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Amine Oxidase Activity of Rat Liver in Riboflavin Deficiency

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(Received 12 October 1951)

The chemical constitution of the enzyme amine oxidase is not known. There are some indications that the closely related enzyme histaminase, which also catalyses the oxidation of amines, is a flavo-protein (Zeller, Stern & Wenk, 1940; Kapeller-Adler, 1949). It is known that D-amino-acid oxidase contains riboflavin in the form of flavin-adenine dinucleotide (FAD) (Warburg & Christian, 1938). In riboflavin deficiency there occurs a marked reduction in the D-amino-acid oxidase activity of rat liver (Axelrod, Soker & Elvehjem, 1939, 1940; Rossiter, 1940), and the FAD content of the liver is reduced (Ochoa & Rossiter, 1939).

In the present work the effect of riboflavin deficiency on the amine oxidase activity of rat liver has been studied. The D-amino-acid oxidase activity has been determined at the same time in order that this might serve as an index of the degree of the riboflavin deficiency obtained. In this study characteristic differences in the two enzymes have been observed.

METHODS

Diet. The method adopted for inducing riboflavin deficiency in rats was based on that described by Carpenter Harris & Kodicek (1948) who used a diet low in protein and containing succinylsulphathiazole. I am grateful to Dr E. Kodicek for his advice on the use of this diet.

The diet was composed as follows: riboflavin-free casein 10.5%, sucrose 80.35%, salt mixture (Steenbock no. 40) 5%, L-cystine 0.15%, arachis oil 3%, and succinylsulphathiazole 1%. This diet was fed to the rats *ad lib.* As supplements, they received daily: 30 µg. aneurin, 30 µg. pyridoxine, 20 mg. choline chloride, 1 µg. biotin, 250 µg. nicotinic acid and 200 µg. calcium pantothenate; three times weekly they were given 40 µg. folic acid dissolved in 1% KH_2PO_4 , and once a week one drop of a 5% solution of toco-

pherol acetate, four drops of cod-liver oil and 1 mg. menaphthone. The animals which received this diet without added riboflavin are called deficient rats. The animals which received a daily supplement of 30 µg. riboflavin are called controls. Modifications of the diet, as used in some of the experiments, will be described later.

Animals. Litters of freshly weaned male and female piebald rats, obtained from Glaxo Laboratories, were used. They weighed about 30 g. on arrival.

About two-thirds of the rats in each litter received the deficient diet. In most litters the remaining animals were kept as controls and received daily supplements of riboflavin.

Tissue preparations. Each liver was homogenized in a small volume of 0.067 M-sodium phosphate buffer, pH 7.4. More buffer was added so that 5.0 ml. homogenate contained 1 g. tissue. About one-half of the homogenate was retained for amine oxidase determination, the remainder was centrifuged at 600g for 5 min. and the supernatant used for D-amino-acid oxidase determination.

Kidney extracts were prepared by first grinding the tissue with sand in a mortar, and then adding 0.067 M-sodium phosphate buffer, pH 7.4, so that 5.0 ml. suspension contained 1 g. tissue. This suspension was centrifuged for 5 min. at 600g and the supernatant was used for D-amino-acid oxidase determination.

Measurement of enzymic activity. All determinations were made at 37° and in O_2 . Conical manometer flasks were used; the inner compartments contained filter paper and 0.3 ml. N-KOH.

Amine oxidase activity. This was determined with tyramine as substrate. The side bulbs contained either 0.4 ml. of 0.05 M-tyramine hydrochloride, or 0.4 ml. of water in the blank. The main compartment of each flask contained 1.0 ml. of rat-liver homogenate plus 0.2 ml. of 0.1 M-semicarbazide plus 0.4 ml. of water, to trap the aldehyde formed during the initial oxidation of tyramine in order to prevent further oxidation.

For the study of D-amino-acid oxidase, DL-proline was chosen, as D-proline was found to be oxidized at a high rate by the rat enzyme. The side bulb contained 0.5 ml. 1.0 M-DL-proline; the main compartment contained 0.5 ml. liver extract plus 0.5 ml. water. In the experiments with kidney the proline concentration was 0.1 M.

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Readings were taken at 5 min. intervals, and the rate of oxidation was calculated from the O_2 uptake during the first 15 min. of the reaction. Enzymic activity is expressed in terms of q_{O_2} (tyramine) and q_{O_2} (proline) respectively, i.e. in μ l. of O_2 /100 mg. fresh wt. tissue/hr.

EXPERIMENTS

Riboflavin deficiency and enzymic activity of liver preparations

Rats reared on the control diet showed a slow but steady growth and appeared healthy; they almost doubled their initial body weight after a fortnight. The deficient animals showed very little or no growth. Apart from diarrhoea, which became noticeable in almost all deficient animals, there were no symptoms of deficiency. The animals on the deficient diet usually survived for a fortnight, but during the third week some deaths occurred. One set of animals was maintained for a fortnight (13–15 days), and a second for 3 weeks (20–22 days). All rats in one litter were killed at one time and the enzymic activities were determined.

Administration of riboflavin and enzymic activity

It is known that on administration of riboflavin to flavin-deficient rats the D-amino-acid oxidase activity of the liver is rapidly restored (Axelrod *et al.* 1940). I have compared the effect on amine oxidase of adding riboflavin to the riboflavin-deficient diet with the effect on D-amino-acid oxidase.

In these experiments about one-third of the animals in each litter were kept as controls, one-third were maintained on the deficient diet throughout, and the remaining third were kept on the deficient diet for 11 or 12 days and then given 1 mg. riboflavin by mouth. In one series the animals received one such dose of riboflavin and were killed 24 hr. later. In a second series the rats were given 1 mg. doses of riboflavin on three successive days, and killed 24 hr. later.

The effect of riboflavin on the growth curve was immediate; the mean gain in wt. after 1 mg. of riboflavin was 4 g., and after three doses of 1 mg. riboflavin it was 7 g. The enzymic activities of the

Table 1. *Liver amine oxidase and D-amino-acid oxidase from control and riboflavin-deficient rats*

(Experimental details are given in the Methods section. In this and subsequent tables values are shown as means $\pm$ s.e. with the number of observations in brackets.)

Time on diet (weeks)	Diet	Amine oxidase (μ l. O_2 /100 mg. wet wt./hr.)	D-Amino-acid oxidase (μ l. O_2 /100 mg. wet wt./hr.)
2	Control	115 $\pm$ 8 (23)	124.5 $\pm$ 11.5 (18)
2	Deficient	61.5 $\pm$ 3.5 (24)	24.5 $\pm$ 1.5 (24)
3	Control	122 $\pm$ 8 (11)	142 $\pm$ 6.5 (11)
3	Deficient	54.5 $\pm$ 5.5 (14)	18 $\pm$ 3 (14)

Table 2. *The effect of riboflavin administration on the enzymic activity of liver extracts from deficient animals*

(Experimental details are given in Methods section.)

Diet	Supplement to diet	Amine oxidase (μ l. O_2 /100 mg. wet wt./hr.)	D-Amino-acid oxidase (μ l. O_2 /100 mg. wet wt./hr.)
Control	None	164 $\pm$ 7.1 (9)	136 $\pm$ 12.8 (9)
Deficient	None	62.5 $\pm$ 3.9 (10)	25 $\pm$ 4.4 (10)
Deficient	One dose 1 mg. riboflavin	48 $\pm$ 7.5 (4)	70 $\pm$ 21 (4)
Deficient	Three daily doses 1 mg. riboflavin	64.5 $\pm$ 2.5 (5)	170 $\pm$ 15 (6)

The enzymic activities of the liver from control and deficient animals are given in Table 1. After 2 weeks on the experimental diet the D-amino-acid oxidase activity in the liver extracts from deficient animals had fallen to one-fifth of that of the controls. The amine oxidase activity was reduced to 53 %. When the animals were maintained on the diet for a third week the D-amino-acid oxidase activity fell to 13 % and the amine oxidase activity to 45 % of the controls.

Changes in the total amount of enzyme present in the liver will be described below.

liver after riboflavin administration are shown in Table 2. It can be seen that the D-amino-acid oxidase activity was increased by one dose of riboflavin, whereas the amine oxidase activity remained essentially unchanged even after three doses of riboflavin. After three doses of riboflavin the D-amino-acid oxidase activity rose to a level higher than that of the controls.

The determination of the enzymic activity per unit weight of liver does not give a full picture of the effect of riboflavin administration on the restoration of the enzymes studied, because administration of

riboflavin causes a rapid increase in liver weight. The body and the liver weights of rats on the different diets are summarized in Table 3. The mean liver weight rose from 1.66 to 2.60 g. after one dose of riboflavin, and to 3.10 g. after three doses. The total enzymic activity can be measured in $\mu\text{l. O}_2$ consumed/hr./liver; this is the q_{O_2} multiplied by the liver weight (in units of 100 mg.). The mean data thus obtained are given in Table 4 which shows that after administration of riboflavin the total amine oxidase of the liver increased. However, this increase appears slight if it is compared with the big rise in total D-amino-acid oxidase activity. Thus, after three daily doses of 1 mg. of riboflavin, amine oxidase had about doubled, whereas D-amino-acid oxidase had increased about 13-fold in these animals.

Other observers have reported that in rats on a fat-free diet low in protein and containing choline, the administration of large amounts of B vitamins resulted in the deposition of fat; this was partly prevented by inositol (McFarland & McHenry, 1948; Best, Lucas, Patterson & Ridout, 1951). Since the basic diet used in my experiments was low in protein and fat and did not include inositol, it was thought desirable to study the effect of inositol on the restoration of enzymic activity in the liver after the administration of riboflavin.

In the first series of experiments inositol was not included in the basic diet. Two litters were used; in one litter three of the deficient animals received three daily doses of 25 mg. inositol on the last 3 days of the experiment. In the second litter three animals received three daily doses of 25 mg.

Table 3. *Body weights and liver weights of rats maintained on deficient and control diets*

Diet	Time on diet (weeks)	Supplement to diet	Mean body wt. (g.)	Liver wt. (g.)	No. of obs.
Control	3	None	66	3.75 $\pm$ 0.08	(12)
Deficient	3	None	42	1.83 $\pm$ 0.11	(13)
Control	2	None	55	3.08 $\pm$ 0.11	(21)
Deficient	2	None	34	1.66 $\pm$ 0.14	(20)
Deficient	2	One dose 1 mg. riboflavin	38	2.60 $\pm$ 0.05	(4)
Deficient	2	Three daily doses 1 mg. riboflavin	41	3.10 $\pm$ 0.15	(6)
Control + inositol	2	None	47	2.85 $\pm$ 0.19	(6)
Deficient + inositol	2	None	35	1.64 $\pm$ 0.08	(11)
Deficient + inositol	2	Three daily doses 1 mg. riboflavin	45	2.68 $\pm$ 0.13	(10)

Table 4. *Total amine oxidase and D-amino-acid oxidase activities of the liver, expressed in $\mu\text{l. O}_2$ /hr./liver*

Diet	Time on diet (weeks)	Supplement to diet	Amine oxidase ($\mu\text{l. O}_2$ /hr.)	D-Amino-acid oxidase ($\mu\text{l. O}_2$ /hr.)
Control	3	None	4560	5350
Deficient	3	None	996	256
Control	2	None	3540	3840
Deficient	2	None	1020	405
Deficient	2	One dose 1 mg. riboflavin	1250	1820
Deficient	2	Three daily doses 1 mg. riboflavin	2000	5270
Control + inositol	2	None	3420	3690
Deficient + inositol	2	None	1130	312
Deficient + inositol	2	Three daily doses 1 mg. riboflavin	2800	5070

Inositol and the restoration of enzymic activity by riboflavin

In the course of the experiments just described, it was noticed that after administration of riboflavin, the livers of deficient rats showed an increase in fat content. This became particularly evident after centrifugation of the liver extracts, when a well-defined layer of fat formed on the surface of the extracts in the centrifuge tubes. This suggested that the rapid increase in liver weight after riboflavin administration might, at least partly, be explained by an increase of the fat content of the liver.

inositol together with 1 mg. of riboflavin. The animals were killed 24 hr. after the last dose and the enzymic activities were determined. Table 5 gives a summary of the results obtained with these litters, and for comparison, the effect of three daily doses of riboflavin alone is also given. The mean enzymic activities of the deficient animals are those from both litters; the values differ little from the mean values given in Table 1. Inositol by itself had no significant effect on either amine oxidase or D-amino-acid oxidase activity, neither did it affect the restoration of D-amino-acid oxidase after riboflavin. The combined administration of riboflavin and

inositol, however, resulted in an increase in the amine oxidase activity of the liver extracts.

In the second series of experiments inositol was included in the basic diet. Table 6 shows that both amine oxidase and D-amino-acid oxidase activities from normal and deficient rats were very much the same as in the absence of inositol. However, when three daily doses of 1 mg. of riboflavin were given to the deficient animals there occurred a rise in the q_{O_2} (tyramine) from 69 to 104.5. In the absence of inositol, the corresponding figures had been 61.5 in the deficient animals and 64.5 after the administra-

D-Amino-acid oxidase activity of the kidney

In a number of experiments the D-amino-acid oxidase activity of the kidney was also determined. The mean enzymic activities found in these experiments are given in Table 7. The activity was high in the kidney extracts from control animals, and there was no significant lowering in the deficient animals. This is in agreement with the observations of Axelrod *et al.* (1940). It seems doubtful also whether the slight increases in q_{O_2} (proline) after three doses of riboflavin were significant.

Table 5. *The effect of inositol and riboflavin on the enzymic activity of liver from animals maintained on a riboflavin-deficient diet*

(Experimental details are given in the Methods section.)

Supplement to diet	Amine oxidase (μ l. O_2 /100 mg. wet wt./hr.)	D-Amino-acid oxidase (μ l. O_2 /100 mg. wet wt./hr.)
None	63.5 $\pm$ 2.2 (5)	30 $\pm$ 3.4 (5)
Three daily doses 25 mg. inositol	69 $\pm$ 8.5 (3)	37 $\pm$ 10.5 (3)
Three daily doses 1 mg. riboflavin + 25 mg. inositol	88.5 $\pm$ 3.5 (3)	163 $\pm$ 24 (3)
Three daily doses 1 mg. riboflavin	64.5 $\pm$ 2.5 (5)	170 $\pm$ 15 (6)

Table 6. *Enzymic activities of liver extract from animals on a basic diet plus inositol*

(Each rat received 8 mg. inositol/day. The experiment lasted 14 days. The corresponding mean enzyme activity of liver from rats on the basic diet alone is given in square brackets.)

Diet	Supplement to diet	Amine oxidase (μ l. O_2 /100 mg. wet wt./hr.)	D-Amino-acid oxidase (μ l. O_2 /100 mg. wet wt./hr.)
Control	None	120 $\pm$ 9.5 [115] (5)	129.5 $\pm$ 19.5 [124.5] (6)
Deficient	None	69 $\pm$ 6.5 [61.5] (11)	19 $\pm$ 2 [24.5] (10)
Deficient	Three daily doses 1 mg. riboflavin	104.5 $\pm$ 6.5 [64.5] (10)	189 $\pm$ 16 [170] (10)

Table 7. *D-Amino-acid oxidase of rat kidney*

(Experimental details are given in the Methods section.)

Diet	Time on diet (weeks)	Supplement to diet	D-Amino-acid oxidase (μ l. O_2 /100 mg. wet wt./hr.)
Control	3	None	251 $\pm$ 27 (5)
Deficient	3	None	243 $\pm$ 12 (8)
Control	2	None	255.5 $\pm$ 19.5 (11)
Deficient	2	None	237 $\pm$ 18.5 (15)
Deficient	2	One dose 1 mg. riboflavin	230 $\pm$ 46.5 (4)
Deficient	2	Three daily doses 1 mg. riboflavin	304.5 $\pm$ 26 (6)
Deficient	2	One dose 1 mg. riboflavin + 25 mg. inositol	203 $\pm$ 31.5 (3)
Deficient	2	Three daily doses 1 mg. riboflavin + 25 mg. inositol	297 $\pm$ 61 (3)
Deficient	2	Three daily doses 25 mg. inositol	236 $\pm$ 28 (3)

tion of riboflavin. No effect of inositol on the restoration of D-amino-acid oxidase activity was seen.

The effect of riboflavin on the total enzymic activities of the liver of rats maintained on the inositol-containing diet is shown in Table 4. On this diet, the D-amino-acid oxidase activity rose from 312 to 5070 μ l. O_2 /hr., about 16-fold; the amine oxidase activity rose from 1130 to 2800 μ l. O_2 /hr., about 2.5-fold.

Enzymic activity on a high-protein diet

In order to find out if the lowered amine oxidase activity was due to a low protein intake, a few animals were reared on a diet which differed from the basic diet in that the casein content was raised from 10 to 20 %, with a corresponding reduction in the sucrose to 70.35 %. On this diet the control animals grew more rapidly than on the low-protein diet: the weights of three control animals kept for

3 weeks on the high-protein diet were 86, 89 and 89 g. as compared with a mean weight of 66 g. after 3 weeks on the low-protein diet (see Table 3). The weight of the riboflavin-deficient rats on the high-protein diet did not remain stationary; the rats put on weight during the first 2 weeks; some of the animals were maintained for as long as 5 weeks without showing any symptoms.

Three of the deficient animals were killed after 21 days and liver extracts were prepared; the mean q_{O_2} (tyramine) was 78 and the mean q_{O_2} (proline) was 19. Eight deficient and three control animals were killed after 30–38 days. Table 8 shows that the fall in both amine oxidase and D-amino-acid oxidase activities had not been prevented by increasing the protein content of the diet; the mean values for q_{O_2} are similar to those of Table 1.

control animals. The data given by Axelrod *et al.* (1940), although not strictly comparable with those reported in this paper, suggest that on the diets used by them the decrease in enzymic activity was less rapid. Thus it seems that the inclusion of succinylsulphathiazole in the diet reduced the time required for establishing a riboflavin deficiency, as indicated by the fall in D-amino-acid oxidase activity. That this fall was due specifically to lack of riboflavin and not to low protein content of the basic diet, is supported by the observation that a similar fall in enzymic activity occurred when the protein content of the diet was doubled.

D-Amino-acid oxidase of the kidneys. The maintenance of both FAD content and D-amino-acid oxidase activity of the kidneys in riboflavin deficiency has already been described (Rossiter,

Table 8. *Enzymic activity of liver extracts from animals maintained for 30–38 days on a diet containing 20% protein*

(Experimental details are given in the Methods section.)

Diet	Amine oxidase (μ l. O_2 /100 mg. wet wt./hr.)	D-Amino-acid oxidase (μ l. O_2 /hr./liver)	Total amine oxidase (μ l. O_2 /hr./liver)	Total D-amino- acid oxidase (μ l. O_2 /hr./liver)
Control	132 $\pm$ 13.4 (3)	64 $\pm$ 4.1 (3)	6700	3270
Deficient	50 $\pm$ 3.5 (8)	19 $\pm$ 4.4 (8)	1260	466
Deficient, then 3 daily doses of 1 mg. riboflavin	86 $\pm$ 12 (5)	134 $\pm$ 13.6 (5)	3948	5895

Some of the animals were maintained on the high protein diet without riboflavin for 5 weeks; they then received three daily doses of 1 mg. of riboflavin before they were killed. The data for enzymic activity of the liver are also given in Table 8. As in the earlier experiments, D-amino-acid oxidase activity was restored; there was, however, also some increase in the amine oxidase activity: the mean figures for q_{O_2} (tyramine) rose from 50 to 86. Table 8 also contains the data for the total enzymic activity of the liver; these show that the amine oxidase had trebled after three doses of riboflavin. The mean liver weight of the deficient animals was 2.51 g.; it increased to 4.56 g. after riboflavin. The mean liver weight of the control animals was 5.12 g.

DISCUSSION

The experiments reported confirm that on a diet free from riboflavin which incorporates succinylsulphathiazole, rats quickly develop a state of deficiency; the rapid increase of body weight which occurs when riboflavin is added to the diet is evidence of the specific character of this condition.

D-Amino-acid oxidase of liver. After about 2 weeks on the riboflavin-deficient diet the enzymic activity dropped to about one-fifth of that of the

1940; Axelrod *et al.* 1940); in my experiments also no fall in enzymic activity could be found. The riboflavin present as FAD in the D-amino-acid oxidase of the kidneys represents such a small fraction of the total flavin content of the body that a great reduction in flavin can occur while the enzymic activity of the kidneys is maintained.

Amine oxidase. The observations on this enzyme stand in marked contrast to those on D-amino-acid oxidase. The loss of amine oxidase in the flavin-deficient animal was slow, as was the rate of increase in activity after riboflavin administration. This makes it impossible to decide whether or not this enzyme contains flavin. Loss of enzymic activity occurred on the high-protein diet as well as on the low-protein diet. The possibility remains, however, that in riboflavin deficiency protein utilization is affected and that this factor is responsible for the low amine oxidase activity in the deficient animals. It seems particularly interesting that with inositol present in the diet, riboflavin was more effective in restoring amine oxidase activity. This may mean that both flavin and inositol are contained in enzyme protein, but it is also possible that amine oxidase is a flavoprotein, and that inositol is required, either for the synthesis of a prosthetic group or of new enzyme protein.

SUMMARY

1. Rats have been made deficient in riboflavin on a diet free from riboflavin and containing 1% succinylsulphathiazole and the amine oxidase and D-amino-acid oxidase of liver extracts have been determined.

2. It has been confirmed that the D-amino-acid oxidase of the liver is rapidly and severely reduced

in riboflavin deficiency and that it is rapidly and fully restored upon the administration of riboflavin.

3. The amine oxidase activity of the liver is about halved in riboflavin deficiency. There is no rapid restoration of enzymic activity on giving riboflavin. If inositol is present in the diet, some restoration of amine oxidase occurs when riboflavin is administered.

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The Effect of Thyroxine and Thiouracil on the Composition of Milk

2. THE CAROTENE AND VITAMIN A CONTENT

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(Received 13 August 1951)

The interactions between the utilization of carotene as a provitamin A and the activity of the thyroid gland have been much studied both for animals and for man (Kunde, 1926; Lindquist, 1938; Thomson *et al.* 1939; Drill, 1943). Experiments with rats provide a basis for the connexion (long known to clinicians and quoted by Lindquist and by Drill) between carotene and the thyroid in certain types of human illnesses. Thus it has been shown in rats (Johnson & Baumann, 1947) that thyroxine accelerates repletion of hepatic stores of vitamin A from dietary carotene, whilst thiourea has the reverse effect. Thyroxine also accelerates the depletion of hepatic stores of rats deprived of dietary carotene whilst thiourea again has the reverse effect (Kelley & Day, 1948). Furthermore, Cama & Goodwin (1949) have correlated these effects with those produced by the hormone and by a goitrogen on the faecal excretion of dietary carotene in the rat.

For many years effects of thyroxine injections on the composition of milk have been studied at this Institute (Smith & Dastur, 1940; Owen, 1948; Chanda & Owen, 1949) and it was thought that the influence of thyroxine on the liver reserves of vitamin A of animals and humans might have a parallel in its effects on the concentration of vitamin

A and carotene in milk. Fellenberg & Grüter (1932) and Fasold & Heidemann (1933) claimed that thyroidectomy caused carotene to appear in goat's milk, but Kon and his co-workers (1944-6) and Smith, Niedermeier & Schultz (1948) could not confirm these observations. The purpose of the present paper is to report experiments which were begun in 1948 on lactating cows and goats in which effects of thyroxine and of the goitrogen thiouracil on the composition of milk were investigated.

EXPERIMENTAL

Experiment 1

This experiment was divided into seven periods. Six lactating Ayrshire cows were used. In period 1 all were deprived of carotene and in period 2 they all received a carotene-containing diet. In period 3 all the cows were again deprived of carotene. In period 4, the diet containing carotene was fed again to all, but cows 17 and 18 were also given subcutaneously 10 mg. DL-thyroxine/day each, while cows 19 and 20 each received 20 mg. thiouracil/day by the same route. The remaining two cows served as controls. In period 5, the carotene-containing diet was continued, but the hormonal treatments were stopped. The experiment was continued into periods 6 and 7, during which the cows all received the carotene-free diet. During period 6 each cow was given the same 'hormonal' treatment as it had received