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The Effect on Mouse-liver Catalase Activity and Blood-haemoglobin Level of a Milk Diet Deficient in Iron, Copper and Manganese

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Recent work has shown that tumours contain some agent capable of rapidly and markedly depressing liver catalase activity in mice (Nakahara & Fukuoka, 1949, 1950; Fukuoka & Nakahara, 1951; Adams, 1950*a*, 1951*a*; Greenfield & Meister, 1951). Further, the importance of adrenal and testicular hormones in the maintenance of mouse-liver catalase activity was demonstrated by Adams (1950*b*, 1952) and may be briefly summarized as follows. There is a sex difference in enzyme activity, males having the higher level, and castration results in a fall in activity to slightly below the normal female level. Testosterone injected into castrated males or normal females produces a rise in catalase activity to approximately that of normal males. A marked fall in enzyme activity follows adrenalectomy in both sexes, and the level is restored to normal by the injection of cortisone. Evidence was then obtained that the tumour agent acts by blocking the normal hormonal stimulation of the liver (Adams, 1951*b*).

In order to gain more information about the normal mechanisms involved in the maintenance of mouse-liver catalase activity it was decided to examine the role of trace metals. Since catalase is an iron-containing enzyme, it is not unreasonable to suppose that trace metals play an important part

in its synthesis, and in fact Schultz & Kuiken (1941) found that rat-liver catalase activity was depressed when the animals were maintained on a diet deficient in iron or copper. Addition of the missing metal restored the activity to normal. Fukuoka & Nakahara (1951) stated that feeding or injecting ferric chloride stimulated liver catalase activity in the tumour-bearing animal. Little or no other work on the influence of trace metals appears to have been done, apart from a report by Day & Skidmore (1947) that catalase activity is depressed in zinc deficiency.

The present work began with an attempt to repeat in mice the effects of iron and copper deficiency noted in rats by Schultz & Kuiken (1941). However, the results with a trace-metal-deficient diet differed so widely from those obtained by these authors that it was found necessary to carry out a more detailed investigation. Accordingly, the effects of deficiencies of iron, copper and manganese, on liver catalase activity have been studied. The degree of iron and copper deficiency was checked by corresponding determinations of haemoglobin level. An attempt was also made to find a connexion between the observed effects and the action of testosterone on catalase level.

EXPERIMENTAL

Diet. Commercially supplied 'homogenized' whole milk supplemented with 3% (w/v) glucose (A.R. quality) was used as the basic diet. A number of milk-glucose samples were analysed for Fe and Cu. The Fe content varied from 0.36 to 0.45 p.p.m., and the Cu content from 0.14 to 0.3 p.p.m. A single Mn estimation on a pooled sample gave a value of 0.03 p.p.m. (Analyses by Dr R. F. Milton.)

Leakage from the drinking bottles was rather troublesome but the mice each consumed 6–8 ml./day. Drinking water was not provided. MnSO_4 , A.R., FeSO_4 (limit of Cu impurity 0.00075%) and CuSO_4 (limit of Fe impurity 0.0028%) were added to the basic diet as required, to provide 5 μg . Mn, 50 μg . Fe and 10 μg . Cu/mouse/day, based on the above consumption figures. As far as could be estimated, during the first 4 or 5 weeks on milk the intake per mouse was largely independent of trace-metal addition. In the later stages, mice on a diet of milk supplemented with Fe, Cu and Mn increased their intake appreciably. Since, however, the important changes to be described occurred during the first 5 weeks, no attempt at paired feeding was made.

Although milk is not a particularly suitable diet (largely because of its liquid nature and low total-solid content), it has been extensively used in trace-metal deficiency studies. Milk supplemented with Fe, Cu and Mn was stated to support good growth to maturity in rats (Kemmerer, Elvehjem, Hart & Fargo, 1932). Ideally, milk should be collected directly from the cow into glass vessels, but owing to the practical difficulties of obtaining regular supplies in this way, the present investigation has been made using 'quick pasteurized' milk provided commercially. One advantage of this procedure is that homogenized milk in which no separation of the cream occurs is readily available. Homogenized milk + 3% (w/v) glucose, supplemented with Fe, Cu and Mn, has in fact given steady growth over periods of up to 7 weeks (the duration of the longest experiment). There have been no obvious signs of thiamine deficiency. It is true that a cube diet supplied *ad lib.* produces a more rapid growth rate (Table 1) but this may well be due to over-eating, since on this diet the animals tend to deposit large masses of body fat after a few weeks. The normal diet used in this work consisted of cubes prepared according to the Rowett Institute formula (Thomson, 1936).

The following abbreviations will be used in referring to the various milk diets: milk + 3% (w/v) glucose without trace metal supplements—milk; milk + 3% (w/v) glucose supplemented with Fe—milk-Fe; with Cu—milk-Cu; with Mn—milk-Mn; with Fe + Cu—milk-FeCu; with Fe + Mn—milk-FeMn; with Cu + Mn—milk-CuMn; with Fe + Cu + Mn—milk-FeCuMn. The amounts of the supplements have been given below.

The mice were kept in heavily galvanized cages, and on heavily galvanized grids which prevented access to the sawdust. These conditions are by no means ideal, but in practice a rapid anaemia could be produced, even with a diet deficient only in Fe.

Animals. Weanling mice (wt. 10–12 g.) of a stock albino strain were used.

Estimation of haemoglobin and liver catalase activity. Blood taken from the neck immediately after decapitation was pipetted into 0.1 N-NaOH, and at least 30 min. allowed for full colour development. The haemoglobin level was then read from a calibrated colorimeter.

Samples of whole liver disintegrated in a homogenizer were allowed to react with 0.025 M- H_2O_2 (A.R.) in 0.2 M phosphate buffer (pH 6.8) at 0°, the reaction being stopped after 4 min. by the addition of H_2SO_4 . The H_2O_2 remaining was estimated by titration with standard $\text{Na}_2\text{S}_2\text{O}_3$ after the addition of excess KI. Corresponding determinations of total N were made (Kjeldahl), catalase levels are expressed in arbitrary units/mg. N. For details, and reason for the use of N as a standard, see Adams (1950a, 1952).

Operative procedure. Castration was carried out under ether- CHCl_3 anaesthesia as previously described (Adams, 1952).

Testosterone. (Commercially supplied for implantation.) It was injected intraperitoneally in 50% (v/v) ethanol-glass-distilled water solution. The ethanol used was redistilled in glass apparatus cleaned with glass-distilled water.

RESULTS

Fig. 1. shows the development of liver catalase activity in weanling mice on a normal cube diet. At the weaning stage the catalase activity was only

Fig. 1. (a) Development of liver catalase activity in weanling albino mice maintained on a normal cube diet (males, $\times$ — $\times$; females, $\bigcirc$ — $\bigcirc$) and on milk supplemented with iron, copper and manganese (males, $\blacktriangle$ — $\blacktriangle$; females, $\bullet$ — $\bullet$). (b) Some corresponding haemoglobin levels. In this and subsequent figures results are given as arithmetic means $\pm$ standard error of means. The number of animals in each group is given at the head of the catalase-standard-error limits.

slightly higher in males than in females, and in both was below adult level. The sex difference in catalase activity became fully developed about 3 weeks after weaning, due to a rapid increase in the male level. The mice were then 5–6 weeks old. Little subsequent change in activity occurred, although there

was a further slight rise in the male level during the next 3 weeks. Fig. 1 also shows the effect of maintaining mice on the milk-FeCuMn diet. The catalase activity in both sexes developed in the same way as on the cube diet, the only difference in the two sets of curves being a tendency for the supplemented-milk animals to maintain a slightly higher level. In both the supplemented-milk and the cube diets the haemoglobin level remained practically unchanged. The results contrasted sharply with those obtained in mice kept on a diet of milk alone (Fig. 2). There was little change in either the male or female catalase level during the first 2 weeks, followed by a sharp rise in activity in both sexes,

Fig. 2. (a) Development of mouse-liver catalase activity on a diet of milk without trace metal supplements. After 5 weeks the animals were transferred to a normal cube diet (males, $\times$ — $\times$; females, $\circ$ — $\circ$). (b) Corresponding haemoglobin levels. Figures refer to number of animals in each group.

the sex difference disappearing entirely. After 5 weeks the level in both sexes had risen to approximately the normal male level, while the blood haemoglobin had fallen to just below 4%. At this stage the animals had cream-coloured livers, and large bald areas of skin. Body weight had increased by only about 30% above the initial value (Table 1) compared with about 150% in mice on normal diet. If the animals were kept on the milk diet beyond this stage, death ensued rapidly. However, they recovered if transferred to a normal cube diet after 5 weeks on milk, and Fig. 2 also shows the effect of this on catalase and haemoglobin levels. Although male catalase activity remained more or less unchanged, the female level fell sharply towards normal. The haemoglobin level rose, but more slowly, reaching 9 g./100 ml. after 3 weeks. Restora-

Table 1. *Mouse body weights at varying times when maintained on normal cube diet, milk, or milk supplemented with iron, copper and manganese*
(Results are given in g. as arithmetic means $\pm$ standard error of means. Mice weighed to the nearest g. The number of animals in the groups are given by the figures in brackets.)

Diet	Sex	Controls	Time on diet (weeks)							
			1	2	3	4	5	6	7	8
Milk	M.	←	10 $\pm$ 1.0 (6)	15 $\pm$ 0.7 (6)	15 $\pm$ 1.1 (6)	16 $\pm$ 0.5 (20)	16 $\pm$ 0.6 (12)	23 $\pm$ 0.4 (6)	26 $\pm$ 1.3 (6)	30 $\pm$ 0.4 (6)
	F.		9 $\pm$ 0.9 (6)	16 $\pm$ 1.1 (6)	18 $\pm$ 1.1 (6)	18 $\pm$ 0.4 (20)	15 $\pm$ 1.0 (12)	22 $\pm$ 1.3 (6)	24 $\pm$ 0.9 (6)	29 $\pm$ 0.7 (6)
Cube	M.	12 $\pm$ 0.3 (24)	14 $\pm$ 0.4 (6)	19 $\pm$ 1.1 (6)	24 $\pm$ 1.7 (6)	26 $\pm$ 1.0 (6)	30 $\pm$ 0.9 (6)	33 $\pm$ 1.6 (6)	—	—
	F.	11 $\pm$ 0.3 (24)	14 $\pm$ 0.5 (6)	20 $\pm$ 0.7 (6)	21 $\pm$ 1.3 (6)	23 $\pm$ 0.8 (6)	26 $\pm$ 1.2 (6)	31 $\pm$ 1.4 (6)	—	—
Milk supplemented with iron, copper and manganese	M.	→	—	—	17 $\pm$ 0.1 (6)	20 $\pm$ 1.1 (6)	25 $\pm$ 1.2 (6)	28 $\pm$ 1.6 (6)	29 $\pm$ 0.8 (6)	—
	F.		—	—	16 $\pm$ 0.6 (6)	19 $\pm$ 0.4 (6)	20 $\pm$ 0.6 (6)	25 $\pm$ 0.8 (6)	26 $\pm$ 1.0 (6)	—

Table 2. *Liver catalase activity and haemoglobin levels in mice maintained on a diet of milk supplemented with iron, and the effect of addition of copper*

(Activities in arbitrary units/mg. N are given as arithmetic means $\pm$ standard error of means. The number of animals in the groups are given by the figures in brackets. Corresponding blood-haemoglobin levels in g./100 ml. as arithmetic means $\pm$ standard error of means.)

		Time on diet (weeks)				
		Controls	2	3	4	5
Catalase	Male	106 $\pm$ 2.6 (24)	102 $\pm$ 4.4 (6)	146 $\pm$ 7.5 (6)	152 $\pm$ 11.0 (6)	143 $\pm$ 8.0 (7)
Haemoglobin	Male	14.3 $\pm$ 0.30	8.6 $\pm$ 0.80	5.6 $\pm$ 0.68	3.5 $\pm$ 0.22	7.4 $\pm$ 0.69
Catalase	Female	88 $\pm$ 3.6 (24)	109 $\pm$ 3.4 (12)	138 $\pm$ 5.6 (12)	149 $\pm$ 5.3 (12)	102 $\pm$ 5.4 (8)
Haemoglobin	Female	13.8 $\pm$ 0.40	8.3 $\pm$ 0.72	7.7 $\pm$ 0.51	5.4 $\pm$ 0.5	7.9 $\pm$ 0.23

tion of hair began, and the liver darkened to its usual colour. Body weight also increased rapidly, more than doubling itself during the 3-week period (Table 1). Table 1 also shows the development of body weight on the milk-FeCuMn diet. On this diet the hair growth and liver colour were normal.

mice kept on milk alone. Copper was then added to the milk-Fe diet of the small group of mice remaining in this experiment. A week later the female enzyme level had fallen to normal, there being little or no change in the males. The haemoglobin in both sexes had risen to nearly 8 g./100 ml.

Fig. 3. (a) Effect of trace metal supplements on the development of catalase activity in weanling mice kept on a milk diet. Copper (males, $\times$ — $\times$; females, $\bigcirc$ — $\bigcirc$); manganese (males, $\blacktriangle$ — $\blacktriangle$; females, $\bullet$ — $\bullet$); Iron + manganese (females only, $\blacksquare$ — $\blacksquare$). (b) Corresponding haemoglobin levels. Figures refer to number of animals in each group.

Addition of iron to the milk (milk-Fe) had little effect on catalase or haemoglobin levels as may be seen from Table 2. The sex difference in catalase activity again disappeared, the level in both sexes approximating to that of normal males, and a haemoglobin level of 5 g./100 ml. was reached after 4 weeks; very pale livers, loss of hair, and failure to gain weight were also observed in these mice, although the effects seemed less marked than in the

Fig. 4. (a) Effect of trace metal supplements on the development of liver catalase activity in weanling mice kept on a milk diet. Iron + copper (males, $\times$ — $\times$; females, $\bigcirc$ — $\bigcirc$); copper + manganese (males, $\blacktriangle$ — $\blacktriangle$; females, $\bullet$ — $\bullet$). (b) Some corresponding haemoglobin levels. Figures refer to number of animals in each group.

The sex difference in catalase activity in animals given the milk-Cu diet developed in a normal way for the first 5 weeks (Fig. 3) (Hb down to 8.6 g./100 ml.). Subsequently, however, the female level rose, reaching the male level after 7 weeks on the diet. The milk-Mn diet was less effective in producing a normal picture (Fig. 3), but the sex difference in catalase activity was apparent for 4 weeks, although the female level was rather higher

than normal. Subsequently, the female level rose still higher, and the male level was almost reached by the fifth week. The experiment was then terminated, the haemoglobin having fallen to 3 g./100 ml. Fig. 3 also shows that females on the milk-FeMn diet behave similarly to those on a manganese supplement only.

The effects of feeding the milk-FeCu and milk-CuMn diets are shown in Fig. 4. The curve obtained with milk-FeCu resembled that with milk-Cu (Fig. 3), the female level being normal for 4 or 5 weeks. The female level then rose, and there appeared to be an upward trend in the male level also. In contrast to the results with milk-Cu, the haemoglobin level in mice on the milk-FeCu diet remained normal for the duration of the experiment. The catalase activity developed normally on the milk-CuMn diet, the results resembling those with milk-FeCuMn. Haemoglobin, however, fell to approximately 6 g./100 ml. after 7 weeks on the diet.

Fig. 5. Variation in liver catalase activity (upper graphs), and haemoglobin level (lower graphs) resulting from the addition of copper and manganese to the diet of mice kept on milk supplemented with iron for 4 weeks. (a) Copper and manganese; (b) copper only; (c) manganese only. Control mice on the left of each graph (the same group being used in each case). (Males, ●—●; females, ○—○.) Figures refer to number of animals in each group.

Addition of copper and manganese to the diet of deficient mice

A number of male and female mice were kept on the milk-Fe diet for 4 weeks. The catalase activity and haemoglobin level of a small group of each sex were then determined, and the remainder divided into three groups. These groups were given copper only, manganese only, and copper plus manganese, respectively, as supplements to the milk-Fe diet. Catalase and haemoglobin were determined after 7 days, after 14 days, and in the case of the copper and manganese supplement, after 21 days. The results appear in Fig. 5.

The copper and manganese supplement caused a sharp fall in the female catalase activity. The normal level was reached after 1 week, and there was no subsequent change. There was also a fall in the male catalase activity after 1 week, but by the second week the level had risen to its normal value. Haemoglobin was restored to normal after 2 weeks, in both sexes. A comparison with Fig. 2 shows that the result of the addition of copper plus manganese to the milk-Fe diet was closely similar to that obtained when mice which had been kept on milk for 5 weeks were transferred to a cube diet.

The addition of copper only, and manganese only, to the milk-Fe diet was not so successful in restoring a normal catalase activity picture. The copper supplement caused a marked fall in the female catalase activity, and a slight fall in the males, after 1 week. The female level was still rather high, however. After 2 weeks the male level had risen again, and the female level also rose slightly. Haemoglobin returned to normal in 2 weeks. Only a slight fall in the female catalase activity resulted from the manganese supplement. There was no change in the males, and the haemoglobin level in both sexes continued to fall.

Experiments with castrated males

The most striking feature of the results so far described is the disappearance of the sex difference in liver catalase activity in copper and manganese deficiency and its reappearance after feeding these metals. In no case has there been any marked variation in the development of male catalase activity—the disappearance of the sex difference being due to a rise in the female level. It has been shown previously (Adams, 1952) that the sex difference is due to lack of testosterone in the females. However, from the general appearance of male mice after 4–5 weeks on the milk or milk-Fe diets, and the small size of the testes, it seemed reasonable to assume that testosterone was not being produced in normal quantities. The diet experiments were therefore repeated in castrated males. The mice were castrated at weaning age, and the control catalase and haemoglobin levels measured 48 hr. later. Control mice were kept on a cube diet for this 48 hr. period. Table 3 shows the result of keeping castrated males on the cube diet, milk only, and the milk-Fe diet. The results were closely similar to those already obtained with females and normal males under the same conditions, i.e. a sharp rise in activity up to the normal male level. On the cube diet there was a very slight rise in catalase level over a period of 6 weeks. After 4 weeks on milk only, the remaining animals were placed on the cube diet. Catalase activity fell steadily, reaching the normal value for castrated males within 3 weeks. Haemoglobin rose during this period from just below 4 to 11.5 g. %. The last group

Table 3. *Liver catalase activity in castrated male mice maintained on various diets*

(Results are given in arbitrary units/mg. N as arithmetic means $\pm$ standard error of means. The number of animals in the groups are given by the numbers in brackets. Corresponding blood-haemoglobin levels in g./100 ml. $\pm$ standard error of means. Details of control experiments given in text.)

Diet	Controls	Time on diet (weeks)						
		2	3	4	5	6	7	
Milk	↑	Catalase 110 ± 7.0 (6)	147 ± 11.2 (6)	158 ± 8.1 (6)	Transferred { to normal diet {	124 ± 6.6 (6)	104 ± 7.1 (6)	91 ± 3.2 (6)
Milk + iron	↑	Haemoglobin 7.3 ± 0.38 (6)	5.9 ± 0.22 (6)	3.9 ± 0.26 (6)	4.5 ± 0.24 (6)	6.4 ± 0.28 (6)	11.4 ± 0.53 (6)	—
		Catalase 100 ± 5.6 (6)	152 ± 9.2 (6)	154 ± 6.2 (6)	147 ± 9.9 (6)	Copper { added to diet {	116 ± 7.0 (6)	—
Milk + copper	Catalase; 84 ± 2.7 (8)	Haemoglobin 12.8 ± 0.22 (6)	9.4 ± 0.48 (6)	7.5 ± 0.62 (12)	6.5 ± 0.38 (6)	10.5 ± 0.55 (6)	—	—
		Catalase 101 ± 5.2 (6)	105 ± 3.9 (12)	113 ± 7.0 (12)	—	133 ± 10.4 (6)	—	—
Milk + iron + copper	Haemo- globin; 13.8 ± 0.5 (8)	Haemoglobin 12.7 ± 0.66 (6)	10.5 ± 0.55 (12)	10.0 ± 0.59 (12)	—	6.7 ± 0.93 (6)	—	—
		Catalase —	—	107 ± 4.7 (6)	127 ± 6.7 (6)	—	148 ± 7.9 (6)	—
Milk + copper + manganese	↑	Haemoglobin —	—	13.3 ± 0.46 (6)	13.4 ± 0.69 (6)	—	16.0 ± 0.36 (6)	—
		Catalase 121 ± 6.9 (6)	128 ± 7.3 (6)	—	—	156 ± 8.1 (8)	—	—
Milk + iron + copper + manganese	↑	Haemoglobin —	9.3 ± 0.52 (6)	8.3 ± 0.51 (6)	—	5.8 ± 0.70 (8)	—	—
		Catalase —	112 ± 8.6 (6)	103 ± 6.7 (6)	—	116 ± 4.7 (8)	—	—
Cube diet	↑	Haemoglobin —	11.7 ± 0.12 (6)	13.0 ± 0.50 (6)	—	12.4 ± 0.41 (8)	—	—
		Catalase —	—	95 ± 6.5 (6)	—	102 ± 6.4 (6)	—	—
		Haemoglobin —	—	13.5 ± 0.62 (6)	—	14.5 ± 0.50 (6)	—	—

Table 4. *A summary of catalase and haemoglobin levels in mice after 4-5 weeks on various diets*

		Milk supplemented with							
	Normal cube diet	—	Iron	Copper	Manganese	Iron + copper	Iron + manganese	Copper + manganese	Iron + copper + manganese
Normal males									
Catalase	High	High	High	High	High	High	—	High	High
Haemoglobin	Normal	Very low	Low	Medium	Very low	Normal	—	Medium	Normal
Normal females									
Catalase	Low	High	High	Low to medium	Medium	Low to medium	Medium	Low	Low
Haemoglobin	Normal	Very low	Low	Medium	Very low	Normal	Low	Medium	Normal
Castrated males									
Catalase	Low	High	High	Low to medium	—	Low to medium	—	Medium	Low to medium
Haemoglobin	Normal	Very low	Low	Medium	—	Normal	—	Medium	Normal

Fig. 6. The effect of testosterone injection and withdrawal on the liver catalase activity (upper graphs) of female mice kept on various diets. Corresponding haemoglobin levels are given in the lower graphs. Six animals per group. (a) Milk + iron, $\circ - \circ$; milk + manganese, $\times - \times$. (b) Normal diet, $\blacksquare - \blacksquare$; milk + iron + copper, $\blacktriangle - \blacktriangle$; milk + iron + copper + manganese, $\bullet - \bullet$.

of mice kept on the milk-Fe diet were given copper as well after 5 weeks. One week later the catalase activity had fallen sharply and the haemoglobin had risen to 10.5 g./100 ml.

Table 3 also shows the effect on catalase activity of maintaining castrated mice on the milk-Cu, milk-

FeCu, milk-CuMn and milk-FeCuMn diets. The last diet was the most successful in maintaining the low level normal for castrated males, although there was a slight upward trend. Milk-Cu and milk-FeCu were less satisfactory, particularly in the later stage (5-6 weeks on diet). However, all these diets were

markedly superior in this respect to milk alone, or to milk-Fe, for at least the first 4 weeks. The haemoglobin level in these mice was maintained by the milk-FeCu or milk-FeCuMn diets. It was expected that there would be no difference between milk-CuMn and milk-FeCuMn in the maintenance of a normal catalase activity, as had been found in females. However, on the former but not on the latter diet the catalase activity in castrated male mice rose steeply, the normal male level being reached within 6 weeks. Table 4 summarizes the catalase and haemoglobin levels observed after 4-5 weeks on various diets.

The influence of testosterone

It appears that under all the dietary conditions described the catalase activity of normal male mice reached approximately the same point, which lies within the range of normal values for this strain of mice. There was a tendency for the enzyme level to be in the high normal range, but values which certainly exceed normal limits have not been observed. The rises in level in castrated male or normal female mice under conditions of copper and manganese deficiency have been approximately equal to the rises which would be expected in these animals after treatment with testosterone. Thus, copper-manganese deficiency appears to be equivalent to testosterone in its effect on liver-catalase level. But it does not follow that the effects are connected. To gain evidence on this question, testosterone was injected into females maintained on various diets. It was soon found that female mice in the early stages of a milk diet would not tolerate the daily injection of 100-150 μ g. of testosterone in 0.03 ml. of 50% (v/v) ethanol-water. After 4 weeks on the diets the injections were taken well, but the reason for this is not known. Testosterone was therefore injected daily in 150 μ g. doses into female mice, which had been maintained for 4 weeks on the cube diet, and on milk-Fe, milk-FeCu, milk-Mn and milk-FeCuMn, respectively. After 4 days, catalase activities were determined, and the testosterone withdrawn from the remaining mice. After a further 4 days the catalase activity of the remaining mice was determined. In the case of the mice receiving milk-Mn, half received testosterone after 4 weeks on the diet and the remainder did not, the catalase activity of both groups being determined after the testosterone had been injected for 4 days. This altered procedure was used because it seemed unlikely that the mice would survive the full length of the experiment. There was no response to testosterone in the mice kept on milk-Fe. A response was obtained in mice kept on the other diets, but in no case did the testosterone injections cause the catalase activity to exceed the level reached in

copper-manganese deficiency (Fig. 6). The catalase activity rose to approximately this level, and fell appreciably when the testosterone was withdrawn. The catalase activity of the manganese-supplemented mice injected with testosterone rose slightly above that of the non-injected controls, but only to the same level as in the other groups.

DISCUSSION

This work has shown that a milk diet deficient in iron or copper, or both, produces a considerable degree of anaemia within about 4-5 weeks. This agrees with the earlier results (e.g. Cohn *et al.* 1927) in showing that iron and copper are essential for the synthesis of haemoglobin, while manganese is not.

Milk supplemented with iron, copper and manganese maintained a normal high catalase level in male mice and a normal low level in female and castrated male mice. On unsupplemented milk or milk-Fe the sex difference disappeared, due to a rise in the female level, the male level remaining substantially unchanged, but castrated males behaved in the same way as females. This rise in catalase level in females and castrated males appeared to be due primarily to a copper and manganese deficiency, suggesting that copper and manganese exert a depressant action on catalase synthesis, contrasting with the stimulating action of copper on haemoglobin synthesis. Castrated males fed milk-CuMn did not behave as expected, however; catalase activity was not held at the normal low level. Possibly this result was due to some other factor, such as defective absorption. Nevertheless, catalase levels over the first 4 weeks were considerably lower than in mice on milk or milk-Fe.

The effect of copper and manganese deficiency in mice has been shown to be approximately the same as that of testosterone. Moreover, testosterone treatment failed to produce a rise above the copper-manganese-deficient level. It is, of course, possible that mice maintained on milk-Fe for some weeks are in too poor a condition to react to testosterone at all. Nevertheless, mice on all other diets have shown some response to testosterone, the maximum levels of catalase activity being similar, and approximately equal, to that reached in copper-manganese deficiency. This suggests that testosterone stimulates catalase synthesis in an indirect way; possibly by inhibiting the depressing action of a copper-manganese system. It would be interesting to see whether cortisone acts through a similar mechanism, although the practical difficulties of using the present method would be very great. Survival of copper-deficient adrenalectomized animals would be unlikely. Cortisone and testosterone certainly exert independent effects on catalase

activity (Adams, 1952). A hypothesis that testosterone acts through a copper-manganese system, while cortisone does not, would explain the observed results.

Iron added to milk produced little effect on catalase level either alone or in combination with other metals, although it was required, in addition to copper, for the maintenance of a normal haemoglobin level. As far as catalase is concerned, an iron-deficiency effect seems difficult to produce. Presumably if the iron content of the body could be reduced to a point at which insufficient was available for catalase synthesis the enzyme level must fall. However, this may not be possible. In the seventh week of the experiment on milk supplemented with copper the catalase activity of the females rose from 130 to 155 arbitrary units, whilst the haemoglobin level fell from 8 to 6 g./100 ml., suggesting that liver catalase is synthesized preferentially to haemoglobin.

The general picture differs so markedly from that suggested by the results of Schultz & Kuiken (1941) with rats, that it is hardly possible to offer an explanation of the discrepancy, beyond the obvious suggestion of a species difference. However, these authors did find that heart catalase activity increased on a copper-deficient diet.

SUMMARY

1. In weanling mice a diet of milk and glucose supplemented with iron, copper and manganese is equivalent to a normal cube diet for the normal development of liver catalase activity. There is a marked sex difference in enzyme activity from about 5 weeks of age.

2. The sex difference in catalase activity disappears in mice kept on a diet of milk alone, the enzyme level in both sexes approximating to that of normal males. The haemoglobin level falls sharply during this process. If the mice are transferred to a normal cube diet after 5 weeks on milk, the haemoglobin and female catalase levels return to normal,

the male catalase activity remaining unchanged and at its normal high level.

3. Castrated males kept on milk supplemented with iron, copper and manganese, maintain their normal low level of catalase activity. On a milk diet unsupplemented with trace metals, the level rises just as in females to approximately that of non-castrated males.

4. Addition of iron to milk produces no change in the catalase levels reached with milk alone, although haemoglobin usually falls less rapidly.

5. Addition of copper or manganese to milk is partially effective in maintaining a normal development of catalase activity. Copper and manganese together are as effective as iron, copper and manganese in normal females.

6. In animals maintained for 4 weeks on a diet of milk supplemented with iron, the addition of copper, or copper and manganese, produces a sharp fall in the female catalase level, leaving the male level substantially unchanged. Manganese added to milk and iron is less effective, and copper and manganese together are more effective than copper alone in restoring the normal level in females. The addition of copper to the diet of castrated males kept on milk and iron causes a sharp reduction in catalase activity, to near the normal level.

7. Testosterone injected into females maintained on various diets gives a rise in catalase activity only to the level obtained in copper manganese deficiency. No further rise is obtained in females in which the level was already raised by copper and manganese deficiency.

8. Copper- and manganese-deficient mice showed other evidence of abnormality, notably loss of hair, and very light-coloured livers.

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