Cholesterol and Liver Vitamin A in Male and Female Rats

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Cholesterol feeding has in recent years been widely used in the experimental approach to a variety of problems, including atherosclerosis. The rat shows a remarkable ability to remove absorbed cholesterol from the plasma and store it in the liver. If cholesterol is fed over a period, much of it, in the rat, is stored in the liver. This animal has only a limited ability to convert exogenous cholesterol into cholic acid and so to remove it from the liver via the bile. Cholesterol esters therefore accumulate, together with other lipid matter, producing the well-known 'cholesterol fatty liver' (Chanutin & Ludewig, 1933).

Okey, Gillum & Yokela (1934) reported that male rats stored significantly more cholesterol in the liver than females fed cholesterol at the same dietary level.

The present paper records another aspect of cholesterol metabolism in the rat in which the sexes differ markedly. It was shown earlier, incidentally to other work (Kantiengar & Morton, 1955), that adult male rats fed on a diet containing 2% cholesterol for a period of 8 weeks had considerably lower liver vitamin A stores than control male rats receiving no cholesterol. In that work, both experimental groups consisted of five rats; the livers of each group were bulked for estimation of vitamin A. Further experiments, using more animals, have been performed, and the results have been analysed statistically. The first two experiments of this type (Green, 1955), using weanling and adult female rats, did not agree with the earlier findings, but the possibility remained that the disagreement was due to a sex difference. Four further experiments have therefore been performed, using weanling and adult rats of both sexes. These six experiments establish a clear-cut difference in the effect of cholesterol feeding on liver vitamin A storage in male and female rats.

EXPERIMENTAL

Animals and diets. Newly weaned or adult hooded rats were given either a synthetic diet (*A*) or one based on a compound cube ration (Animal diet no. 4, British Extracting Co. Ltd., Bromborough, Cheshire). The basal diets were made up as follows. Diet *A*: casein (Genatosan Ltd., 'low vitamin content') 18, maize starch 65, arachis oil 5, Marmite water-soluble yeast extract 8, salt mixture (Osborne &

Mendel, 1913) 4%. Diet *B*: food cubes were ground to powder, exhaustively extracted with diethyl ether, and to the dried product was added arachis oil (10%) containing α -tocopherol (3 mg. per 100 g. of diet). Diet *C*: food cubes were ground (but not extracted) and arachis oil (5%) was incorporated.

Procedure. Experiments were carried out with separate groups of newly weaned and adult rats of both sexes. In each experiment there were two experimental groups. Group I received the basal diet, whilst group II received the same diet plus 2% of crystalline cholesterol (Croda Ltd.). This was dissolved in an amount of arachis oil equivalent to 5% of the diet. The groups were of equal size, as nearly as was practicable with available animals, and each rat in group I had a corresponding litter-mate in group II. The individual experiments were planned as follows. (They are described in chronological order.) (i) Six females in each group were fed on diet *A*, from weaning, for 5–6 weeks. (ii) Ten adult females in each group were fed on diet *B* for 6 weeks. (iii) Female rats, eight in group I and six in group II, were fed on diet *C*, from weaning, for 8–9 weeks. (iv) Male rats, four in group I and five in group II, were fed on diet *C*, from weaning, for 8–9 weeks. (v) Fourteen adult males in each group were fed on diet *C* for 8–9 weeks. (vi) Twelve adult females in each group were fed on diet *C* for 8–9 weeks.

The rats were housed separately in wire-mesh cages with raised screen floors to prevent coprophagy. They were fed a weighed amount of food in individual pots. This was initially 8 g./day for weanling rats, increasing to 10 g. after 3 weeks. The adult ration was always 12 g./day. Water was freely provided.

Vitamin A dosage. Diets *A* and *B* were vitamin A-free. In Expts. (i) and (ii) all animals were therefore given a tested cod liver oil, suitably diluted in arachis oil and administered orally by pipette (0.2 ml.). In Expt. (i) the dose provided 100 i.u. of vitamin A daily. In Expt. (ii) the same dose was administered once every 5 days.

The unextracted food cubes used in diet *C* contained approximately 5 i.u. of vitamin A/g. As the animals consistently ate all their food, it was unnecessary to give vitamin A by mouth in the four experiments in which diet *C* was fed.

Analysis of livers

At the end of the feeding period the rats were killed by chloroform anaesthesia and the livers were removed, washed, dried on filter paper, and weighed.

Saponification. Each liver was separately saponified by the following method. To the moist tissue, cut into small pieces, 60% (w/v) KOH (0.5 ml./g.) was added and digestion continued on a boiling-water bath until the mixture was homogeneous. Ethanol (95%, 2 vol.) was then added, and the mixture boiled under reflux for a further 45 min. Water (4 vol.) was then added and the mixture extracted (five or

six times) with ether (freshly distilled over reduced Fe). The combined ether extracts were washed with water until free from alkali, dried over anhydrous Na_2SO_4 , filtered under suction through a sintered-glass funnel, taken to dryness under N_2 , and weighed.

Vitamin A analyses. The liver unsaponifiable material was dissolved in spectroscopically pure cyclohexane, and the liver vitamin A content determined by the method of Cama, Collins & Morton (1951), with a Unicam SP. 500 spectrophotometer.

Liver cholesterol analyses. In Expts. (i) and (ii) liver cholesterol content was determined on each liver unsaponifiable fraction by the method of Kenny (1952) as modified by Green, Lowe & Morton (1955).

RESULTS

In all six experiments, livers from group II animals were markedly larger than those of the corresponding group I animals. Group I livers were normal in appearance, but group II animals invariably had typical pale pink-grey 'cholesterol fatty livers'. These contained greatly increased amounts of unsaponifiable material. There was a similar increase in cholesterol content (Expts. (i) and (ii)). These findings are shown in Tables 1 and 2.

A direct comparison can be made between the increases in liver unsaponifiable material of young males and females of group II in Expts. (iv) and (iii) respectively, as these animals received the same diet for the same length of time. Both sexes showed the same increase in group II total unsaponifiable

matter when compared with their group I controls. The increase was 10.0 times for the males and 9.7 times for the females.

A similar comparison is possible between the adult males and females of Expts. (v) and (vi). In the case of adults a sex difference was observed. The increase in total liver unsaponifiable material of group II over group I animals in these cases was 6.5 times for the males and 9.5 times for the females.

The mean liver vitamin A contents for each group and the statistical analyses derived from the individual values for each animal within a group are shown in Tables 3 and 4.

DISCUSSION

The data given in Tables 3 and 4 show first that feeding cholesterol does not affect liver vitamin A storage in adult female rats, but lowers the liver vitamin A levels of adult males. In earlier work in this Laboratory (Kantiengar & Morton, 1955) the cholesterol-fed adult males had 35 % less vitamin A in bulked livers than had the controls. In the present work (Expt. (v), Table 4) single livers were examined and the average drop was less dramatic (16 %), but highly significant even at the 1 % level of probability.

Secondly, the sex difference observed with adults also applies to young rats fed on cholesterol from weaning. Here the results are less clear-cut. In Expts. (i) and (iii) (Table 3) the cholesterol-fed

Table 1. *Liver weights, liver unsaponifiable material, and liver cholesterol contents of control (group I) and cholesterol-fed (group II) young male and female rats*

	Males		Females			
	Expt. (iv)		Expt. (i)		Expt. (iii)	
	Group I	Group II	Group I	Group II	Group I	Group II
No. of rats	4	5	6	5*	8	6
Mean liver wt. (g.)	6.14	7.29	4.85	6.48	5.93	6.31
Percentage of unsaponifiable matter	0.55	4.65	0.96	10.75	0.43	4.06
Total unsaponifiable matter (mg.)	33.8	339	47	695	26	253
Total cholesterol (mg.)	—	—	16.4	483	—	—

* One animal in group II was incorrectly sexed at the time of weaning; therefore its analyses have been omitted from the mean values.

— signifies not determined.

Table 2. *Liver weights, liver unsaponifiable material, and liver cholesterol contents of control (group I) and cholesterol-fed (group II) adult male and female rats*

	Males		Females			
	Expt. (v)		Expt. (ii)		Expt. (vi)	
	Group I	Group II	Group I	Group II	Group I	Group II
No. of rats	14	14	10	10	12	12
Mean liver wt. (g.)	8.18	9.60	6.83	7.28	7.49	8.43
Percentage unsaponifiable matter	0.50	2.77	0.99	3.33	0.56	4.74
Total unsaponifiable matter (mg.)	41	266	68	243	42	401
Total cholesterol (mg.)	—	—	25	206	—	—

Table 3. *Comparison of liver vitamin A levels in control (group I) and cholesterol-fed (group II) young male and female rats*

Expt.	Sex	No. of rats		Mean liver vitamin A (i.u.)		Percentage difference $\left(\frac{I-II}{I} \times 100\right)$	Statistics	
		I	II	I	II		<i>t</i> (found)	<i>t</i> (tables)
(iv)	M.	4	5	1699 (± 87)*	924 (± 58)	46	8.171	3.499
S.								
(i)	F.	6	5	796 (± 73)	646 (± 93)	19	0.953	3.250
N.S.								
(iii)	F.	8	6	1249 (± 119)	952 (± 78)	24	1.930	3.055
N.S.								

* Standard deviation from the mean. S.=significant. N.S.=not significant. For significance, *t* (found) must exceed *t* (tables) at the desired level of probability (0.01) (Snedecor, 1946).

Table 4. *Comparison of liver vitamin A levels in control (group I) and cholesterol-fed (group II) adult male and female rats*

Expt.	Sex	No. of rats		Mean liver vitamin A (i.u.)		Percentage difference $\left(\frac{I-II}{I} \times 100\right)$	Statistics	
		I	II	I	II		<i>t</i> (found)	<i>t</i> (tables)
(v)	M.	14	14	3714 (± 494)*	3117 (± 524)	16	3.100	2.779
S.								
(ii)	F.	10	10	5438 (± 740)	5806 (± 890)	-7	0.437	2.878
N.S.								
(vi)	F.	12	12	5003 (± 484)	5300 (± 854)	-6	0.651	2.819
N.S.								

* See footnote to Table 3.

young females have liver vitamin A stores lowered, on the average, by 19 and 24 % respectively, but these changes are not significant at $P=0.01$. The mean depression in Expt. (i) is not significant even at the 10 % level of probability. The mean depression in Expt. (iii) is just significant at the 10 % level [*t* (found)=1.93, *t* (tables)=1.78], but not at the 5 % level [*t* (tables)=2.18]. With young males, however, a dramatic drop of 46 % is highly significant at the 1 % level of probability.

If the possibility that dietary cholesterol affects intestinal absorption of vitamin A is excluded, two alternative reasons can be advanced why cholesterol-fed rats should store less vitamin A in their livers than do controls: (1) cholesterol-fed rats require more of the vitamin, i.e. they metabolize it more rapidly; (2) absorbed cholesterol competes with vitamin A for storage in the liver, and may even displace it.

If the production of a 'cholesterol fatty liver' is itself a metabolic hazard, the first suggestion can be supported. Thus the stress of sterile abscesses (induced by subcutaneous injection of turpentine) caused a marked lowering of serum and liver vitamin A level (Kagan & Kaiser, 1955). Ershoff

(1950) showed that the stress of sustained exposure to cold accelerated the production of vitamin A deficiency. Clark & Colburn (1955) demonstrated that the stress of inanition mobilized liver vitamin A and suggested that the effect was mediated through the adrenal glands.

There is indirect support for the second idea, namely that absorbed cholesterol competes with vitamin A for liver storage 'space'. Cholesterol and vitamin A ester both pass from the plasma to the parenchymal cells via the reticulo-endothelial system. For example, Friedman & Byers (1954) blocked the reticulo-endothelial system with colloidal indian ink and then administered cholesterol, which accumulated in the plasma. Glover & Morton (1948) showed that chylomicrons containing vitamin A are ingested from the blood by the Kupffer cells. Several workers (e.g. Lasch & Roller, 1936) have reported that reticulo-endothelial system blocking agents displace vitamin A into the plasma. Therefore excessive ingestion of cholesterol-bearing chylomicrons by the Kupffer cells may cause ejection of vitamin A from these cells.

When the animals in Expt. (i) were killed, the kidneys, lungs and spleen were examined to see

whether vitamin A had been displaced from the liver to other organs. No such effect was observed. As females were used in that experiment, it is now necessary, in view of our later findings, to examine the same organs from cholesterol-fed males.

Although Okey *et al.* (1934) reported a difference in the amounts of exogenous cholesterol stored in the liver by males and females, these amounts were large in both sexes. Therefore the depression of liver vitamin A in only males cannot readily be explained in terms of a direct displacement by cholesterol. Cholesterol feeding must have some indirect metabolic effect on the liver vitamin A levels of male rats.

A well-known sex-difference in the rat is the much higher requirement of males for the essential fatty acids (see e.g. Greenberg, Calbert, Savage & Deuel, 1950). One of these acids (linoleic) appears to be the main acid present in plasma cholesteryl esters (Deuel, Alfin-Slater, Wells, Kryder & Aftergood, 1955), and Peifer & Holman (1955) showed that a dietary cholesterol supplement accelerated the rate of induction of essential fatty acid deficiency. The sex difference in liver vitamin A storage may therefore be no less closely linked with the essential fatty acids than with cholesterol.

Sexually immature males fed on cholesterol showed a greater depression of liver vitamin A than adult males. Coleman, Chen, Alfin-Slater & Deuel (1956) castrated mature male rats and then fed them on a diet containing cholesterol (1%) and cottonseed oil (15%). After 6 weeks the castrated males had less liver cholesterol than intact male controls. However, ovariectomized females showed the reverse effect. If rats of either sex were castrated at weaning, and subsequently, at an age when their intact controls were sexually mature, given the same diet, no significant differences in liver cholesterol levels were observed for either sex, when compared with intact controls.

The reason for the sex difference in essential fatty acid requirement is not yet known. Panos, Klein, Wall & Finerty (1956) showed that the retardation of growth which is typical of fat deficiency is less marked in ovariectomized females, or intact females given testosterone, than in intact females, but is more marked in ovariectomized females given oestrone. Similar investigations with males are necessary before an interpretation of these results or of those of Coleman *et al.* (1956) can be profitably attempted. The recent work emphasizes the inter-relationship of cholesterol, the essential fatty acids and gonadal function in both sexes.

We are now carrying out experiments to deter-

mine whether there is a sex difference in liver vitamin A storage in fat-deficient rats, and in fat-deficient rats fed cholesterol.

SUMMARY

1. The effect of a dietary supplement of cholesterol (2% by weight) on liver vitamin A storage has been determined for young and adult rats of both sexes.

2. Cholesterol feeding had no statistically significant effect on liver vitamin A stores in young or adult female rats, but the liver vitamin A levels of males were significantly lowered.

3. The mean depression of liver vitamin A was more marked in sexually immature males (46%) than in adult males (16%).

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REFERENCES

- Cama, H. R., Collins, F. D. & Morton, R. A. (1951). *Biochem. J.* **50**, 48.
- Chanutin, A. & Ludewig, S. (1933). *J. biol. Chem.* **107**, 207.
- Clark, I. & Colburn, R. W. (1955). *Endocrinology*, **56**, 232.
- Coleman, R. D., Chen, Y.-M., Alfin-Slater, R. B. & Deuel, H. J. jun. (1956). *Fed. Proc.* **15**, 234.
- Deuel, J. H. jun., Alfin-Slater, R. B., Wells, A. F., Kryder, G. D. & Aftergood, L. (1955). *J. Nutr.* **55**, 337.
- Ershoff, B. H. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 586.
- Friedman, M. & Byers, S. O. (1954). *Amer. J. Physiol.* **179**, 201.
- Glover, J. & Morton, R. A. (1948). *Biochem. J.* **43**, xii.
- Green, B. (1955). Ph.D. Thesis: University of Liverpool.
- Green, B., Lowe, J. S. & Morton, R. A. (1955). *Biochem. J.* **61**, 447.
- Greenberg, S. M., Calbert, C. E., Savage, E. E. & Deuel, H. J. jun. (1950). *J. Nutr.* **41**, 473.
- Kagan, B. M. & Kaiser, E. (1955). *J. Nutr.* **57**, 277.
- Kantiengar, N. L. & Morton, R. A. (1955). *Biochem. J.* **60**, 28.
- Kenny, A. P. (1952). *Biochem. J.* **52**, 611.
- Lasch, F. & Roller, D. (1936). *Klin. Wschr.* **15**, 1636.
- Okey, R., Gillum, H. L. & Yokela, E. (1934). *J. biol. Chem.* **107**, 207.
- Osborne, T. B. & Mendel, L. B. (1913). *J. biol. Chem.* **15**, 317.
- Panos, T. C., Klein, G., Wall, R. L. & Finerty, J. C. (1956). *Fed. Proc.* **15**, 141.
- Peifer, J. J. & Holman, R. T. (1955). *Arch. Biochem. Biophys.* **57**, 520.
- Snedecor, G. W. (1946). *Statistical Methods*, chap. 3, p. 62. Ames, Iowa: Iowa State College Press.