholesta-3:5-dien-7-one (out of 3 g. administered) was recovered.

A second, very similar, substance with $\lambda_{\max}$ 281 m μ . (cyclohexane) and 355 m μ . (conc. H₂SO₄) was accumulated. The final 10% ether-light petroleum eluate (fraction 2B) of Table 4 was again rechromatographed to give a fraction (25 mg.) $\lambda_{\max}$ 280 m μ . $E_{1\text{cm}}^{1\%}$ 484. A further chromatography gave 21 mg. $E_{1\text{cm}}^{1\%}$ 513. This material gave a positive Zimmermann (1935, 1936) test. A small amount of crystalline

material m.p. 81° was obtained, and it was concluded that the second substance was cholesta-4:6-dien-3-one (see Discussion).

Experiment 8. The object of this experiment was to ascertain whether administration of cholesta-3:5-dien-7-one to rats affected the liver vitamin A reserves. Three rats (180, 184, 176 g.) were kept on the 'sterol-free' diet for 12 days, followed by 0.3 mg. dienone/rat/day for 12 days. Three control rats (175, 176 and 184 g.) were kept on the 'sterol-free' diet over the whole period. The rats were then killed and the livers studied. The following results were obtained (results for control rats in parentheses): livers 28 g. (26 g.), unsaponifiable matter, 127 mg. (89 mg.), digitonin-precipitable sterol 72 mg. (43 mg.), vitamin A, 916 i.u./rat (819 i.u./rat); absorption spectra of chromatographed fractions: cholesta-3:5-dien-7-one (278 m μ . ethanol) *ca.*

Table 4. *Faecal lipids of rats on a 'sterol-free' diet for 12 days (after a preparatory period of 9 days) with and without addition of cholesta-3:5-dien-7-one (0.25 g./36 g. diet)*

Control group: 3 rats; 21 days on sterol-free diet. Experimental group: 3 rats; 9 days on sterol-free diet followed by 12 days on the same diet plus dienone: 3 g. in all.

	Controls	Experimental
Dry wt. faeces (12 days) (g.)	16.9	18.4
Ether-soluble portion (g.)	0.5	2.6
Unsaponifiable fraction (g.)	0.308	2.221*
Ultraviolet absorption of unsaponifiable	General	$\lambda_{\max}$ 278 m μ . $E_{1\text{cm}}^{1\%}$ 405 (ethanol)
Digitonin-precipitable sterol (g.)	0.15	0.4
'Dienone' excreted (g.)	Nil	<i>ca.</i> 1.29

* For details of chromatographic examination see Table 5.

Table 5. *Chromatographic separation carried out on unsaponifiable fraction from faecal lipids of rats on a 'sterol-free' diet supplemented with cholesta-3:5-dien-7-one (83 mg./rat/day for 12 days)*

45 g. alumina + 5 g. water.

Fraction	Eluent	Wt. of eluate (g.)	$\lambda_{\max}$ (m μ)
1	2% Ether-light petroleum	1.036	{ 278 (ethanol) (approx. 0.9 g. 'dienone')
2	5% Ether-light petroleum	0.516	{ 278 (ethanol) (approx. 0.4 g. 'dienone')
3	10% Ether-light petroleum	0.242	{ 235, 285 (ethanol) 230, 280 (light petroleum)
4	20% Ether-light petroleum	0.048	230, 285
5	Ether	0.134	General end absorption
6	Ethanol	0.030	—
5% Ether fraction (2) rechromatographed			
2A	2% Ether-light petroleum	0.44 (Very largely cholesta-3:5-dien-7-one)	278 (ethanol)
2B	10% Ether-light petroleum (mainly cholesta-4:6-dien-3-one)	0.052	{ 285 (ethanol) $E_{1\text{cm}}^{1\%}$ 230 280 (light petroleum) 281 (cyclohexane)
2C	Ether	0.005	General absorption
10% Ether fraction (3) rechromatographed			
3A	10% Ether-light petroleum	0.17*	235, 285 (ethanol)
3B	20% Ether-light petroleum	0.036	General absorption
3C	Ether	0.014	General absorption

* In conc. H₂SO₄ $\lambda_{\max}$ 310; inflexions, 298 and 355 m μ .

2.6 mg. (trace only); derivative $\lambda_{\max}$ 284 m μ . (ethanol) was present (none found); derivative, $\lambda_{\max}$ 235 m μ . (ethanol) present (absent).

DISCUSSION

The first experiment showed that 4 hr. after dosing, more than 80 % of the cholesta-3:5-dien-7-one could be recovered from the intestinal washings. The amount of dienone in the gut wall was about 2.3 % of the dose and only a trace had reached the liver. When the rats were killed 6 hr. after dosing, about 60 % of the dienone had disappeared, 32 % was still unabsorbed and about 6 % was in the gut wall. A small fraction (which was not pure dienone) in the liver accounted for less than 2 % of the dose. The unabsorbed material was found on chromatography to be a mixture of cholesta-3:5-dien-7-one and a companion substance (probably cholesta-4:6-dien-3-one), the former predominating. The two dienones differ in respect of the position of $\lambda_{\max}$ and are eluted with 2 % ether, and 5 % ether in light petroleum respectively. Both show $\lambda_{\max}$ 355 m μ . in conc. H_2SO_4 .

The identification of the companion substance with cholesta-4:6-dien-3-one rests on strong but not conclusive evidence; although a small amount was crystallized (m.p. 81°), and had the correct m.p., no mixed melting point was done. Prelog *et al.* (1943) obtained the 4:6-dien-3-one apparently contaminated with cholest-4-en-3-one and noted that this substance does not depress the m.p. of the dienone. Cholesta-3:5-dien-7-one and cholesta-4:6-dien-3-one have been found together in arteriosclerotic aorta (Hardegger *et al.* 1943) the former predominating greatly (1.2 % of unsaponifiable matter as compared with 0.02 %). Both substances were also found in pig spleen (Prelog *et al.* 1943), the former again being more plentiful.

Comparing the properties of cholesta-3:5-dien-7-one, and those of our companion substance, we have: (a) similar absorption curves in respect of position and intensity except that $\lambda_{\max}$ for the congener (285 m μ .) is 7 m μ . greater (solvent ethanol); (b) both substances show in conc. H_2SO_4 a broad band at $\lambda_{\max}$ 355 m μ . indistinguishable in intensity; (c) the companion substance gives a positive Zimmermann (1935, 1936) test, whereas the 3:5-dien-7-one does not. It may be added that consideration of the ultraviolet absorption spectra of steroids (Dorfman, 1953) provides no alternative to the cholesta-4:6-dien-3-one for the substance derived from cholesta-3:5-dien-7-one. Cholesta-4:6-dien-3-one has $\lambda_{\max}$ 284–285 m μ . in ethanol $\epsilon_{\max}$ 26 000 (m.p. 81°; $[\alpha]_D + 33^\circ$) (Dane, Wang & Schulte, 1937; Wilds & Djerassi, 1946).

When the rats were killed 8 hr. after dosage, about 88 % of the administered dienone had disappeared. From the gut wall only 2.6 % was recovered. The

intestinal washings yielded similar amounts (2.5 and 3.4 %) of each of the isomeric dienones and the liver contained a little of both (about 3.4 %) with a small amount of another compound (not identified) with two peaks ($\lambda_{\max}$ 268 and 330 m μ .).

In the 18 hr. experiment, 84 % of the administered dienone had disappeared totally. The intestinal washings contained about 1.6 % of the administered cholesta-3:5-dien-7-one and about 7.2 % as the isomeric ketone. About 4 % of the dose was recovered from the gut wall as dienone and about 3.3 % from the liver.

It is evident that a small proportion of the dose is slowly changed to the isomeric dienone, apparently in the lumen of the gut. The rise in faecal unsaponifiable matter which occurred in the animals killed 18 hr. after dosage suggests that a portion of the administered steroid is reduced in the gut (since there is no rise in selective absorption) and excreted.

The experiment in which rats received daily doses of cholesta-3:5-dien-7-one for 12 days showed that as compared with the control group these rats had considerably raised liver unsaponifiable matter and a doubled digitonin-precipitable sterol content. There was also definite accumulation of the two isomeric dienones in small amounts. It seems likely that both dienones were reduced to sterols exhibiting no selective absorption in the ultraviolet region.

Expt. 6, in which two rats were placed on a 'sterol-free' diet showed that after 9 days faecal lipid constituents (Table 3) had all become low. Dosage with cholesta-3:5-dien-7-one (100 mg./rat/day) caused marked increases in faecal lipid, titratable acidity, unsaponifiable matter, mixed dienones and sterols precipitable by digitonin. When the rats were again put on the 'sterol-free' diet the faecal lipid constituents again fell to low values.

The administered dienone thus results in an increase in faecal organic acids and in unsaponifiable constituents including sterols precipitated by digitonin but not exhibiting the ultraviolet absorption characteristic of the dienones. Expt. 7 with three rats given cholesta-3:5-dien-7-one for 12 days, showed a very marked rise of faecal unsaponifiable matter compared with the controls. The chromatographic separation was consistent with cholest-4-en-3-one, cholesta-4:6-dien-3-one and unchanged dienone being all excreted.

Schoenheimer, Rittenberg & Graff (1935) fed cholest-4-en-3-one to a dog and found that cholesterol and coprostan-3 β -ol were excreted as derivatives. Bloch (1949) found that cholestenone (containing stably bound deuterium) was well absorbed by rats, and that on analysis of tissues and excreta isotopic dihydrocholesterol (cholestan-3 β -ol) was found in relatively large amount and isotopic cholesterol in small amount. Cook (1938) and Cook,

Polgar & Thompson (1948) found that feeding cholesterol to rats caused increased excretion of fatty acids of rather high molecular weight. The final experiment (8) confirmed the effect of dienone feeding in respect of liver lipids; but the vitamin A reserves were about the same.

It seems very plausible to regard the following sequence as fitting the results

The results of experiments on cholesterol feeding are not inconsistent with this scheme, but it seems from a closer scrutiny of the absorption curves that a possibly important component is missing from this scheme. It is clear enough that conversion of one dienone into the other is unlikely to occur without intermediates.

SUMMARY

1. Cholesta-3:5-dien-7-one administered to rats at dosages between 25 and 100 mg./day is slowly and partially absorbed from the lumen of the gut.

2. Some of it is isomerized very probably to cholesta-4:6-dien-3-one and subsequently reduced to cholest-4-en-3-one, but the major part disappears to form unidentified products.

3. Some of the administered dienone is converted into digitonin-precipitable steroids showing no selective absorption in the region 220–400 m μ . and other constituents of faecal unsaponifiable matter, together with organic acids.

4. Small amounts of dienones are found in the livers of the dosed rats.

5. Since cholestadienones are formed by rats on

a diet supplemented with cholesterol, the metabolic sequence,

may have some significance in normal conditions.

6. There is some evidence that the suggested partial metabolic scheme is oversimplified.

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The Effects of Administering Cholesterol and Cholesta-3:5-dien-7-one to Cockerels

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The occurrence of cholesta-3:5-dien-7-one in plaques from human atherosclerotic aortas, together with its absence from brain (Kantiengar & Morton, 1955) raises interesting questions. The 'dienone' has also been found in the unsaponifiable fraction of livers from aged horses (Cain & Morton, 1955), but it has

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not been found in human gall-stones (unpublished observations).

The aetiology of atherosclerosis is an important and debatable problem. The fact that, in small amount, cholesta-3:5-dien-7-one is a regular congener of cholesterol in plaques makes it interesting to study the effect of continuous feeding of the dienone side by side with cholesterol. The possibility that