Dietary Cholesterol and Liver Vitamin A in Normal and Castrated Male Rats

By A. A. HORNER AND R. A. MORTON

Department of Biochemistry, The University of Liverpool

(Received 18 August 1959)

Green, Horner, Lowe & Morton (1957) showed that a dietary supplement of cholesterol brought about a marked and significant depression of liver vitamin A stores in male rats but not in female. Further experiments on similar lines are now reported in which normal and castrated male rats were compared. In these experiments, as in the earlier ones, the cholesterol might have depressed either absorption or liver storage of vitamin A (or perhaps both). Alternatively, the vitamin might have been 'stored' to the same extent as that shown by normal animals on a stock diet but utilized (i.e. mobilized and destroyed) more rapidly. Throughout the feeding periods vitamin A and cholesterol were given together in the diets so that there is no presumptive reason to reject either suggestion. Additional experiments were therefore planned with normal and castrated males in which liver vitamin A stores were built up before feeding with cholesterol in a diet which provided no further vitamin A. Any depression of liver vitamin A in such experiments would be due either to increased utilization of liver vitamin A stores or to their displacement by cholesterol accumulating in the liver.

EXPERIMENTAL

Diets. All diets were based on a stock compound cube ration (Animal diet no. 4, British Extracting Co. Ltd., Bromborough, Cheshire). In Expt. (i) diet A contained ground cubes (95%) blended with arachis oil (5%). Diet B contained ground cubes (93%), arachis oil (5%) and crystalline cholesterol (Croda Ltd.) (2%). The food cubes contained vitamin A (approx. 5 i.u./g.). In Expt. (ii) diet C was composed of defatted cubes (i.e. ground to a powder and then exhaustively extracted with diethyl ether) (90%), blended with arachis oil (10%). Diet D contained similarly extracted food cubes (88%), arachis oil (10%) and cholesterol (2%).

Procedure. Expt. (i). Twenty-two newly weaned male hooded rats were selected from five litters born within 4 days of each other, and fed on the stock cube diet for 3 days without restriction. The 12 heaviest rats were then surgically castrated under ether anaesthesia. Two days after the operation the four experimental groups were formed. In group I five normal males received diet A, and five others constituting group II received diet B. In group III six castrated males were fed on diet A and six more, constituting group IV, received diet B. Litter mates were

evenly distributed between the groups, as far as possible. All animals were offered 12 g. of food/day throughout the experiment. Three rats from each of groups I and II were killed after 28 days. The remaining 16 animals were all killed after 56 days.

Expt. (ii). Thirty-one male hooded rats from nine litters born within 4 days of each other were weaned and given a diet of ground food cubes without restriction for 10 days. Each rat was given vitamin A (2500 i.u., synthetic acetate) by mouth on each of the last 4 days of this period; this daily dose also contained 2 mg. of DL- α -tocopheryl acetate, and was administered in 0.2 ml. of glycerol-ethanol (9:1, v/v). On the eleventh day after weaning, 19 rats were surgically castrated under ether anaesthesia. All the animals were fed on diet C for the next 3 days and then were divided into four experimental groups. In group I six normal males were fed with diet C and another six in group II received diet D. In group III nine castrated animals received diet C and ten castrated animals in group IV were fed on diet D. All the rats were given 10 g. of food/day for the first 20 days and then 12 g./day until they were killed after 42 days on the experimental diets.

Analyses of livers, kidneys and plasma. Expt. (i). The rats were killed by cardiac puncture under chloroform anaesthesia. Livers were saponified separately and vitamin A was measured as reported previously (Green *et al.* 1957). Kidneys were bulked within each experimental group and saponified to measure the group-mean kidney vitamin A levels. Blood from a pair of rats was bulked and centrifuged to obtain about 5 ml. of plasma for vitamin A estimation by the method of Glover, Goodwin & Morton (1947).

Expt. (ii). Each liver was ground with five times its own weight of anhydrous Na_2SO_4 and a little silver sand. The mixture was extracted with five 100 ml. portions of boiling ether and the bulked extracts were evaporated to dryness under reduced pressure on a warm-water bath. Liver vitamin A was determined spectrophotometrically on a small portion of the extracted lipid dissolved in cyclohexane, the correction formulae given by Cama, Collins & Morton (1951) for vitamin A acetate being used. Kidneys and plasma were treated as in Expt. (i).

RESULTS

Expt. (i). The results are shown in Table 1. Originally the whole experiment was planned to be of 4 weeks' duration. Accordingly, three animals from each of groups I and II were killed after 28 days. Liver vitamin A in group II was only slightly lower (4%) than in group I and the difference has no significance (t 0.3163, $P > 0.5$).

The data from these six animals show that at 28 days cholesterol-feeding had not caused a gain in liver weight, although liver unsaponifiable material was already four times the control value. The control rats had gained 49 g. in weight in this period, and those given cholesterol had gained only 38 g.

The rest of the animals were killed after 8 weeks. The normal males in groups I and II were of very similar mean weight and their actual gains in weight were identical (90 g.). Similarly, the castrates in groups III and IV both gained 77 g. during the experiment. At 42 days, groups I-IV had gained 78, 72, 62.5 and 59 g. respectively. As was expected, both cholesterol-fed groups had larger livers than their respective controls. The liver weights (after 56 days) of groups I-IV expressed as percentages of body weights were 5.08, 5.94, 5.55 and 6.64 % respectively.

The ratios of total unsaponifiable material in the livers of groups I-IV (animals killed after 56 days) were 1.00:4.64:0.54:3.00. Group II livers contained 32 % less vitamin A than their group I controls. The difference was significant at the 5 % level of probability (t 5.499). Similarly, liver vitamin A in group IV was 31 % lower than in

group III, the difference being highly significant (t 3.212, $P < 0.01$). A difference of 4.5 % between groups I and III had no significance (t 0.3949, $P > 0.5$).

Expt. (ii). The results are summarized in Table 2. The gains in weight of groups I-IV in the 42 days were 79.5, 65.9, 71.2 and 62.2 g. respectively. Liver weights represented 4.15, 5.40, 3.63 and 4.77 % of total body weight respectively. Liver-lipid concentrations in the four groups were 26.8, 99.3, 27.6 and 104.7 mg./g. respectively.

Liver vitamin A contents in groups I and II were practically identical, the latter being greater by 0.6 %, a difference of no significance (t 0.0822, $P > 0.5$). The group III value exceeded that of group IV by 2.2 %. Again the difference had no significance (t 0.4896, $P > 0.5$). Dietary cholesterol had not affected liver vitamin A in either normal or castrated animals, as compared with their controls. Therefore the mean liver vitamin A value for groups I and II combined (4029 i.u.) may be compared with the corresponding value for groups III and IV (4529 i.u.). This represents a level in the castrates 12.4 % higher than that in intact rats. This difference is highly significant (t 3.023 $P < 0.01$).

Table 1. *Expt. (i): vitamin A given throughout the experimental feeding period*

Body and liver weights, liver unsaponifiable material and liver, kidney and plasma vitamin A levels of control (group I) and cholesterol-fed (group II) young normal male rats, and of control (group III) and cholesterol-fed (group IV) young castrated male rats. Standard deviations of the means, or standard errors, are given in parentheses. N.D., Not determined.

	Animals killed after 28 days		Animals killed after 56 days			
	Group I	Group II	Group I	Group II	Group III	Group IV
No. of rats	3	3	2	2	6	6
Final mean body wt. (g.)	91	82	139	136	130	129
Mean liver wt. (g.)	3.98	3.89	4.57	5.35	4.27	5.11
Mean total liver unsaponifiable material (mg.)	28.0	111.7	42.8	198.7	23.2	128.6
Mean total liver vitamin A (i.u.)	463	445	1257	859	1201	832
	(± 48)	(± 30)	(± 58)	(± 48)	(± 83)	(± 86)
Mean plasma vitamin A (i.u./100 ml.)	41.3	34.7	39.6	40.9	37.6	32.4
Mean kidney-pair vitamin A (i.u.)	N.D.	N.D.	6.0	3.9	6.4	8.8

Table 2. *Expt. (ii): vitamin A administered before the feeding period*

Body and liver weights, liver-lipid material and liver, kidney and plasma vitamin A levels of control (group I) and cholesterol-fed (group II) young normal male rats and of control (group III) and cholesterol-fed (group IV) young castrated male rats. Standard deviations of the means are given in parentheses.

	Normal rats		Castrated rats	
	Group I	Group II	Group III	Group IV
No. of rats	6	6	9	10
Final mean body wt. (g.)	123	111	121	112
Mean liver wt. (g.)	5.08	5.98	4.38	5.34
Mean total liver-lipid material (mg.)	136	594	121	559
Mean total liver vitamin A (i.u.)	4017	4041	4581	4483
	(± 241)	(± 166)	(± 180)	(± 100)
Mean plasma vitamin A (i.u./100 ml.)	34.9	45.2	43.2	57.8
Mean kidney-pair vitamin A (i.u.)	13.7	17.7	7.5	9.1

DISCUSSION

The results of each experiment showed the comparative effects of two kinds of treatment, cholesterol-feeding and castration. In Expt. (i) castration had depressed growth more effectively than dietary cholesterol after 42 days. In Expt. (ii) the reverse effect was observed. At the end of Expt. (i) only castration depressed growth, the earlier effect of cholesterol having been overcome. In the last 14 days the cholesterol-fed rats actually grew more rapidly than their controls. The period of feeding seems to be critical in these growth effects.

In both experiments cholesterol-feeding caused an increase over control liver weights to about the same extent in normal and castrated animals. In Expt. (i) castrates (group III) had smaller livers than normal rats (group I) but the organ constituted a slightly larger percentage of body weight in the former. In the second experiment group III had smaller livers than normal animals both on an actual weight and percentage body-weight basis. This again illustrates changes which occurred in an extra 2 weeks of feeding in the first experiment.

In Expt. (i) cholesterol caused a rather larger increase over the control value for total liver unsaponifiable material in castrates than in normal rats. Comparison of groups I and III shows that castration caused a marked drop of 46% in liver total unsaponifiable matter. In the second experiment cholesterol-feeding increased liver-lipid concentration to 3.7 times the control value in normal rats and 3.8 times in castrates. Liver-lipid concentration was not altered by castration. These lipids were analysed by chromatography on silicic acid and alkali-isomerization of their fatty acids.

Cholesterol depressed liver vitamin A in both castrated and normal rats in Expt. (i). The results with intact animals confirmed earlier findings (Green *et al.* 1957). The cholesterol-vitamin A effect is not controlled by male gonadal hormones, for it was equally marked in the castrated animals. It was shown in the second experiment that liver vitamin A stores formed before cholesterol was given were not affected by such treatment. Therefore vitamin A utilization was not increased, nor did cholesterol which accumulated in the liver displace vitamin A from the organ. The livers of castrated rats in Expt. (ii) contained 12.4% more vitamin A than those of normal animals. All rats received equal doses of vitamin A before some of them were castrated. The castrates would therefore have at the operation liver stores of the vitamin approximately equal to those of the normal rats. Therefore in spite of the operative stress the castrates utilized their stores less rapidly than did the normal animals. This finding is supported by

Bult & Sorgdrager (1938), who showed that males utilized vitamin A stores less rapidly during a depletion period if they had been castrated. Booth (1952) confirmed this.

In the first experiment the livers of castrated and normal males contained similar amounts of vitamin A. If both groups laid down liver vitamin A with the same efficiency, and the deposits were utilized more slowly by the castrates, one would have expected the castrates to have had the larger net vitamin A storage. That this was not the case suggests that liver accumulation was less efficient in the castrated males. The results of Moore, Sharman & Ward (1951) do not support this idea for they found that in castrated males liver vitamin A storage was double the normal value. However, they worked at a very low dosage level, at which kidney storage of the vitamin was qualitatively more important than liver storage, so that their experiments and ours are not strictly comparable.

The fate of vitamin A other than that in the liver after feeding with cholesterol and vitamin A concomitantly as in Expt. (i) was not elucidated. The kidney is generally accepted to be an important extrahepatic site of vitamin A storage. If the vitamin was displaced from (or prevented from entering) the liver by cholesterol, a rise in kidney or plasma vitamin A or both should have been observed. The results show that no such changes occurred. Castration did not increase kidney vitamin A levels in the present experiments, nor in those of Moore *et al.* (1951).

Green *et al.* (1957) postulated that absorbed cholesterol and vitamin A competed for liver storage space. Expt. (ii) showed that liver vitamin A stores formed before cholesterol was given were not displaced by subsequent cholesterol infiltration. Cholesterol and vitamin A might, however, compete for retention by liver when fed concomitantly. If this were to occur the vitamin might be stored by other tissues or destroyed in the plasma.

As Expt. (i) established that vitamin A utilization was not affected by cholesterol, two other possible explanations of the effect of cholesterol-feeding remain to be considered. One is that vitamin A is absorbed less efficiently from the intestine in the presence of cholesterol; the alternative is that if absorption is normal vitamin A is not removed from the plasma by the liver to the usual extent, presumably because of competition from abnormally large numbers of cholesterol-containing chylomicra. In the latter case the vitamin could be taken up by other tissues or degraded by oxidative-enzyme systems in the plasma. Conventional feeding experiments of the type used here cannot discriminate between the two possibilities but the use of radioactive vitamin A may allow the problem to be solved.

The present work with castrates has shown that the effect of cholesterol in males is not controlled by their secondary sex hormones. However, it is of primary importance to elucidate the precise mechanism of the cholesterol-vitamin A effect in male rats before attempting to discover why it is not manifested in females.

SUMMARY

1. The effects of a dietary supplement of cholesterol on liver vitamin A stores have been determined for normal and castrated young male rats.

2. When vitamin A and cholesterol were given in the diet together, liver vitamin A stores were significantly lowered to the same degree in normal and castrated animals. No significant changes in kidney or plasma vitamin A levels were observed.

3. If the animals were given large doses of vitamin A before cholesterol was given in a vitamin A-free diet, liver vitamin A stores were not affected in either normal or castrated rats.

4. Castration significantly decreased the rate of utilization of liver vitamin A in young male rats.

5. In castrates receiving a cholesterol-free diet liver unsaponifiable material was markedly reduced.

We thank Professor R. G. Harrison for castrating the rats. The present study formed part of work which was assisted by a grant from the Nuffield Foundation.

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The Chemical Composition of Snail Gelatin

By A. P. WILLIAMS

The British Gelatine and Glue Research Association, 2a Dalmeny Avenue, London, N. 7

(Received 7 July 1959)

Early work on the distribution of collagen in the animal kingdom, mainly based on X-ray studies, was considered by Bear (1952) and Rudall (1955). Watson (1958*b*) has reviewed the chemical analyses available for invertebrate collagens, which at that time were restricted to the cuticle of earthworm (*Lumbricus* sp.) (Watson, 1958*a*), the cuticle of *Allobophora longa* (Singleton, 1957), the byssus threads of *Mytilus edulis* (Jackson *et al.* 1953), and the spongin fibrils from the mesogloea of sponges (Gross, Sokol & Rougvie, 1956).

Since Watson's review, analyses have appeared of the cuticle of *Ascaris lumbricoides*, and of the cuverian tubules of sea cucumber (*Holothuria forskali*), both by Watson & Silvester (1959), and a preliminary study was made of the body wall of the garden snail (*Helix aspersa*) by Melnick (1958). More recently, Piez & Gross (1959) have reported the analyses of *Thyone* body wall (Echinodermata), of *Metridium* skin and *Physalia* float (Coelenterata), and of spongin A and spongin B from *Spongia graminiae* (Porifera). This present paper is concerned with the chemical composition of gelatin derived from the intact collagen of the body wall of the garden snail.

EXPERIMENTAL

Materials

Snail gelatin. The 200 mg. (air-dried weight; moisture 12.4%) sample of gelatin used was prepared from the body walls of 30 garden snails by Melnick (1958). The snails were killed by immersion in aqueous 10% (v/v) ethanol for 2 hr. The shells were removed and each body was opened by cutting along the dorsal ridge from the mouth to the collar. The viscera were removed and the body walls soaked in 5% (w/w) hydrochloric acid to coagulate the mucus. The material was then soaked in 0.5% sodium chloride solution for a week followed by a short treatment in saturated lime water. The material was extracted by warming in water for 2 hr. at 70°, 80° and 90°. Each extract was filtered and concentrated *in vacuo* over phosphorus pentoxide. The gelatin used for analysis was that extracted at 90°.

Analytical methods

Hydrolysis. The gelatin (100 mg.) was hydrolysed with 20 ml. of 20% (w/w) hydrochloric acid at 100° in a sealed tube for 24 hr.

Amino acid analysis. The analysis was carried out by the method of Moore & Stein (1951) with the modification suggested by Eastoe (1955); the corrections applied were those used by Eastoe. Hydroxyproline was estimated as by