3. Among the unsaturated acids, octadecenoic acid was present together with eicosenoic acid.

4. Polyunsaturated acids were absent, eicosatetraenoic acid was present only in traces in diamond snake oil but docosenoic acid occurred to the extent of 3 % in python fat.

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Liver Catalase in the Riboflavin-deficient Mouse

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Work so far carried out in this laboratory has shown that testicular and adrenal hormones and trace metals are of importance in the maintenance of liver-catalase activity in the normal mouse (Adams, 1950*a*, 1952, 1953*a*). In the search for additional factors influencing the catalase activity the effects of riboflavin deficiency have been studied, and some results of this work are described in the present paper. It had been hoped to pursue the subject further before publication, but work has been held up for some time by an infection of the liver in the strain of mice used ('Tyzzer's disease'). Previous experience here has indicated that a variety of infectious diseases in which the liver is directly involved ('Tyzzer's disease', salmonella infections, and ectromelia) result in a lowered liver-catalase activity, and a reduction or disappearance of the normal sex difference in enzyme level. Further, under such conditions, attempts to change the liver catalase level by hormone infection are quite unsuccessful. Since two brief reports of the present work on riboflavin deficiency have already appeared (Adams, 1953*b*, 1954) it seemed desirable to publish in full the experimental results obtained up to the outbreak of the epidemic. These results suggest that testosterone, which is responsible for the sex difference in catalase activity in normal mice, requires the presence of riboflavin for its action on the enzyme level.

EXPERIMENTAL

Animals. Mice of a stock albino strain were used. Water was provided *ad lib*.

Diets. Mice fed normal diet were given commercial 'rat'

cubes prepared according to the Rowett Institute formula (Thomson, 1936).

A diet designed to induce riboflavin deficiency was prepared by British Drug Houses Ltd. according to the following recipe:

	%
Casein, 'fat and vitamin free'	21
Sucrose	32.5
Rice starch	32.5
Arachis oil	9.1
Agar	1.0
Salt mixture	2.0
Succinyl sulphathiazole*	1.0
Vitamin mixture	0.85

* Altered to succinyl sulphathiazole, 0.75 %, streptomycin sulphate, 0.25 %, in later experiments.

The salt mixture was prepared according to the method of Hubbel, Mendel & Wakeman (1937).

The vitamin mixture per 5 kg. of diet was as follows: thiamine 30 mg., pyridoxine 30 mg., biotin 1 mg., nicotinic acid 250 mg., calcium pantothenate 200 mg., folic acid 17 mg., inositol 200 mg., *p*-aminobenzoic acid 100 mg., vitamin B₁₂ 1 mg., ascorbic acid 500 mg., tocopherol acetate 350 mg., 2-methyl-1:4-naphthoquinone (Menaphthone) 150 mg., choline chloride 20 g., cod liver oil 20 g. These substances were mixed in with the rest of the diet. Since the average amount of diet eaten by the mice was about 3 g. each/day, each mouse received approximately 1/1700 of these quantities/day. The powder was moistened with sufficient water to give a rather dry dough (approximately 1 ml./5 g. of diet). The diet of pair-fed control groups receiving a riboflavin supplement was prepared by moistening the powder with water containing 25 µg. of riboflavin/ml. (i.e. providing 15 µg./mouse/day).

Mice were placed on the diets at weanling age (weight about 12 g.).

Riboflavin antagonist. A sample of the riboflavin antagonist 6:7-dichloro-9-D-1'-ribitylisoalloxazine (RA) was kindly provided by Roche Products Ltd. This was injected intraperitoneally in saline solution.

Estimation of liver-catalase activity. As previously described (Adams, 1950*b*, 1952).

Operative procedure. Castration was carried out as previously described (Adams, 1952).

Hormone. This was testosterone, commercially supplied for implantation. This substance was injected in 100 μ g. doses, dissolved in the minimum quantity of aqueous ethanol (50%, v/v).

RESULTS

Use of the diet

Weanling mice placed on the riboflavin-free diet showed an almost complete failure of growth. Other symptoms of deficiency were rarely observed but mortality was high after about six weeks. Fig. 1

Fig. 1. Growth curves of weanling male mice placed on the riboflavin-deficient diet (●—●), the deficient diet supplemented with riboflavin, pair-fed (×---×), the cube diet pair-fed (○...○), and the cube diet *ad lib.* (▲---▲). Results in this and the next three figures are given as arithmetic means $\pm$ s.e.m. with 6-8 animals/group.

shows typical growth curves of mice kept on the deficient diet, pair-fed on the deficient diet supplemented with riboflavin, pair-fed on the cube diet, and fed *ad lib.* on the cube diet. The mice on the riboflavin-deficient diet ate about 3 g. each/day compared with 6-7 g. each/day eaten by the *ad lib.* cube-diet groups. The graph shows that no growth was obtained in the deficient group, while there was a steady growth rate in the pair-fed groups. In

these latter mice the cube diet produced a slightly higher growth rate than the synthetic diet and added riboflavin. This appeared to be due largely to very slow growth in the first week of feeding the synthetic diet. The mice seemed to require a few days to settle down but subsequently took the diet well. It was found in preliminary experiments that unless agar was included, the mice suffered from diarrhoea. The results of catalase estimations on the mice described in Fig. 1 are given in Fig. 2. However, since catalase measurements were not always made when the mice were weighed, more points appear in Fig. 1 than in Fig. 2.

Catalase levels in riboflavin-deficient mice

Fig. 2 shows liver-catalase activities in three groups of male mice at intervals after being placed respectively on the riboflavin-free diet, the riboflavin-free diet supplemented with riboflavin (pair-fed), and the cube diet (pair-fed). A group of mice

Fig. 2. Liver-catalase activities in weanling male mice kept on the riboflavin-deficient diet (●—●), the deficient diet supplemented with riboflavin, pair-fed (×---×), the cube diet pair-fed (○...○), and the cube diet *ad lib.* (▲---▲). Testosterone was injected into the first three groups after 4 weeks.

receiving the cube diet *ad lib.* was also included. During 4 weeks the catalase activity fell in the first three groups, although the usual rise to adult level occurred in the *ad lib.* cube-diet group. Normally the catalase activity in male weanling mice rises to adult level within 2-3 weeks. It was considered that the fall in activity in the experimental mice might be caused by an interference with testosterone secretion due to the decrease in total food intake, and so, after 4 weeks on the diets, daily injections of testosterone were given. A rise to the normal adult

level was obtained in the pair-fed groups, there being no change in the riboflavin-deficient group. In an entirely separate experiment using male mice (Fig. 3) daily injections of testosterone were started earlier. Under these conditions the catalase level in pair-fed controls rose to normal, while that in the riboflavin-deficient animals fell as in the previous experiment. Riboflavin was then added to the diet of the deficient animals, testosterone still being injected. The dietary intake was held down to the amount eaten by the riboflavin-deficient group immediately prior to the addition of riboflavin. Within a week the catalase activity of the group which had been deficient rose to normal. A rapid weight increase also followed the feeding of riboflavin to deficient mice.

In this experiment haemoglobin estimations were also made in parallel with the catalase measurements. The results are shown in Table 1. No significant variations in haemoglobin level were observed, the values in all groups being approxi-

mately equal to that of the *ad lib.* cube-diet mice at all stages.

Groups of female mice were also placed on the riboflavin-free diet. No change in catalase activity occurred in either the deficient or pair-fed control groups (Fig. 4), the level in both cases being normal. However, once again testosterone injection produced a rise in catalase activity only in the groups receiving either a riboflavin supplement, or pair-fed on the cube diet.

Fig. 3. Liver-catalase activities in weanling male mice kept on the riboflavin-deficient diet (●—●), the deficient diet supplemented with riboflavin pair-fed (×—×), the cube diet pair-fed (○...○), and the cube diet *ad lib.* (▲—▲). Testosterone was injected daily after 3 weeks into the first 3 groups and riboflavin given to the deficient mice after 4-5 weeks.

Fig. 4. Liver-catalase activity in weanling female mice placed on the riboflavin-deficient diet (●—●), the deficient diet supplemented with riboflavin pair-fed (×—×), the cube diet pair-fed (○...○), and the cube diet *ad lib.* (▲—▲). Testosterone was injected into the first 3 groups after 5 weeks.

Results using the riboflavin antagonist

The experiments already described suggest that testosterone does not exert its normal action on catalase activity in the absence of riboflavin. Further, the normal catalase level in female mice suggests that the adrenal factor affecting catalase level (Adams, 1952) does not need the intervention of riboflavin. Confirmatory evidence was sought by following the change in catalase level occurring in mice injected with RA. Fig. 5 shows the catalase

Table 1. Haemoglobin levels in normal male mice compared with those in riboflavin-deficient and pair-fed mice

Haemoglobin levels are given in g./100 ml. as arithmetic means±s.e.m. Eight animals/group.

Mice	Weeks on diet				
	0	2	3	4-5	5-5
Riboflavin-deficient diet	—	16.5±0.3	16.8±0.2	16.0±0.4*	14.8±0.5
Riboflavin-deficient diet supplemented with riboflavin (pair-fed)	14.5±0.5	15.9±0.2	16.3±0.4	16.0±0.4	14.5±0.5
Cube diet (pair-fed)	—	17.0±0.6	—	16.2±0.6	—
Cube diet <i>ad lib.</i>	—	—	15.8±0.6	—	16.2±0.5

* Riboflavin added to diet.

activities of normal male mice after single injections of 100 μ g. of RA. A further group of these mice was castrated to determine the castration level. The graph shows that 100 μ g. of RA reduced the catalase activity of normal males to the castration level after 48 hr., the normal level being restored after a further 48 hr. Increasing the dose of RA to 500 μ g. produced the same effect. Fig. 5 also shows that little or no change in the catalase level of castrated males resulted from the injection of 500 μ g. RA.

The continuous daily injection of 250 μ g. RA into normal males and normal females gave the result shown in Table 2. Little change occurred in the

females although there was possibly a slight late fall in catalase activity. In the males, the enzyme activity fell after 48 hr., rose to normal at the seventh day, and subsequently began to fall again, reaching a constant level about 14 days after the start of the experiment.

DISCUSSION

In all investigations involving the deprivation of an essential food factor, two separate processes are involved: the deficiency of the factor itself, and the concomitant fall in total food intake. It has been

Fig. 5. (a) Liver-catalase activities in normal young adult male mice after a single injection of riboflavin antagonist, compared with the catalase activities of castrated males from the same batch. Injected with 100 μ g. of antagonist (○---○), with 500 μ g. (●---●). Normal controls at 0 day, ●, castrated controls, ▲. The crosses represent the arithmetic mean values of the groups, and the points, (●, ○, ▲), individual mice. (b) Liver-catalase activities of castrated male mice after a single injection of 500 μ g. of riboflavin antagonist.

Table 2. Catalase activities in young adult male and female mice during a course of daily injections of 200 μ g. of riboflavin antagonist

Catalase levels are given in arbitrary units as arithmetic means $\pm$ s.e.m. Eight animals/group.

	Days of injection						
	0	2	4	7	11	17	22
Males	139 $\pm$ 5.3	103 $\pm$ 5.5	122 $\pm$ 7.2	135 $\pm$ 9.2	116 $\pm$ 6.0	92 $\pm$ 7.8	104 $\pm$ 5.2
Females	95 $\pm$ 3.9	94 $\pm$ 3.8	92 $\pm$ 7.8	—	80 $\pm$ 3.2	78 $\pm$ 6.4	87 $\pm$ 7.5

known for some years that rat-liver catalase activity is decreased in starvation (Miller, 1947), but the effects of a chronic food deficiency on catalase activity do not appear to have been studied. It has also been shown in the rat that chronic underfeeding results in a diminution of testosterone secretion (Mulinos & Pomerantz, 1941). The results already described have shown that weanling male mice whose food intake is reduced, by pair-feeding with riboflavin-deficient groups, to 40–50 % of normal do not exhibit the rapid rise in catalase activity to adult level which normally occurs after about two weeks. In fact some decrease in catalase activity below the weanling level may be observed. Male mice kept on a riboflavin-free diet show the same effects as far as their liver-catalase level is concerned. After the injection of testosterone, however, the catalase level in pair-fed mice receiving a riboflavin supplement (or normal diet) rose to normal, but remained unaltered in riboflavin-deficient mice. The results have also shown that feeding riboflavin to deficient mice receiving testosterone is followed by a restoration of catalase activity to normal.

The catalase activity of female mice on the various diets remained unchanged. Again, however, a rise in catalase activity after testosterone injection was seen only in the groups receiving riboflavin.

These results suggest that testosterone cannot exert its normal effect on catalase in the absence of riboflavin. The experiments with single doses of riboflavin antagonist were consistent with this view, by showing that the catalase level of normal males, but not of castrated males, fell after injection. Continuous daily injections of riboflavin antagonist produced little or no change in female catalase level. The result in males was unexpected, in that although their catalase activity was depressed 48 hr. from the beginning of the experiment, the level rose to normal by the seventh day; a further fall in enzyme level was then observed. This might be explained by supposing that, to meet the stress of the antagonist, reserves of riboflavin are brought into play, but that its continued administration eventually overcomes the liver's attempt to restore normal conditions. In any event, this phenomenon of a return to normal of catalase activity during the continuous injection of a catalase depressing substance is not confined to the riboflavin antagonist. Adams & Roe (1953) found that 4-dimethylaminoazobenzene, and hydroxylamine, gave the same effect during continuous daily injections; these experiments did not however extend beyond 4 days. The catalase activity of female mice was reduced,

over a 4-day injection period, by these two substances, but, as the present experiments show, the riboflavin antagonist did not have this effect.

SUMMARY

1. The catalase activity of weanling male mice placed on a riboflavin-deficient diet, and of pair-fed animals receiving a riboflavin supplement, does not show the rise to adult level which normally occurs after 2–3 weeks.

2. The injection of testosterone into mice kept for 3 or more weeks on the diets results in a rise in catalase activity only in groups receiving riboflavin. No change in catalase level occurs in the riboflavin-deficient groups.

3. The catalase level of female mice is not altered either by riboflavin deficiency or the restriction in total dietary intake involved in pair-feeding. Again, however, the catalase activity of riboflavin-deficient groups is not raised by testosterone injection.

4. Blood haemoglobin level remains normal in riboflavin deficiency.

5. A single injection of a riboflavin antagonist reduces the catalase activity of normal males to the castration level within 48 hr. After a further 48 hr. the catalase level returns to normal. There is no change in the catalase level of castrated males after the injection of antagonist.

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