SUMMARY

1. Young male chicks (Light Sussex breed) placed on a vitamin A-deficient diet became unsteady on their legs in the fourth week and soon afterwards died. The kidneys were pale and showed deposits of urates. There was, however, no loss of appetite or weight, no xerophthalmia or lesions in the trachea or oesophagus.

2. Older birds (Brown Leghorn breed) of both sexes were put on a vitamin A-deficient diet at 16 weeks of age. Control birds were given vitamin A as a supplement. Deficiency symptoms began to appear after 7 weeks of deprivation; the males suffered recurring brief loss of consciousness and the females found difficulty in standing. Epithelial lesions were a late symptom. Sexual development was greatly retarded in both sexes.

3. Various tissues (liver, kidney, intestine) were obtained from the young chicks and from control birds and the unsaponifiable fractions were examined by chromatographic and spectrophotometric methods. The rise in SA and SC which occurs in vitamin A-deficient rat tissue did not appear in the chick tissues.

4. Similar study of tissues (liver, kidney, intestine, gizzard, oesophagus and crop, trachea) from older birds of both sexes made vitamin A deficient revealed no real difference in respect of SA, SC, total sterol, 7-dehydrocholesterol and cholesta-3:5-dien-7-one between the controls and the deprived birds.

5. Preliminary work on gizzard muscle and lining showed the presence of cholesta-3:5-dien-7-one in both tissues.

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Vitamin A Deficiency in the Rat, With and Without Added Dietary Cholesterol

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Certain aspects of the vitamin A-deficiency syndrome in the rat have been studied by Heaton, Lowe & Morton (1955, 1957). These workers examined the unsaponifiable portions from various tissues of normal, control and vitamin A-deficient rats and drew attention to marked increases in two unidentified substances, designated SA and SC, in

the livers of the vitamin A-deficient rats. Lowe, Morton & Harrison (1953) had earlier put forward, as a working hypothesis, the idea that vitamin A exerts a restraining influence on the dehydrogenation of cholesterol or steroid hormones, such restraint being lifted in vitamin A deficiency. The hypothesis that this might account for the appearance of SA

and SC was tested when Green, Lowe & Morton (1955) studied in rats the plasma and liver cholesterol levels during vitamin A deficiency. They found no significant difference between plasma cholesterol levels of the deficient and control rats at any stage during the depletion.

The present work is concerned with a study of the vitamin A-deficiency syndrome in rats subjected to the additional hazard of cholesterol feeding.

EXPERIMENTAL

The preparation of unsaponifiable extracts and the chromatographic procedures are described by Heaton *et al.* (1957).

Experiment 1

Animals. Forty weanling rats were divided into two groups (*A* and *B*) of twenty each. The aim was to match each rat in group *A* with a litter mate of the same sex and approximately the same weight in group *B*, but one female in group *A* had to be balanced by a male from another litter, so that group *A* was actually made up of eight males and twelve females and group *B* of nine males and eleven females.

Diet. The experimental diet was as follows: casein ('low vitamin content', Genatosan Ltd.) 18, maize starch 60, arachis oil 10, Marmite 8, salt mixture (Osborne & Mendel, 1913) 4 parts by weight. To each 100 g. of the diet were added α -tocopherol (3 mg.), calcium pantothenate (1 mg.) and choline chloride (80 mg.), the tocopherol being dissolved in the arachis oil before mixing the food. The rats in group *B* were also given crystalline cholesterol to the extent of 2% (w/w) of diet. The cholesterol was dissolved in the arachis oil before the final mixing of the dietary constituents.

Each rat was given 8 g. of diet/day for the first 3 weeks and 10 g./day afterwards. Water was provided without restriction.

Experiment 2

A second experiment was carried out using 20 newly weaned rats divided into two groups I and II with five males and five females in each. Both groups were placed on the vitamin A-free diet supplemented with α -tocopherol, calcium pantothenate and choline, and all the rats received the addition of 2% of cholesterol to the diet. Group I also received 100 i.u. of vitamin A every 5 days during the first

41 days and then for 9 days subsequently five rats (batch 2) received the same dose every day. When a rat in group II showed vitamin A deficiency it was killed and a group I animal was killed at the same time. The animals were dissected and the tissues stored at -20° until sufficient quantities were obtained for examination.

Preparation of tissues

The test of vitamin A deficiency was the appearance of xerophthalmia accompanied by marked loss of weight. The deficient animals were anaesthetized and killed by cardiac puncture with removal of blood by means of a syringe. The liver, small intestine and kidneys in each case were removed, washed, wiped dry, weighed and stored under ethanol in the refrigerator until saponified.

The livers and intestines in Expt. 1 were saponified in three batches from six, seven and seven animals for the two groups and the kidneys were saponified in two lots of ten for each group. In Expt. 2 the tissues were saponified in batches each from five animals.

RESULTS

Experiment 1

The deficiency symptoms appeared clearly in both groups after 5-6 weeks, although growth had slackened after 4 weeks. Of the twenty rats not given cholesterol, three showed grossly distended stomachs and three grossly distended bladders (cf. Heaton *et al.* 1957). The livers of three of these rats had yellow patches on the surface and exhibited fatty infiltration. Out of the twenty rats given cholesterol one had a distended stomach and another a distended urinary bladder; all the livers in the group were grey in colour, heavy and 'fatty'. It could not be said, however, that the rats under the double risk of vitamin A deprivation and cholesterol feeding were at any very obvious disadvantage compared with the others.

Livers. Table 1 shows how liver weights varied in the two groups as the animals were killed off, in turn, on showing signs of deficiency. In every case the absorption spectrum of the total unsaponifiable fraction showed selective absorption near $272\text{ m}\mu$.

Table 1. *Livers of vitamin A-deficient rats (A) compared with livers from cholesterol-fed vitamin A-deficient rats (B)*

Batch	No. of livers	Mean wt. (g.)	Percentage unsaponifiable matter (calc. on tissue wt.)	$E_{1\text{ cm.}}^{1\%}$ 273 $\text{m}\mu$ (calculated on unsap.)
<i>A</i> ₁	6	2.79	0.86	8.9
<i>B</i> ₁	6	4.84	7.3*	0.644
<i>A</i> ₂	7	3.0	0.78	10.6
<i>B</i> ₂	7	5.06	6.4*	0.613
<i>A</i> ₃	7	3.63	0.84	11.37
<i>B</i> ₃	7	5.48	9.7*	0.513
Average <i>A</i>		3.15 ₆	0.82 ₆	—
<i>B</i>		5.14	7.93	—

* A second 'saponification' with ethanolic KOH was necessary to complete the hydrolysis of cholesteryl esters.

Table 2. Comparison of unsaponifiable constituents of livers from vitamin A-deficient (group A) rats with those from livers of cholesterol-fed vitamin A-deficient (group B) rats

Batch	...	...	Absorption units/liver*					
			Group A			Group B		
			1	2	3	1	2	3
Gross absorption			211	247	344	329	271	273
Fraction 1 (petrol eluate $\lambda_{\max}$ 260 m μ)			27	34	40	40	31.6	59
Fraction containing SA			118	109	118	113	?	??
Fractions containing SC			15.5	29	27	32.8	33	49
Cholesta-3:5-dien-7-one (mg.)			0	Trace	0	0.019	—	0.04
7-Dehydrosterol (mg.)			0	0	0	0.02	0.017	0.037

* Absorption units per liver given by the following:

$$\frac{E_{1\text{ cm.}}^{1\%} \lambda_{\max} (\text{calc. on wt. of unsap.}) \times \text{wt. of unsap. in mg.}}{\text{no. of rats in batch}}$$

† In order to complete the hydrolysis of cholesteryl esters it was necessary to prolong the refluxing with alkali; this resulted in destruction of much of the SA present. This loss of SA incidentally unmasked vitamin E in the group B livers; it was eluted by means of 6% ether-light petroleum from Brockman Grade 3 alumina.

If for each batch, the $E_{1\text{ cm.}}^{1\%}$ 272 m μ value of the intensity of absorption is multiplied by the weight (in mg.) of unsaponifiable matter obtained and divided by the number of rats in the batch, the result is a measure of the intensity of absorption per liver. The average figure was 270 for the group A rats and 290 for the group B rats. This means that, despite the great increase in liver size which accompanied cholesterol ingestion, the total amount of selectively absorbing material remained substantially unchanged.

Table 2 summarizes the results of six chromatographic separations on three batches from groups A and B. Within the limits of experimental error the two groups do not differ in respect of (a) the material eluted by light petroleum and (b) SA, and SC. The livers from the cholesterol-fed group contained small but definite amounts of cholesta-3:5-dien-7-one (eluted by 4% ether-light petroleum, $\lambda_{\max}$ 280 m μ in cyclohexane and $\lambda_{\max}$ 355 m μ in conc. H₂SO₄) which was not detected in the corresponding fraction from the livers of the other group.

The main cholesterol fraction from the group B rats (eluted by means of 20% ether-light petroleum) exhibited weakly the characteristic absorption of the 5:7-diene chromophore (7-dehydrocholesterol). This absorption was not detected in the cholesterol from the group A rats.

The liver tissue from the vitamin A-deficient rats was readily hydrolysed by alkali and the unsaponifiable material was obtained easily. Liver tissue from cholesterol-fed rats contains a good deal of esterified sterol which is only saponified with difficulty. The substance SA is, however, destroyed if the refluxing with ethanolic KOH is continued long enough to complete the hydrolysis of the cholesteryl esters. The cholesteryl esters are

highly transparent to ultraviolet light, and if there had been any significant rise in SA or SC in group B as compared with group A it would have been detected even if the 'unsaponifiable' matter from the former still contained sterol esters. In Table 2 the ? sign reflects the failure to determine SA accurately after the rather prolonged heating with alkali. The disappearance of SA, however, had the incidental advantage of permitting α -tocopherol to appear in all three group B batches as a fraction eluted from Brockmann Grade 3 alumina by means of 6% ether-light petroleum, i.e. immediately before SC, which begins to be eluted with 8% ether-light petroleum. In the fractions obtained from group A livers the tocopherol tends to accompany SA and SC and to distort the absorption curves.

Kidneys. Table 3 indicates no real difference between groups A and B in respect of kidney weights, percentage unsaponifiable matter or ultraviolet absorption.

The chromatographic separations indicated the presence of very small amounts of cholesta-3:5-dien-7-one. Each pair of kidneys contained on the average 0.165 mg. of SA and 0.03 mg. of SC. In every separation, 20% ether-light petroleum eluted a material with two maxima, at 261 and 270 m μ respectively. This fraction has been shown in other work (Mervyn, 1956) to be α -tocopherylquinone. It is interesting that α -tocopherol itself could not be detected in these vitamin A-deficient kidneys, but 0.012 mg. of the quinone was found per pair. The quinone is, however, much more strongly absorbing than tocopherol so that minute amounts of the latter might have escaped detection.

Intestines. Table 4 shows that the rats of groups A and B did not differ appreciably in respect of weights of intestine, unsaponifiable matter or

intensity of ultraviolet absorption. Six chromatographic separations were carried out on Brockman Grade 3 alumina.

Light petroleum eluted a small amount of material (about 13 mg. altogether) showing a weak maximum ($E_{1\text{ cm.}}^{1\%}$ approx. 25) at 258–260 $m\mu$. Cholesta-3:5-dien-7-one was eluted by means of 2% ether–light petroleum, but the total amount in all the rat intestines probably did not exceed 0.5 mg. SA was eluted by means of 4% ether–light petroleum and an approximate estimate for the amount present was 3 mg. for the forty rat intestines. α -Tocopherol was clearly seen in some but not all of the separations; when found it was eluted by means of 6%

ether–light petroleum, i.e. between SA and SC. The total amount of SC was about 1 mg. After SC, with 12–20% ether–light petroleum a substance was eluted with two peaks 260 and 270 $m\mu$ which are characteristic of α -tocopherylquinone. In only one batch did the sterol fraction show clearly the 271, 282, 293 $m\mu$ group of bands due to the 7-dehydro steroid structure.

Experiment 2

In this experiment all the animals received 2% of cholesterol in the diet and half the animals were given prophylactic doses of vitamin A (group I). The vitamin A-deficient animals (group II) showed

Table 3. *Kidneys of vitamin A-deficient rats (A) compared with kidneys from cholesterol-fed vitamin A-deficient rats (B)*

Batch	No. of kidneys (pairs)	Mean wt./pair (g.)	Percentage unsaponifiable matter (on tissue wt.)	$E_{1\text{ cm.}}^{1\%}$ (270–272 $m\mu$) (cyclohexane) calc. on unsaponifiable matter
A_1	10	0.911	0.70	3.25
B_1	10	0.971	0.88	2.91
A_2	10	0.975	0.84	4.12
B_2	10	0.942	0.86	3.95
	Means A	0.943	0.77	3.68
	B	0.956	0.87	3.43

Table 4. *Intestines of vitamin A-deficient rats (A) compared with intestines from cholesterol-fed vitamin A-deficient rats (B)*

Batch	No. of intestines	Mean wt. (g.)	Percentage unsaponifiable matter (on tissue wt.)	$E_{1\text{ cm.}}^{1\%}$ (270–273 $m\mu$) (cyclohexane) calc. on unsaponifiable matter
A_1	6	2.62	0.38	4.6
B_1	6	2.65	0.50	3.44
A_2	7	3.35	0.61	4.27
B_2	7	4.28	0.57	3.67
A_3	7	4.30	0.46	2.47
B_3	7	3.86	0.64	2.68
	Means A	3.42	0.49	3.78
	B	3.59	0.59	3.36

Table 5. *Comparison of livers from cholesterol-fed vitamin A-deficient (group II) rats with cholesterol-fed rats given prophylactic doses of vitamin A (group I)*

Batch	No. of livers	Mean wt. of livers (g.)	Total wt. of unsaponifiable matter (g.)	Percentage of unsaponifiable matter	$E_{1\text{ cm.}}^{1\%}$ (270 $m\mu$) (cyclohexane) calc. on unsaponifiable matter	Vitamin A (i.u./g. of liver)
I_1	5	6.68	1.78	5.35	0.425	4.7
II_1	5	5.83	1.72	5.88	0.79	0
I_2	5	7.51	2.91	7.75	0.383	19.0
II_2	5	5.53	1.93	7.12	0.90	0
		Group I	4.69 (total)	6.55 (mean)		
		Group II	3.65 (total)	6.50 (mean)		

xerophthalmia, loss of weight, four had distended stomachs and one a distended bladder. The livers in both groups were markedly fatty.

Tissues rich in cholesteryl esters, as has been pointed out, are fully saponified only after prolonged refluxing with ethanolic alkali. Nevertheless, it was found that the intensity of absorption at 270 $m\mu$ was higher by 66% in the vitamin A-deficient livers than in those from animals given protective doses. This rise occurred (Table 5), although SA may have been largely destroyed.

In Table 2 the average gross absorption units/liver in group B rats was 290, and in this experiment it was 310 for the group II rats as compared with 180 for the rats given prophylactic doses of vitamin A.

Chromatographic separations on the liver unsaponifiable matter from group I resulted in the detection of traces of cholesta-3:5-dien-7-one, of SA, and little else other than, of course, large amounts of cholesterol. SC was not definitely found in the group II unsaponifiable fractions, although it may have accompanied the unhydrolysed sterol esters in one batch and the 7-dehydrocholesterol in the other, more completely saponified, batch. Small quantities of vitamin E were found in the livers. Perhaps the most interesting result was that the amount of cholesta-3:5-dien-7-one was not appreciably different in the two groups, nor in fact very different from the figure of 0.034 mg./liver found for adult male stock colony rats.

These weanling rats did not show the rise in 'dienone' content of liver on feeding cholesterol previously recorded by Kantiengar & Morton (1955) for adult male rats.

The intestines of both groups I and II were studied, and chromatographic separations were carried out on the unsaponifiable fractions. Traces of dienone of SA and α -tocopherylquinone were detected in groups I and II, but no important differences emerged.

DISCUSSION

Cholesterol levels are unresponsive to hepatic vitamin A stores in young rats of both sexes (Green, *et al.* 1955) or adult females, but there is a significant effect in young and adult males of cholesterol feeding on hepatic vitamin A (Kantiengar & Morton, 1955; Green, Horner, Lowe & Morton, 1957).

The accumulation of SA and particularly of SC in the livers of vitamin A-deficient rats might well have been the result of a derangement of sterol metabolism. The subjection of rats to the double hazard of excessive cholesterol intake and vitamin A deprivation neither diminished nor enhanced the

accumulation of SA and SC as compared with the effects seen in uncomplicated vitamin A deficiency. The absorbed cholesterol seems to be deposited in the liver as ester and not to be in itself a direct cause of a relevant metabolic abnormality. Similarly, vitamin A deprivation superimposed on cholesterol excess does not appreciably modify the consequences of the latter.

This work has not revealed any direct or major connexion between vitamin A metabolism and sterol metabolism. No evidence has emerged that cholesterol is a direct precursor of SA or SC.

When cholesterol-fed rats given just enough vitamin A for small storage to occur in the liver are compared with cholesterol-fed vitamin A-deficient animals it is seen that the vitaminosis again results in an increased intensity of absorption near 270 $m\mu$. The problem of obtaining unsaponifiable fractions from cholesterol fatty livers without decomposition has not been fully solved, but it seems clear from the chromatography that the increased absorption at 270 $m\mu$ in avitaminosis is not due to cholesta-3:5-dien-7-one, nor to 7-dehydrocholesterol nor to any very marked extent to SA. It is probably due in the main to SC, but this is not quite certain.

SUMMARY

1. Weanling rats on a vitamin A-deficient diet were compared with litter mates on the same diet to which cholesterol (2%) had been added. The tissues (liver, kidney, intestine) from both groups were examined when the deficiency syndrome became manifest. SA and SC (substances recognizable by their absorption spectrum) were produced in the liver in both groups. Cholesta-3:5-dien-7-one and 7-dehydrocholesterol were found in the liver unsaponifiable fraction of the group given cholesterol. The effects of vitamin A depletion and cholesterol feeding seemed additive and largely independent. No important differences were seen in respect of ultraviolet absorption shown by unsaponifiable fractions from kidney and intestine.

2. Weanling rats on a vitamin A-deficient diet containing 2% cholesterol were compared with rats on the same diet supplemented with enough vitamin A to permit small liver storage. The animals were killed when avitaminosis A was shown in the first group. The examination of tissue unsaponifiable fractions again showed little evidence of interaction between cholesterol feeding and vitamin A deprivation.

One of us (B.G.) participated in this work as holder of an M.R.C. Scholarship and another (J.S.L.) as an I.C.I. Fellow.

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Unsaponifiable Constituents of Kidney in Various Species

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Two substances, designated SA and SC respectively pending determination of their structures, have been found in unsaponifiable fractions from various organs, e.g. liver and intestine, from both vitamin A-deficient and normal rats (Heaton, Lowe & Morton, 1955, 1957). The purpose of the present investigation was to study their occurrence in normal kidneys from various species, one of the objectives being to find a convenient source of either SA or SC. In the course of the work a constituent was encountered in some species, particularly cat, which was provisionally denoted SE. It was later identified as vitamin E, probably mainly α -tocopherol. In the domestic cat, the kidney was found to differ from the other species studied in having unusually large amounts of both vitamin A and vitamin E.

The substance SA occurs in the kidneys of all eleven species studied, the concentration being of the order of 20 μ g./g.; it also occurs in human kidney (Mervyn, 1956). The substance SC is almost equally widely distributed, the concentration being of the order of 5 μ g./g. These estimates are based on the properties (in preparation) of the purest preparations of SA and SC so far obtained.

MATERIALS

Kidneys from pig, sheep and ox were obtained as fresh as possible from the Stanley Abattoir, Liverpool. Kidneys from three piglets suspected of suffering from vitamin A deficiency were supplied by Mr Brooksbank, Veterinary Investigation Officer. Dog kidney was obtained from the Veterinary School, University of Liverpool and from the Dogs' Home, Liverpool. Hen kidneys from normal laying pullets were supplied by J. Bibby and Sons Ltd., Liverpool. Cat kidneys were obtained (a) from the Department of Physiology, University of Liverpool, and (b) through the Royal Society for the Prevention of Cruelty to Animals (R.S.P.C.A.), Liverpool Branch. When it became necessary

to obtain larger quantities of material, permission was obtained from W. Smith and Sons, Lymm, to dissect carcasses bought by them from the R.S.P.C.A. The cats were unwanted and had been taken to the Society's Branches by the public and had there been painlessly killed. The dissection was done at W. Smith and Sons' premises. The material was admittedly not all entirely fresh, but no other method of obtaining adequate supplies was possible. Rabbit kidney was obtained from laboratory animals. Two batches of mouse kidney were obtained, one through the co-operation of the Wellcome Laboratories of Tropical Medicine, London, and the second with the help of The Distillers Co. Ltd., Liverpool. Lasco Ltd., Liverpool, provided kidneys from two healthy horses killed after being injured in accidents. Guinea-pig kidneys were obtained from normal laboratory animals. Kidneys from a goat came from the Department of Veterinary Pathology, University of Liverpool; the animal had been suffering from John's disease, but this is not known to make the kidney abnormal.

The authors wish to record their gratitude for much help in obtaining materials and to acknowledge the courtesy extended to them by many persons acting on behalf of institutions, laboratories or industrial firms.

METHODS

Preparation of unsaponifiable matter. The kidney tissue was subjected to digestion with ethanol and KOH as follows: 60% (w/v) aqueous KOH (0.5 ml./g. of tissue) was added to moist kidney and the mixture was heated on the water bath until it became homogeneous; ethanol (1 ml./g. of tissue) was then added and the mixture was boiled under reflux for 30–45 min. After cooling, water (2 vol.) was added and the solution was extracted 5 or 6 times with ether (freshly redistilled over reduced iron). The combined ether extracts were washed free from alkali, dried over Na_2SO_4 , filtered through a sintered-glass funnel and evaporated to dryness under nitrogen. Any kidneys and any unsaponifiable fractions which it was necessary to store were kept at -20° .

Chromatography. The unsaponifiable matter was chromatographed on alumina (Brockmann Grade 3) in the manner described in previous papers (cf. Heaton *et al.* 1957).