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

cholesta-3:5-dien-7-one... → cholesta-4:6-dien-3-one → cholest-4-en-3-one → coprostan-3β-ol... organic acids.

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 mμ. 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,

cholesta-3:5-dien-7-one... → cholesta-4:6-dien-3-one → cholest-4-en-3-one → coprostan-3β-ol... organic acids

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fraction of livers from aged horses (Cain & Morton, 1955), but it has not been found in human gall-stones (unpublished observations).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

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a cholesterol metabolite rather than cholesterol itself plays a critical part in atherosclerosis clearly needs to be settled one way or the other.

Atherosclerosis can be readily brought about in cockerels. Dauber & Katz (1942, 1943), Rodbard, Bolene, Pick, Lowenthal, Gros & Katz (1950)—see also Katz & Stamler (1953)—have described how the incorporation of cholesterol in the diet of chicks can result in atherosclerosis. During the first 2 months of life there was little evidence of atheromatosis or even of hypercholesterolaemia in chicks, despite a considerable cholesterol intake. After the 8th week, which marks the onset of puberty in cockerels, the plasma cholesterol increased sharply, although the diet was unchanged. The resistance to processes culminating in atheromatosis and in vascular lesions declined until by the 15th week atherogenesis was very marked.

The findings of Katz and his colleagues led us to try similar experiments in which the effects of cholesterol and cholesta-3:5-dien-7-one could be compared. The dose level of the latter was fixed at 1/6 that of the cholesterol on the assumption that if the 'dienone' did possess any special biological activity a very high dosage would not be necessary.

EXPERIMENTAL

Birds. Nine Light Sussex cockerels, 8 weeks old, were obtained from the experimental farm of Messrs J. Bibby and Sons, Liverpool. They were housed separately in large cages in a room kept at about 68° F (20° C).

Food. The stock diet consisted of a meal (supplied by Messrs Bibby and Sons) with the following percentage composition: extracted soya bean meal, 2·5; extracted sunflower seed meal, 1·25; fish meal, 5·0; fine wheat bran, 20·0; barley, 10·0; maize, 35·0; oats, 10·0; sorghum, 10·0; dried grass, 2·5; mineral mixture, 3·75; plus riboflavin concentrate to supply 1 mg. riboflavin/500 g. meal. (The mineral mixture was made up of NaCl, 10%; sterilized bone flour 27·5%; limestone flour, 62·5%; and manganese, 77 p.p.m.)

Vitamins A and D were supplied by adding a little cod liver oil daily to the mash.

The amounts of food given were: 70 g./cockerel/day from the 8th to 10th weeks, 80 g. from the 11th to 12th weeks and 100 g. from the 13th to 16th weeks.

Plan of experiment. The cockerels were divided into three groups of three. Group *A* received the stock diet plus arachis oil (5 ml./bird/day), sufficient water being added to give a thick moist paste. Group *B* received the same diet as group *A* with the addition of cholesta-3:5-dien-7-one at the rate of 0·33 g./bird/day. Group *C* received the same diet as group *A* with the addition of cholesterol at the rate of 2 g./bird/day.

The birds were fed as indicated for 8 weeks from the beginning of the 9th week after hatching.

Cholesterol. This was a commercial sample highly transparent to ultraviolet light; it may have contained a little cholest-7-en-3β-ol (Fieser, 1953), but it was as 'pure' as any cholesterol available to us in the necessary amounts.

Cholesta-3:5-dien-7-one. This was prepared in batches following in essentials the procedure described by Daniel, Lederer & Velluz (1945).

All the cockerels ate well and remained outwardly normal. At the end of the experimental period they were killed and samples of blood were withdrawn from the heart and used for the determination of cholesterol (King, 1951). The livers, intestines and aortas were dissected out, washed with cold water and roughly dried between sheets of filter paper before weighing. Small portions of liver and aorta were used for histological examination and the main portions were used for analysis. The tissues were digested with ethanolic KOH and the unsaponifiable fractions were extracted and studied by a combination of spectrophotometric and chromatographic methods. The alumina (P. Spence and Co., grade 0) was always weakened by the incorporation of water (5%, w/w) with stirring, under light petroleum. Spectra, unless otherwise stated, were obtained using light petroleum as solvent.

RESULTS

Post-mortem appearances. The aortas of the cholesterol-fed group showed some thickening consistent with early atherosclerotic change; the livers showed typical fatty degeneration. The organs of the 'dienone'-fed group were not obviously different from those of the normally fed birds.

Histological changes. The aortas of the cholesterol-fed group exhibited conspicuous patches of intimal lipid deposition. Very small lipid droplets appeared at points of thickening. The lipid stained strongly by the Schultz reaction for cholesterol, and by Sudan black, but less markedly by Sudan red. The livers showed the same fatty infiltration as is seen with rats maintained on a diet containing added cholesterol. The Schultz reaction indicated that almost every liver cell contained much cholesterol, sometimes it appeared as short columns and occasionally as rather large droplets (1–2 μ.). The 'dienone'-fed birds and the normally fed controls differed but little in the histological appearances of aortas or livers.

Tissue weights. Table 1 shows that liver weights, percentages of unsaponifiable matter and vitamin A contents were not appreciably different for the 'dienone'-fed birds as compared with those normally fed. On the other hand, the livers of the cholesterol-fed birds were enlarged, their unsaponifiable content had increased more than tenfold and their vitamin A store had considerably decreased in absolute terms as well as in terms of i.u./g. liver. The weights of intestinal tissue were greater for the sterol-fed birds, particularly for those on the 'dienone' diet. This is an interesting result which merits further study. The unsaponifiable matter in the intestinal tissue increased particularly for the cholesterol-fed birds. There was no hypercholesterolaemia in the 'dienone'-fed birds, but the values for the blood cholesterol were notably high for the birds given the parent sterol.

Table 1. *Livers, intestines and blood of cockerels on (A) normal diet, (B) diet enriched with cholesta-3:5-dien-7-one (0·33 g./bird/day) and (C) diet enriched with cholesterol (2 g./bird/day) all from the 9th to 16th weeks (inclusive) after hatching*

	(A) Normal diet	(B) 'Dienone' diet	(C) Cholesterol diet
Liver wt. (g.)	111·5 (33·1, 40·8, 37·6)	122·5 (37·1, 39·4, 46·0)	160·8 (60·8, 49·9, 50·1)
Liver unsaponifiable matter (as % of liver wt.)	0·56	0·47	5·29
Sterol portion (as % of liver wt.)	0·34	0·34	5·19
Vitamin A from three livers (i.u.)	62 600	62 400	38 100
Vitamin A from three livers (i.u./g. liver)	562	510	237
Intestines wt. (g.)	78·7 (24·4, 25·1, 29·2)	134·5 (40·1, 45·2, 49·2)	105·7 (41·2, 36·3, 28·2)
Unsaponifiable matter from intestinal tissue (g.)	0·352	0·751	1·03
Cholesterol portion (approx.) (g.)	0·15	0·33	0·9
Blood cholesterol of individual birds; (mg./100 ml. whole blood)	152 105 144	140 126·5 123	860 560 526

Chromatographic separations

The results of examining the various unsaponifiable fractions are recorded briefly in Tables 2–4.

Normal animals. The normal livers contained a small amount of β-carotene and a few mg. of material showing an absorption peak at 271–273 mμ. This substance (SA of Festenstein, Heaton, Lowe & Morton, 1955) is a normal minor constituent of liver unsaponifiable matter and is quite distinct from cholesta-3:5-dien-7-one. The normal intestine unsaponifiable fraction probably contained a little SA together with detectable amounts of 7-dehydrocholesterol. Cholesta-3:5-dien-7-one was not present in detectable amount.

'Dienone'-fed animals. The livers from the 'dienone'-fed cockerels gave a fraction (DL 2) with $\lambda_{max.}$ 270 mμ., which seems to have been a mixture of SA and cholesta-3:5-dien-7-one. The latter, dissolved in conc. H_2SO_4 shows $\lambda_{max.}$ 356 mμ., whereas DL 2 gave a more complicated spectrum. DL 2 was therefore rechromatographed (see Table 3). DL 10 showed a very sharp peak at 267 mμ. (light petroleum) 277 mμ. (ethanol) and a sharp peak at 354 mμ. in conc. H_2SO_4. The ultraviolet absorption curve also showed weak selective absorption at 295–375 mμ., but on allowing a solution in light petroleum to stand in daylight for 2 weeks this absorption disappeared, leaving untouched the absorption on the short-wave side of 290 mμ. In conc. H_2SO_4 the 354 mμ. peak persisted. The main constituent of DL 10 was therefore cholesta-3:5-dien-7-one, which unlike the congener is not destroyed by daylight. DL 11 showed peaks at 330

Table 2. *Chromatography of liver and intestine unsaponifiable material from normal cockerels*

(a) Liver (30 g. weakened alumina)

Fraction	Wt. (g.)	Eluent: ether (%) in light petroleum	Absorption spectrum
NL1	0·021	0	β-Carotene maxima (425, 450, 470 mμ.) and general u.v. absorption
NL2	0·014$_5$	2	$\lambda_{max.}$ 271–274 mμ., $E^{1\%}_{1cm.}$ 103
NL3	0·026	5	$\lambda_{max.}$ 275 mμ. weaker band 325 mμ.

NL 4, 5 and 6 eluted with increasing amounts of ether contained mainly vitamin A and probably decomposition products and optically transparent substances.

(b) Intestine (30 g. weakened alumina)

Fraction	Wt. (g.)	Eluent: ether (%) in light petroleum	Absorption spectrum
NI1	0·04	0	Inflexion 275 mμ. $E^{1\%}_{1cm.}$ 4·1
NI2	0·006	2	Inflexion 230 mμ. $E^{1\%}_{1cm.}$ 4·1
NI3	0·008	5	$\lambda_{max.}$ 275 mμ. $E^{1\%}_{1cm.}$ 45
NI4	0·046	10	General absorption
NI5	0·105	20	$\lambda_{max.}$ 272, 281 and 291 mμ. (7-dehydrocholesterol)

Table 3. *Chromatography of liver and intestine unsaponifiable matter from cockerels fed a diet containing cholesta-3:5-dien-7-one* (0·33 *g./bird/day*)

(*a*) Liver (25 g. weakened alumina)

Fraction	Wt. (g.)	Eluent: ether (%) in light petroleum	Absorption spectra
DL1	0·048	0	β-Carotene maxima
DL2	0·018	2	$\lambda_{max.}$ 270 mμ., $E^{1\%}_{1\,cm.}$ 204

DL3, 4, 5 and 6 eluted with increasing amounts of ether contained vitamin A in quantity and apart from vitamin A decomposition products, traces of other absorbing substances would be masked.

DL2 rechromatographed and developed with 2% ether–light petroleum gave DL 10, 11 and 12, estimated to contain 1·46 mg. cholesta-3:5-dien-7-one. (See text, p. 36).

(*b*) Intestine

Fraction	Wt. (g.)	Eluent: ether (%) in light petroleum	Absorption spectra
DI1	0·069	0	Inflexion 240 mμ.
DI2	0·090	2	$\lambda_{max.}$ 268 mμ., $E^{1\%}_{1\,cm.}$ 64·5*
DI3	0·072	5	270 mμ., $E^{1\%}_{1\,cm.}$ 0·53
DI4	0·058	10	Rising end absorption
DI5	0·275	20	Inflexions 270, 281, 296 mμ.

* Equivalent to 8 mg. cholesta-3:5-dien-7-one.

Table 4. *Chromatography of liver and intestine unsaponifiable material from cockerels fed a diet containing cholesterol 2 g./bird/day*

(*a*) Liver. The unsaponifiable matter (8·05 g.) was extracted with light petroleum; 6·37 g. insoluble sterol (mainly cholesterol) remained. The soluble portion was chromatographed on alumina weakened by water (5% w/w).

Fraction	Wt. (g.)	Eluent: ether (%) in light petroleum	Absorption spectrum: $\lambda_{max.}$ mμ.	Absorption spectrum: $E^{1\%}_{1\,cm.}$
CL1	1·233	0	425, 440, ~345, ~280	—
CL2	0·035	2	268	356*
CL3	0·033	5	271–273	—
CL4	0·019	10	274, 329	36, 14·7
CL5	0·357	10	325	21·4
CL6	0·595	20	280	0·83
CL7	0·239	50	271, 328	2·35, 1·21
CL8	0·011	100	270, 330, 440	39·5, 43·4, 119
			CL3 rechromatographed:	
CL9	<0·001	0	End absorption	—
CL10	0·006	2	272	153
CL11	0·023	2	274–275	29
CL12	0·002	5	End absorption	—
			CL1 resaponified and rechromatographed:	
CL13	0·037	0	Not examined	—
CL14	0·595	100	Inflexion 270–275	—

(*b*) Intestine (unsaponifiable). 1·03 g. was dissolved in light petroleum and left overnight at 0°; crystals (0·648) were filtered off; soluble material (0·38 g.) chromatographed. The sterol showed inflexions at 271, 280 and 295 mμ., $E^{1\%}_{1\,cm.}$ 0·20, 0·19 and 0·13 respectively.

Fraction	Wt. (g.)	Eluent: ether (%) in light petroleum	Absorption spectrum: $\lambda_{max.}$ mμ.	Absorption spectrum: $E^{1\%}_{1\,cm.}$
CI1	0·0008	0	Not tested	—
CI2	0·0082	2	267	640*
CI3	0·011	5	274	52·3
CI4	0·045	10	End absorption	—
CI5	0·235	20	Inflexions at 270, 280 and 295	—

* Pure cholesta-3:5-dien-7-one shows $\lambda_{max.}$ 268 mμ. $E^{1\%}_{1\,cm.}$ about 700.

and 272 mμ., with $\lambda_{max.}$ 354 mμ. in conc. H_2SO_4. In light petroleum and exposed to daylight for 25 days, DL 11 lost the 330 mμ. peak, but the final solution showed $\lambda_{max.}$ 267 mμ. (354 mμ. in conc. H_2SO_4). DL 11 was thus originally cholesta-3:5-dien-7-one with unknown congeners. DL 12 showed a 272 mμ. peak (8·7 mg., $E^{1\%}_{1\,cm.}$ 272 mμ. = 111), but in conc. H_2SO_4 the absorption maximum occurred at 313 mμ. The 272 mμ. peak was displaced to 275 mμ. when ethanol was used as solvent instead of light petroleum. The absorbing entity is indistinguishable from SA. Unlike the 267 mμ. peak of the 'dienone' the 272 mμ. peak diminishes in definition and intensity on exposing the solution to daylight, but the capacity to give a 313–315 mμ. peak in conc. H_2SO_4 does not decrease at the same rate as the 272 mμ. absorption. This supports the doubt expressed by Festenstein *et al.* (1955) that the 272 mμ. substance is alone responsible for 313–315 mμ. band.

The substance responsible for the 313–315 mμ. peak in conc. H_2SO_4 might possibly have been cholesterol, but a quantitative Liebermann–Burchard test indicated that no more than 0·6 mg. out of the 8·7 mg. was cholesterol. Moreover, $E^{1\%}_{1\,cm.}$ 313 mμ. for the fraction was 366, whereas under the same conditions purified cholesterol showed $\lambda_{max.}$ 320 mμ. $E^{1\%}_{1\,cm.}$ 230. This means that SA is accompanied by a material more transparent in the ultraviolet which is responsible for the selective absorption in conc. H_2SO_4.

Cholesterol-fed animals. The examination of the liver unsaponifiable matter from the cholesterol-fed cockerels resulted in a fraction (CL 2) which is estimated to have contained about 16–18 mg. of cholesta-3:5-dien-7-one ($\lambda_{max.}$ 278 mμ. in ethanol, 352 mμ. in conc. H_2SO_4). Fraction CL 10 ($\lambda_{max.}$ 272 mμ. in light petroleum, 310 mμ. and ~350 mμ. in conc. H_2SO_4) seems to be mainly SA and CL 11 was similar but contained much more of the material giving rise to a peak at 315 mμ. in conc. H_2SO_4. For CL 10 $E_{310\,m\mu.}/E_{272\,m\mu.}$ was about 2, whereas for CL 11, $E_{315\,m\mu.}/E_{272\,m\mu.}$ was about 10. This again supports the view that the 272 mμ. substance (SA) does not specifically give rise to the band in conc. H_2SO_4. The crystalline cholesterol from the intestinal unsaponifiable fraction of cholesterol-fed cockerels (Table 4) showed quite clearly the absorption bands of 7-dehydrocholesterol. The cholesterol from the corresponding livers was chromatographed (300 g. of weakened alumina). Ether–light petroleum (20%, v/v, 2 l.) eluted 0·13 g. of sterol, m.p. 144°, $\lambda_{max.}$ 274 mμ. $E^{1\%}_{1\,cm.}$ 0·41. Ether (0·5 l.) eluted 4·54 g. of sterol showing the 7-dehydrocholesterol bands. Ether (0·5 l.) then eluted 0·02 g. of material containing a little 'xanthophyll' ($\lambda\lambda_{max.}$ 443, 470 mμ.). On extracting the alumina with boiling ethanol, 1·7 g. of sterol were obtained which again showed the 7-dehydrocholesterol bands weakly. The total 7-dehydrocholesterol content was of the order 1–2 mg.

DISCUSSION

The cholesterol-fed birds showed the expected hypercholesterolaemia, fatty livers and atheromatosis. The livers were greatly enlarged and had more than 10 times as much unsaponifiable matter as the livers of the untreated controls. The vitamin A liver reserves had fallen substantially. This observation is complementary to the decrease in liver cholesterol which occurs as the vitamin A store increases.

The high unsaponifiable content of the liver of the cholesterol-fed birds was mainly made up of cholesterol itself, but 16–18 mg. of cholesta-3:5-dien-7-one were present in the liver and 6–7 mg. in the intestines.

This is very striking when compared with the results obtained after feeding in all 56 g. of cholesta-3:5-dien-7-one itself to three birds. Of this intake only about 1·5 mg. were recovered from the livers and 8 mg. from the intestines. There was no evidence of hypercholesterolaemia, increased storage of cholesterol in the liver or diminution in vitamin A reserves. It seems difficult to avoid the conclusion that the amount of cholesta-3:5-dien-7-one, which can be absorbed unchanged, is very small. On the other hand, the 'dienone' content of the intestinal tissue, although small, was measurable, and there was a definite increase in intestinal unsaponifiable matter including cholesterol (Table 1). There was also marked hypertrophy of intestinal tissue.

In all the groups substance SA (Festenstein *et al.* 1955) was found together with a congener not showing selective absorption in the ultraviolet region.

From the results of the experiment as a whole the small but quite measurable amounts of cholesta-3:5-dien-7-one found in the tissues of the cholesterol-fed birds are most unlikely to be laboratory artifacts. (Even if, as is conceivable, the 'dienone' is derived from 7-oxocholesteryl esters, the fundamental problem would only be shifted to accounting for the origin of such a precursor of the 'dienone'.) The ingestion of large quantities of cholesterol results in a detectable metabolic anomaly which gains in significance when it is remembered that cholesta-3:5-dien-7-one is the selectively absorbing contaminant of the cholesterol from atherosclerotic aortas.

The only tissue on which the ingested cholesta-3:5-dien-7-one has had much chance to act is the gut, which is the only grossly hypertrophied tissue. The fact that the 'dienone' cannot be esterified may contribute to its poor absorption. Nevertheless, plant sterols, which can of course be esterified, are

absorbed as badly or worse. Some 'dienone' may be absorbed in chylomicrons but, if so, the liver must destroy it quickly, in which case it is difficult to account for the storage of dienone in the liver by the cholesterol-fed birds.

The occurrence of cholesta-3:5-dien-7-one in atherosclerotic aortas could have been the result of a metabolic anomaly peculiar to the aorta and secondary to cholesterol accumulation. This is made less likely by the clear proof that the 'dienone' (or possibly a precursor) is present in liver and intestinal tissue of the cholesterol-fed birds. Its accumulation in detectable amounts suggests that the exogenous cholesterol is overloading a disposal mechanism. In man, the endogenous cholesterol is in negative balance to the extent of 0·3 g./day (Frazer, 1953). Whether cholesta-3:5-dien-7-one is on a normal pathway of synthesis or degradation cannot be stated; too little is known about cholesterol biosynthesis or metabolism.

More work on the origin and biological properties of cholesta-3:5-dien-7-one is necessary.

SUMMARY

1. Cockerels maintained from the 8th to the 16th week of age on diets containing cholesterol (2 g./bird/day) or cholesta-3:5-dien-7-one (0·33 g./bird/day) have been compared with control birds on the unsupplemented diet.

2. The blood cholesterol remained between 105 and 152 mg./100 ml. whole blood for the controls and the 'dienone'-fed birds, but reached 526–860 mg./100 ml. for those given cholesterol.

3. The cholesta-3:5-dien-7-one was poorly absorbed and there was no significant change, compared with the controls, in liver unsaponifiable matter, liver cholesterol or liver vitamin A. The intestines were, however, much heavier than those of the controls, and contained a larger amount of total unsaponifiable matter and of cholesterol.

4. The cholesterol-fed birds had enlarged fatty livers with a more than tenfold rise in unsaponifiable matter (nearly all due to cholesterol) compared with the controls. Nearly 40% of the liver vitamin A store had been lost. The liver and intestine unsaponifiable matter contained respectively 16–18 mg. and 6–7 mg. of cholesta-3:5-dien-7-one. This material is regarded as a metabolite and not a laboratory artifact.

5. The presence of a substance showing $\lambda_{max.}$ 272 mμ. (in light petroleum) in the livers of all three groups of cockerels provides additional evidence that the material is a normal constituent. It is accompanied by a substance of similar chromatographic properties which gives rise to an absorption band near 315 mμ. in concentrated sulphuric acid.

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The Partial Purification of Leaf Ribonuclease

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Nucleic acids have been found in every virus preparation that has so far been made and they are widely held to play a part in protein synthesis. For these reasons, and also because of their intrinsic interest, the nucleases have been extensively studied. They are generally thought to split nucleic acids into oligo- or mono-nucleotides and this may well be part of their function *in vivo*; but it is also possible that they take part in the synthesis of nucleic acid and in the rearrangement of components in it or in polynucleotide fragments.

Ribonucleases (RNase), that is enzymes that degrade ribonucleic acid so that it no longer shows its typical macromolecular properties, are widely distributed. They have, for example, been studied in pancreas and other tissues, snake venom, bacteria,