EFFECTS OF RIBOFLAVIN DEFICIENCY AND REALIMENTATION ON FLAVIN ENZYMES OF TISSUES*

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This study was undertaken to determine how flavin enzymes are distributed in different tissues and their fate with riboflavin restriction of greater or lesser severity. Such a study might be expected to uncover differences in metabolic patterns of different tissues and to expose enzymatic defects which must underlie the functional deficits and physical changes of riboflavin deficiency. Tissues differ as much as 15-fold in their concentrations of FAD[1] and FMN (1) and probably, therefore, in their total content of flavin enzymes. Specific flavoproteins may vary even more from organ to organ. For the understanding of normal function it may be well to know more about the distribution of these specific enzymes.

In this paper, concentrations of FAD and FMN and activities of xanthine oxidase, D- and L- amino acid oxidases, glycine and glycolic acid oxidases, and DPNH-oxidizing enzymes are reported for various normal and deficient rat tissues. The rates at which some of these enzymes decrease in liver and kidney during riboflavin deficiency and increase during realimentation are also given.

EXPERIMENTAL

Source of Tissue—Sprague-Dawley weanling male rats were used in all the experiments unless otherwise specified. The basal diet consisted of 20 per cent glucose, 20 per cent casein, 47 per cent cornstarch, 3 per cent Osborne-Mendel salt mixture supplemented with 1.09 gm. of $Na_2MoO_4 \cdot 2H_2O$ per kilo of salt, 8 per cent peanut oil, 2 per cent cod liver oil, and 1 per cent of a vitamin mixture[2] in glucose. The depleted rats received this

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[1] The abbreviations include flavin adenine dinucleotide, FAD; flavin mononucleotide, FMN; diphosphopyridine nucleotide, oxidized or reduced, DPN^+ or DPNH, respectively.

[2] The vitamin mixture contained choline chloride 7.5 gm., thiamine hydrochloride 50 mg., nicotinamide 200 mg., pyridoxine hydrochloride 60 mg., calcium pantothenate 60 mg., biotin 1 mg., folic acid 20 mg., inositol 1 gm., vitamin B_{12} 2 mg., with glucose to make 100 gm.

diet *ad libitum;* the weight control animals received the basal diet, enriched with 15 mg. of riboflavin per kilo, in daily quantities just sufficient to maintain weight equal to that in the deficient group. The intermediate and high riboflavin groups were fed *ad libitum* the basal diet enriched with 1 mg. and 15 mg. of riboflavin per kilo, respectively.

For study of the regeneration of flavin enzymes, twenty-six rats were fed the basal diet for 5 to 6 weeks. Litter mate controls were fed the high riboflavin diet. Depleted rats were sacrificed to establish the initial levels of riboflavin enzymes in the tissues. Other depleted rats were each given 600 γ of riboflavin intraperitoneally and sacrificed at $\frac{1}{2}$, 2, 6, or 24 hour intervals after the dose. The animals for the 24 hour period were given an additional 400 γ of riboflavin 12 hours after the first dose. Because of the number of analyses involved, it was necessary to conduct the experiments in realimentation over a period of a week. However, on each test day rats from each group were sacrificed to keep the treatment of all groups as nearly uniform as possible.

Rats maintained 8 to 13 weeks on the riboflavin-free diet, until the eyes and other tissues showed typical signs of severe deficiency, were used with litter mate controls for attempts to demonstrate regeneration of glycolic acid oxidase in liver slices. These slices were kept in rat serum saturated with 95 per cent oxygen and 5 per cent CO_2 at 0°. They were blotted, weighed, and immediately dropped into flasks containing 3 ml. of rat serum, previously saturated with gases as above and containing riboflavin, FMN, or FAD. The flasks were incubated in an atmosphere of 95 per cent oxygen and 5 per cent CO_2 in a metabolic shaker at 37° for 1 hour. After incubation, the slices were rinsed in serum without flavin, blotted on filter paper, homogenized in glass grinders with 0.02 M pyrophosphate buffer, pH 8.3, and analyzed for enzyme activity, FAD, and FMN. Homogenates of liver stored at 0° during the incubation of the slices were analyzed simultaneously.

Preparation of Homogenates—When tissues were to be taken for analysis, the animals were sacrificed by bleeding from the heart as completely as possible under light ether anesthesia. Tissue samples were weighed rapidly and either kept at 0° for a few hours until analyzed (unstable enzymes) or stored for a short time in liquid nitrogen (stable enzymes). Homogenates were prepared in glass grinders at 0° in 0.02 M phosphate buffer, pH 7.4, or in 0.02 M pyrophosphate buffer, pH 8.3. Aliquots were removed at once for FMN and FAD measurements, and DPNH dehydrogenase measurements were made as soon as possible after the preparation of homogenates. Glycolic acid and glycine oxidase assays were made within 3 hours and D- and L-amino acid oxidase assays within 6 hours after sacrifice of the animal, except that the tissue used for D-amino acid oxidase as-

says of Table I was stored in liquid nitrogen for a few days before analysis. Xanthine oxidase was measured on homogenate aliquots stored at −20°.

Coenzyme and Protein Measurement—FAD and FMN determinations were made on 10 per cent trichloroacetic acid extracts prepared at 0° with 1:100 dilutions of tissue and measured according to the fluorometric method of Bessey *et al.* (1). Protein of the homogenates was measured by a published colorimetric method (2).

DPNH Dehydrogenase—The enzymes which oxidize DPNH were measured with three different electron acceptors; cytochromc *c*, by the increase in absorption at 550 mμ, $\Delta\epsilon = 18{,}500$; trichlorophenol-indophenol (3), by the fall in absorption at 660 mμ, $\Delta\epsilon = 25{,}000$; and ferricyanide, by the fall in absorption at 350 mμ, $\Delta\epsilon = 5990$ (5670 + 320 correction for ferricyanide absorption). Fresh unfrozen homogenates were used at final dilutions of 1:16,000 for liver, 1:6000 for kidney and heart, and 1:1600 for brain. The three buffer-substrate reagents were composed of (1) 6.8×10^{-5} M cytochrome *c* (Sigma Chemical Company, St Louis), 2×10^{-4} M DPNH (Sigma), 0.05 M sodium phosphate buffer, pH 7.4, 0.025 M nicotinamide, and 10^{-4} M NaCN; (2) 10^{-4} M 2,6-dichlorobenzenoneindo-3′-chlorophenol (No. 3467, Distillation Products Industries, Rochester) instead of cytochrome *c* without other change, except that the DPNH was added separately to start the reaction; and (3) 2.5×10^{-4} M $K_3Fe(CN)_6$ instead of cytochrome *c*, with the DPNH concentration decreased to 10^{-4} M and added separately to start the reaction. Appropriate blanks without tissue or DPNH were treated in a similar way for all three acceptors. Since this enzyme has a large temperature coefficient, the Beckman spectrophotometer was equipped with a water jacket to maintain the temperature of the cell chamber near 27°. The rate of change in optical density was measured at 30 second intervals for 3 minutes.

Xanthine Oxidase—This enzyme was measured by following the increase in fluorescence which occurs when 2-amino-4-hydroxypteridine is oxidized in phosphate buffer to isoxanthopterin (4). As adapted to the analysis of homogenates, the procedure is as follows. A 5×10^{-4} M stock solution of the substrate (stored frozen) is diluted 1:100 with 0.2 M phosphate buffer, pH 7.2 to 7.3. 1 ml. of this buffer-substrate in a fluorometer tube is brought to 30° in a water bath, and enough homogenate is added to oxidize half the substrate in 30 to 90 minutes. For example, 50 μl. of 1:50 rat liver homogenate or 100 μl. of 1:50 rat kidney homogenate would suffice. An initial reading is made as soon as possible at a fluorometer setting which permits a full scale reading when 100 per cent of the substrate is oxidized. Corning glass No. 5860 is the primary fluorometer filter; No. 5113 and No. 3389 form the secondary filter. Several readings are made

at timed intervals between 0 and 50 per cent oxidation. The tube is kept in a 30° bath and is mixed and wiped clean before each reading. A final reading is taken after oxidation is complete; *i.e.*, after an interval 3 or 4 times that required for 50 per cent oxidation. If available, 5 μl. of an active xanthine oxidase preparation are added to complete the reaction in a few minutes. Care is taken that the working standard (quinine sulfate in 0.1 N H_2SO_4) does not change temperature over the experimental period.

If the change in fluorescence is not linear with time, the initial velocity is calculated graphically. (The rate may fall off, due to product inhibition (4).) 1 ml. of substrate is 5×10^{-6} mmole. Thus, if 1 mg. of liver caused a change in fluorescence of 1 galvanometer division per minute and the change for 100 per cent oxidation was 80 divisions, the tissue enzyme activity would be $1/80 \times 5 \times 10^{-6} \times 60 \times 10^{6} = 3.75$ mmoles per kilo per hour.

The Michaelis constant for xanthine oxidase with aminohydroxypteridine is quite small, so that the enzyme is saturated throughout most of the course of the reaction (4). Therefore, the time required to oxidize a given fraction of the substrate will vary with the substrate concentration. The substrate level chosen is low enough to permit a good percentage rate without requiring an amount of tissue that would produce marked turbidity and consequent quenching. It is also low enough to prevent self-quenching. On the other hand, the substrate level is high enough to provide strong fluorescence and avoid troublesome optical blanks. The procedure used and the method of calculation whereby each sample is its own standard automatically correct for possible quenching or contribution of fluorescence from the enzyme sample.

In comparison with methods based upon oxygen consumption, this procedure does not suffer from difficulties arising from large endogenous blanks (5), and, in comparison with methods based on uric acid formation or disappearance of xanthine (6, 7), it is not complicated by tissue uricase. Technically it is simple and rapid. Because it is a fluorometric method, it is more sensitive than other current procedures, in spite of the fact that the absolute rate of oxidation with the pterin substrate is only about a fifth of the rate with xanthine (4).

Amino Acid and Glycolic Acid Oxidases—The oxidases for D-amino acids, L-amino acids, glycine, and glycolic acid were all measured by similar spectrophotometric procedures. The keto acids formed from D-alanine, L-leucine, glycine, or glycolic acid were condensed with quinolylhydrazine and measured in the Beckman spectrophotometer at 305 mμ (8, 9).

The activities fall during prolonged incubation, particularly in riboflavin-deficient tissues. This is probably due in part to dissociation of the

prosthetic groups from the enzymes. Analytical conditions were therefore established which permit short incubation periods to minimize this effect. It is believed that the result is a much closer approximation to the activity in the original tissue than was possible with earlier methods. The details will be published separately.

Results

Normal Levels of Flavin Enzymes and Coenzymes—The FAD and FMN normal values are slightly lower, but in general agree with earlier data (1). The very low levels of both coenzymes in brain are striking (Table I), and the high level of FMN in kidney and the low level in heart are particularly noteworthy. None of the enzymes measured thus far in kidney account for a function of the high level of renal FMN.

Flavin enzyme levels differ greatly from tissue to tissue (Tables I to III). Liver and kidney have the highest levels of the enzymes measured and brain the lowest, while heart is intermediate. D-Amino acid oxidase is highest in kidney, with liver about one-tenth and both heart and brain about 100th as high. L-Amino acid oxidase is 3 times as high in kidney as in liver. Xanthine oxidase is too low to measure in rat brain, but in heart there is one-tenth as much as in liver. Glycolic acid oxidase is relatively high in liver (Table III), 100 times lower in kidney (not shown), and not measurable in other tissues. In contrast, glycine oxidase is twice as high in kidney as in liver.

The absolute activity of DPNH dehydrogenase as measured with ferricyanide is greater than that of any other flavin enzyme studied (Table II). Liver contains twice as much DPNH dehydrogenase as either kidney or heart, and 10 times as much as brain. The low value in brain is particularly surprising since the oxygen consumption of brain (0.08 mole or 0.32 electron equivalent per kilo, wet weight, per hour (10)) is comparable to that of liver (0.07 mole or 0.28 electron equivalent per kilo, wet weight, per hour (11)). This might be interpreted as indicative of some difference in electron transport in brain and liver. The DPNH dehydrogenase activity as measured in brain with ferricyanide is equivalent to about 3 times the oxygen consumption, whereas in liver it is equivalent to 25 times the oxygen consumption. Therefore, the DPNH dehydrogenase has enough capacity to handle the electron transport in brain, although there is much less margin of safety than in other tissues. It is perhaps not surprising that DPNH dehydrogenase activities measured with different electron acceptors are not strictly proportional from tissue to tissue. With cytochrome *c* as acceptor, liver is 3.3-fold as active as kidney, whereas with ferricyanide as acceptor it is only 1.8 times as active. Differences in the activity of intermediate components between cytochrome *c* and the de-

TABLE I

Levels of Riboflavin Enzymes and Coenzymes in Rat Tissues

Each value is the average obtained on tissues from four rats in each group ± the standard error of the mean. Calculations are based on wet weight. FMN and FAD are expressed as riboflavin in this and in Tables II to V.

Group*	FMN	FAD	DPNH dehydrogenase with		D-Amino acid oxidase	Xanthine oxidase	Protein
			Cytochrome *c*	Dye†			
	γ per gm.	*γ per gm.*	*moles per kg. per hr.*	*moles per kg. per hr.*	*mmoles per kg. per hr.*	*mmoles per kg. per hr.*	*gm. per kg.*
Brain							
High riboflavin	0.68 ± 0.4	2.4 ± 0.13	0.28 ± 0.02	0.19 ± 0.01	3.1 ± 0.2		96 ± 2.7
Low riboflavin	0.56 ± 0.02	2.3 ± 0.04	0.29 ± 0.01	0.18 ± 0.01	2.3 ± 0.3		100 ± 1.7
Deficient	0.54 ± 0.01	2.1 ± 0.05	0.29 ± 0.03	0.17 ± 0.01	2.8 ± 0.1		102 ± 1.5
Weight control	0.71 ± 0.06	2.6 ± 0.08	0.29 ± 0.02	0.18 ± 0.01	2.6 ± 0.2		101 ± 2
Liver							
High riboflavin	5.7 ± 1.0	27.0 ± 1.2	4.0 ± 1.6	1.9 ± 0.7	264 ± 41	5.5 ± 0.5	182 ± 4.7
Low riboflavin	0.68 ± 0.1	11.8 ± 0.5	4.3 ± 1.1	2.2 ± 0.4	82 ± 4	4.5 ± 0.3	174 ± 3.7
Deficient	0.48 ± 0.1	11.8 ± 0.2	4.5 ± 1.1	2.6 ± 0.6	65 ± 14	3.1 ± 0.3	187 ± 8.9
Weight control	3.4 ± 0.3	30.2 ± 0.7	4.1 ± 1.3	2.2 ± 0.8	193 ± 5	4.0 ± 0.4	185 ± 5.6
Kidney							
High riboflavin	16.0 ± 0.3	19.1 ± 0.9	1.8 ± 0.29	1.1 ± 0.2	2070 ± 58	2.0 ± 0.1	174 ± 9.0
Low riboflavin	10.2 ± 0.7	17.4 ± 0.7	1.6 ± 0.32	1.1 ± 0.4	1973 ± 33	2.0 ± 0.2	170 ± 2.8
Deficient	6.7 ± 0.5	16.5 ± 0.5	1.7 ± 0.28	1.0 ± 0.1	1461 ± 72	1.3 ± 0.1	159 ± 3.1
Weight control	11.4 ± 0.5	20.4 ± 1.0	1.9 ± 0.37	0.9 ± 0.2	1751 ± 86	1.6 ± 0.2	161 ± 3.4
Heart							
High riboflavin	1.6 ± 0.2	19.7 ± 1.3	1.3 ± 0.16	1.0 ± 0.4	2.7 ± 0.8	0.5 ± 0.02	144 ± 7.2
Low riboflavin	1.2 ± 0.1	13.8 ± 1.1	1.0 ± 0.06	0.8 ± 0.2	1.5 ± 0.2	0.6 ± 0.03	139 ± 9.0
Deficient	1.2 ± 0.1	11.7 ± 0.2	1.0 ± 0.04	0.8 ± 0.3	1.7 ± 0.3	0.6 ± 0.04	142 ± 9.0
Weight control	1.2 ± 0	17.2 ± 0.7	1.3 ± 0.26	0.9 ± 0.1	1.6 ± 0.5	0.6 ± 0.04	129 ± 2.6

* The average initial weight of each group was 60 gm. and the average weight gain per rat per day was 1.7 gm. for the deficient, 1.8 for the weight control, 4.3 for the low riboflavin, and 6.0 for the high riboflavin groups. Food intakes of 4.1, 3.4, and 2.1 gm. produced a weight gain of 1 gm. in the deficient, weight control, low, and high riboflavin groups, respectively.

† The dye was 2,6-dichlorobenzenoneindo-3′-chlorophenol.

hydrogenase might easily explain this discrepancy, since ferricyanide taps into the electron transport chain below the point at which antimycin A is inhibitory (12).

Effects of Deficiency, FMN, and FAD—The findings confirm and extend

TABLE II

Electron Transport Enzymes in Tissues of Rats Depleted of Riboflavin for 12 to 13 Weeks

Each value is the average of several analyses from the tissues of four or five rats. Enzyme activities are expressed as moles per kilo of wet weight per hour.

Tissue	FMN	FAD	DPNH dehydrogenase measured with		
			Cytochrome *c*	Dye*	Ferricyanide
	γ per gm.	*γ per gm.*			
Brain					
High riboflavin	0.79	2.27	0.31	0.19	0.97
S.e.m.	0.05	0.11	0.04	0.02	0.07
Deficient	0.42	1.95	0.29	0.20	0.81
S.e.m.	0.13	0.06	0.01	0.02	0.03
Liver					
High riboflavin	4.00	26.52	4.63	2.06	7.43
S.e.m.	0.53	0.60	0.33	0.37	0.44
Deficient	0.19	8.03	3.89	2.21	6.63
S.e.m.	0.08	0.25	0.16	0.22	0.29
Kidney					
High riboflavin	15.25†	20.65	1.56	0.92	3.28
S.e.m.	0.69	0.69	0.18	0.11	0.30
Deficient	2.76	13.74	1.51	0.66	2.85
S.e.m.	0.48	0.58	0.13	0.11	0.10
Heart					
High riboflavin	1.68	21.45	1.40	0.83	4.16
S.e.m.	0.31	0.78	0.14	0.13	0.14
Deficient	1.01	7.41	0.92	0.64	3.56
S.e.m.	0.12	0.31	0.10	0.09	0.17

S.e.m. = the standard error of the mean.

* The dye was 2,6-dichlorobenzenoneindo-3′-chlorophenol.

† These are the results on three rats.

an earlier report (1). Tissues differ a great deal in the rate and extent of fall in FAD and FMN levels. FMN, in general, falls more rapidly than FAD (Table I, Fig. 1). By 12 weeks (Table II) hepatic FMN has almost disappeared. Curiously, cardiac FMN, which was initially low, falls only by a third even in severe deficiency. Liver suffers the greatest loss in FAD (70 per cent), whereas brain FAD is diminished by only 15 per cent. The inclusion of sufficient riboflavin in the diet to give 70 per cent maximal

TABLE III

Regeneration of Flavin Enzymes in Depleted Rats after Administration of Riboflavin

Each value is the average of duplicate analyses on the tissues of five or six rats. Enzyme activities are expressed as millimoles per kilo per hour.

Group*	Weight of organ	FAD	FMN + free riboflavin†	Enzyme activity				
				Glycolic acid oxidase	Glycine oxidase‡	D-Amino acid oxidase	L-Amino acid oxidase	Xanthine oxidase
Liver								
	gm.	*γ per gm.*	*γ per gm.*					
Deficient control	3.8	10.0	0.4	17	1.7	34	4.8	2.2
S.e.m.	0.2	0.5	0.1	2	0.3	18	0.4	0.4
After dose, 0.5 hr.	3.3	15.1	4.8	39	2.0	110	6.4	3.3
S.e.m.	0.3	0.9	0.8	6	0.6	22	0.8	0.5
2 hrs.	3.7	18.9	1.5	70	3.7	90	7.0	3.1
S.e.m.	0.1	0.4	0.4	9	0.3	8.8	0.7	0.5
6 hrs.	4.0	20.3	1.1	68	4.0	63	7.6	4.4
S.e.m.	0.2	0.7	0.3	6	0.5	12	0.3	0.5
24 hrs.	5.0	19.1	1.4	75	5.7	127	5.6	4.8
S.e.m.	0.5	0.4	0.1	5	0.3	15	0.6	0.2
Weight control	2.9	28.6	2.6	121	6.7	175	6.6	4.3
S.e.m.	0.2	1.5	0.2	14	0.4	17	0.6	0.2
High riboflavin	9.8	26.2	3.6	226	8.1	198	7.1	6.0
S.e.m.	0.8	0.6	0.1	20	0.4	11	0.7	0.2
Kidney								
	mg.							
Deficient control	333	16.3	4.9		11.4	1271	11.4	1.73
S.e.m.	8	0.5	0.9		1.5	106	1.3	0.02
After dose 0.5 hr.	331	17.5	13.7		14.2	1532	12.4	1.77
S.e.m.	12	0.6	1.9		1.2	115	0.9	0.04
2 hrs.	338	18.9	6.6		11.2	1436	13.3	1.74
S.e.m.	17	0.4	0.6		1.4	96	1.0	0.07
6 hrs.	356	17.9	5.2		13.5	1309	12.6	1.81
S.e.m.	14	0.7	0.7		1.6	91	1.1	0.02
24 hrs.	366	19.1	7.8		13.1	1626	14.0	2.01
S.e.m.	30	0.4	0.4		1.6	123	0.8	0.03
Weight control	317	21.2	7.7		11.0	1589	12.7	2.41
S.e.m.	14	0.4	0.5		1.6	115	0.7	0.1
High riboflavin	784	20.0	14.9		17.5	2155	22.8	2.69
S.e.m.	52	0.6	0.6		1.0	123	1.2	0.08

TABLE III—*Concluded*

S.e.m. = the standard error of the mean.

* The average initial weights of the deficient, weight control, and high riboflavin groups were 44, 43, and 45 gm., respectively, the gains in weight per rat per day were 0.79, 0.81, and 4.79 gm., and the food consumed per gm. of weight gain was 5.4, 4.8, and 3.0.

† Duplicate determinations for free riboflavin on tissues from one rat of each group showed that a large amount of free riboflavin was present in liver after 0.5 hour, 3.69 γ per gm.; 2 hours, 0.66; and 6 hours, 0.65; in kidney, after 0.5 hour, 7.8 γ per gm.; 2 hours, 0.54; and 6 hours, 0.92.

‡ Glycine oxidase samples were incubated at pH 8.3, which is below the optimum.

growth gave tissue coenzyme levels at 23 days which were but little higher than those of completely deficient animals (Table I). More total flavin was present, of course, because of the larger size. Weight control animals had significantly lower FMN levels in liver, kidney, and heart than the controls on a full diet, although there was no appreciable difference in FAD (Tables I and III).

Protein—Differences observed in tissue enzyme or coenzyme levels were uncomplicated by changes in protein concentrations, since changes of significance were not observed among the various experimental groups (Table I).

Enzymes—DPNH dehydrogenase activities were remarkably stable in spite of prolonged omission of riboflavin from the diet. At 3 weeks none of the deficient tissues tested was demonstrably lower in this enzyme activity. Restriction of food intake was also without demonstrable effect. By 12 weeks a slight fall in tissue levels was apparent, particularly when measured with ferricyanide as electron acceptor. This acceptor would be expected to measure most nearly the DPNH dehydrogenase itself, rather than intermediate links in the electron transport chain (12). Not all of the differences were statistically significant, but as a whole a trend downward is unmistakable.

After 23 days depletion, the *xanthine oxidase* level of riboflavin-deficient liver had dropped to 63 per cent of that of the weight control, but other tissues failed to show any significant change (Table I). Since previous investigators (13), using manometric techniques and other methods of measurement, have reported that the xanthine oxidase of liver falls to 25 per cent or less in riboflavin depletion, longer depletion periods were induced. After 6 and 12 to 13 weeks without riboflavin, the hepatic xanthine oxidase was 35 per cent of that in litter mate controls (Fig. 1, Table III). Renal xanthine oxidase was less affected (Table III).

Hepatic D-*amino acid oxidase* of rats on a deficient diet 23 days fell to 34 per cent of the weight control animals, but the renal enzyme dropped

to only 80 per cent. In the partially deficient groups, a similar discrepancy between the changes in hepatic and renal D-amino acid oxidase occurred. Further depletion for nearly 8 weeks resulted in a hepatic level 20 per cent of that of well nourished animals.

Hepatic *glycolic acid oxidase* activity declined to 10 per cent of normal by 6 weeks of depletion, and only 1 or 2 per cent was left after 12 weeks. After 6 weeks depletion 20 per cent of the initial *glycine oxidase* and 70 per cent of L-*amino acid oxidase* remained in liver. In kidney, neither of

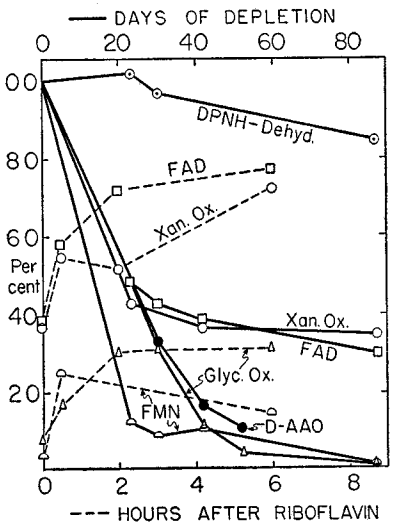

FIG. 1. Depletion of hepatic enzymes and coenzymes during riboflavin deficiency and regeneration after injection of riboflavin. The values are all expressed as per cent of those found with a high riboflavin diet. The enzymes represented are DPNH dehydrogenase measured with cytochrome *c* (DPNH-Dehyd.); xanthine oxidase (Xan. Ox.); D-amino acid oxidase (D-AAO); and glycolic acid oxidase (Glyc. Ox.). The regeneration data are calculated from Table III.

these last two enzymes was demonstrably lower after 6 weeks of deficiency than after a like period of restricted food intake (Table III).

Weight control animals experienced some loss in enzyme activity in comparison to the high riboflavin group (Tables I and III). DPNH dehydrogenase remained the same in both groups, but other enzymes were lower in weight controls. For example, in liver after 7 weeks D-amino acid oxidase was 88, L-amino acid oxidase 93, glycine oxidase 83, xanthine oxidase 72, and glycolic acid oxidase 53 per cent of the high riboflavin group. The effect of food and protein restriction in depressing xanthine oxidase has been repeatedly observed by others. Glycolic acid oxidase has been

shown to be even more sensitive to protein restriction.[3] In the kidney, food restriction had almost as great an effect on the three amino acid oxidases measured as did riboflavin deficiency. Conceivably, these changes reflect the decrease in substrates that would result from the lowered protein intake. The decrease in certain enzyme activities with food restriction is mirrored by a decrease in FMN but not in FAD (Tables I and III).

Realimentation—Rates of regeneration of the coenzymes and several flavin enzymes in depleted rats were measured, following a dose of 600 γ of riboflavin (Table III). The FAD increased rapidly, so that within 2 hours it was back to two-thirds of the control levels in liver and 95 per cent of normal in kidney. FMN is slower to recover in both liver and kidney. The high level of FMN plus free riboflavin at the 30 minute interval represents an unusual situation in which liver and kidney have a large amount of free riboflavin. The content of free riboflavin in the tissues is normally very low. By 6 hours after injection the free riboflavin had virtually disappeared, and, after 24 hours, FMN was still only 50 per cent or less of normal.

Hepatic *glycolic acid oxidase* exhibited a rapid rise, doubling in 30 minutes and again in 2 hours, after which it remained almost stationary for the next two periods at about 60 per cent of the weight control level and only about one-third of the high riboflavin level. FMN, the coenzyme of glycolic acid oxidase, after 24 hours, was likewise only about a third of the level in the high riboflavin group.

Hepatic D-*amino acid oxidase* increased rapidly in 30 minutes but decreased in the later intervals, possibly owing to a shift of the FAD to other enzymes. At the 24 hour period, due to a second injection of riboflavin at 12 hours, the level rose to 70 per cent of the weight control. *Glycine oxidase* showed an increase almost to the weight control level by 24 hours. Hepatic *xanthine oxidase* returned to the weight control level within 6 hours and to 80 per cent of the high riboflavin level in 24 hours. Xanthine oxidase followed closely the FAD rise (Fig. 1).

Changes in the kidney enzymes were less dramatic since the initial decreases were less than in liver. D-*Amino acid oxidase*, which had fallen to only 50 per cent of the control level, returned to 70 per cent at the 30 minute interval. Little additional change occurred at 24 hours. Glycine oxidase, L-amino acid oxidase, and xanthine oxidase showed a slow increase during 24 hours.

Livers and kidneys of weight control rats were smaller than those of depleted animals of equal body weight. This finding suggests utilization of the limited food available to weight control animals for building other

[3] Data of R. A. Pesch and J. H. Clark, now partially published (*J. Pharmacol. and Exp. Therap.*, **117**, 202 (1956); *Proc. Soc. Exp. Biol. and Med.*, **91**, 510 (1956)).

tissues at the expense of these organs. The liver of deficient animals weighed progressively more following the injection of riboflavin, until, at the 24 hour interval, the average weight was 30 per cent above that of the deficient group. Since the protein concentration did not change significantly, the increase is due to growth of liver tissue. The total quantity of the enzymes in the whole liver, therefore, increased more than is revealed by the activity per unit weight.

Enzyme Regeneration in Vitro—Addition of FMN to deficient rat liver homogenates during enzyme assay resulted in an increase in the observed

TABLE IV

Effect of Coenzyme Addition during Measurement of D-*Amino Acid or Glycolic Acid Oxidase*

Each value is the average of several analyses from the tissues of four or five rats. Activities are expressed as millimoles per kilo per hour.

Addition*	Glycolic acid oxidase				D-Amino acid oxidase			
	Liver				Liver		Kidney	
	None	Riboflavin	FMN	FAD	None	FAD	None	FAD
High riboflavin	225	224	235	242	135	154	1543	1711
S.e.m.	12.3	11.0	12.7	14.0	10	14	51	43
Deficient, 7 wks.	6	7	27	17	17.4	24.8	1147	1227
S.e.m.	1.7	1.3	2.5	1.5	2.0	2.4	70	105
Deficient, 9–13 wks.	3	3	17	12				
S.e.m.	0.7	0.9	1.5	1.5				

S.e.m. = the standard error of the mean.

* 6 γ of riboflavin per ml. of substrate or equimolar amounts of FMN or FAD were added for the glycolic acid oxidase measurements. 3.2 γ of FAD per ml. were added for the D-amino acid oxidase.

activity of glycolic acid oxidase (Table IV), indicating the presence of a certain amount of apoenzyme. However, even with FMN addition the activity was far below normal. The activity was only a little greater if homogenates were preincubated with FMN, even for long periods, prior to enzyme assay. The values found indicate that, after 9 to 13 weeks of depletion, less than 2 per cent of the original enzyme is present in active form and only 6 per cent is present as the apoenzyme. Thus 92 per cent of the original enzyme is no longer demonstrable. Added riboflavin did not increase the activity of glycolic acid oxidase. The activity of FAD is attributed to the presence of a small amount of FMN in the FAD preparation plus the FMN liberated enzymatically by the tissue.

In an analogous way, there was found to be only a limited restoration

of D-amino acid oxidase activity upon the addition of the coenzyme, FAD, to deficient homogenates (Table IV). The data indicate that in the liver, after 7 weeks deficiency, about 11 per cent of the original enzyme remains in active form and only about 5 per cent is present as the apoenzyme, whereas about 84 per cent is no longer demonstrable. Similarly in the

TABLE V

Regeneration of Glycolic Acid Oxidase in Liver Slices from Riboflavin-Deficient Rats

Activities are expressed in millimoles per kilo wet weight, per hour. Each value is the average of four to eight determinations except as noted. All incubations were for 1 hour.

Group		FMN	FAD	Protein	Glycolic acid oxidase		DAAO*
					No FMN added to substrate	With FMN added to substrate	
		γ per gm.	*γ per gm.*	*mg. per gm.*			
Normal	Homogenate not incubated	3.50	28.5	170	222	229	112
	S.e.m.	0.53	0.60	0.8	20	20	
	Slices incubated in rat serum	4.80	20.8	140	164		90
	S.e.m.	0.14	0.13	0.8	21		
	Slices incubated in rat serum + 6 γ FMN per ml.	10.80	17.2	134	160		84†
	S.e.m.	0.03	0.10	0.7	12		
Deficient	Homogenate not incubated	0.19	7.29	190	4.6	15.3	21
	S.e.m.	0.02	0.24	0.1	0.01	0.05	
	Slices incubated in rat serum	0.50	4.95	145	9.2	15.1	11
	S.e.m.	0.01	0.21	0.1	0.11	0.15	
	Slices incubated in rat serum + 6 γ FMN per ml.	8.21	5.98	143	21.3	25.1	11†
	S.e.m.	0.63	0.40	0.8	1.0	0.5	

S.e.m. = the standard error of the mean.

* These are average values for D-amino acid oxidase on two samples of liver slices.

† These slices were incubated in rat serum plus 19 γ of FAD per ml.

kidney there is little apoenzyme shown to be present, although the total enzyme loss is much less than in liver.

In deficient liver slices, regeneration of glycolic acid oxidase was demonstrated to a limited extent (Table V) after incubation of the slices in rat serum with added FMN. The level attained was only one-tenth that in normal rat liver. A variety of incubation media with different salt mixtures was tried without inducing greater regeneration of the enzyme activity. Slices incubated without FMN addition showed a decrease in FAD

and an increase in FMN, with some flavin leaking from the tissue into the serum. The protein of both normal and deficient slices decreased 25 per cent during incubation, which accounts for the fall in enzyme activity of normal slices.

D-*Amino acid oxidase* in deficient liver slices showed no evidence of regeneration upon incubation with FAD or FMN (not shown). Nor was there any means found to increase the FAD of liver slices through incubation with riboflavin or FMN.

DISCUSSION

The values reported here for D-amino acid oxidase are of an order of magnitude higher than most of the values in the literature.[4] The average normal renal value is 20 times that reported by Hawkins (15) and 6 times that of Axelrod *et al.* (16). The normal hepatic levels are 5 to 12 times those found by previous investigators (15–18). These discrepancies may help to explain the different results upon addition of FAD *in vitro*. Rossiter (17) found that FAD added to deficient tissue during incubation raised D-amino acid oxidase back to normal, which led to the conclusion that the apoenzyme was still present undiminished. The present study indicates that the apoenzyme as well as the FAD disappears in riboflavin deficiency. The discrepancy may result from analytical difficulties with older methods. The low values suggest that something other than the enzyme itself may have been limiting. FAD is rapidly destroyed in homogenates of kidney and liver. The measurement of oxygen consumption may have reflected the amount of FAD present rather than the amount of D-amino acid oxidase available. The findings presented indicate that, in the case of at least two flavin enzymes, both the protein and the prosthetic group disappear in riboflavin deficiency. This is of some theoretical interest. It would indicate that either the apoenzymes are less stable in the cell than the holoenzymes or that the rate of synthesis of the protein part of the enzymes is diminished in the absence of the prosthetic groups. The latter might imply a type of adaptive process concerned with synthesis of these flavoproteins.

Attempts to induce regeneration of glycolic acid oxidase, D-amino acid oxidase, or FAD *in vitro* were rather abortive, and suggest that suitable conditions were not discovered since regeneration *in vivo* is quite rapid.

Glycolic acid oxidase of mammalian tissue has been studied very little. Dohan (19) reported its presence in liver of rats and rabbits. Kun *et al.* (20) purified it from rat liver and showed its coenzyme to be FMN.

Ratner *et al.* (21) were unable to obtain glycine oxidase from rat kidney,

[4] Umbreit and Tonházy (14), however, using methionine as the substrate, found activities for D-amino acid oxidase of normal rat liver about 2.5 times the present values obtained with D-alanine.

although they demonstrated its presence in kidneys of other species. Krebs (22) had found earlier that rat kidney slices slowly deaminated glycine but that extracts were inactive. The use of less sensitive manometric methods, requiring longer incubation and more concentrated tissue preparations, may explain earlier difficulties in demonstrating the presence of this enzyme. Rat kidney is twice as rich as liver in this enzyme, but so far tests for its presence in other tissues have been negative. L-Amino acid oxidase is also much higher in rat kidney than in liver.

The decrease in xanthine oxidase in deficiency is less than that described by other investigators (13). Inclusion of molybdenum in the diet and the specificity and sensitivity of the analytical method used may account for the differences.

One of the most striking findings is the range in sensitivity to deficiency among the various flavin enzymes. Each enzyme decreases in activity at a different rate and to a different degree with the progression of deficiency, so that the enzymatic pattern changes to a considerable extent with time. The order of sensitivity to deficiency in liver, with the per cent of original activity remaining after 6 weeks, is glycolate oxidase 8, D-amino acid oxidase 17, glycine oxidase 21, xanthine oxidase 33, L-amino acid oxidase 67, and DPNH dehydrogenase over 90. One might anticipate that the enzymes with more readily dissociable prosthetic groups would be the first to suffer. It is true that the first three enzymes listed have all been reversibly dissociated from their prosthetic groups, whereas the last three have not. Nevertheless, in the kidney, glycine oxidase and L-amino acid oxidase are about equally affected by deficiency, although only the first has been shown to be dissociable (23). It remains to be explained why the same enzyme activities are affected quite differently in different tissues by riboflavin deficiency. Thus nearly all renal enzyme activities measured are more resistant to deficiency than are the same activities in liver.

SUMMARY

1. Four tissues of rats on four different dietary régimes have been analyzed for two riboflavin coenzymes, FMN and FAD, and for six enzymes. Levels have been determined for animals receiving (*a*) a high riboflavin diet *ad libitum*; (*b*) the same diet in restricted quantity; (*c*) a riboflavin-free diet; and (*d*) a diet low in riboflavin.

2. Of the normal tissues, the liver was richest in FAD, in DPNH dehydrogenase, xanthine oxidase, and glycolic acid oxidase. The kidney was richest in FMN, in D- and L-amino acid oxidases, and in glycine oxidase. Brain was lowest in all these enzymes. It contains only a tenth as much DPNH dehydrogenase as liver, even though its oxygen consumption is at least as great.

3. Remarkable differences were found in the rates at which the coen-

zymes and enzymes fall during depletion. In the brain changes were negligible. In liver FMN was lost more rapidly than FAD and some enzymes showed rapid and large decreases. In kidney and heart changes were much smaller.

4. DPNH dehydrogenase had the highest activity in all tissues and showed no decrease in moderate deficiency. In severe deficiency it showed a downward trend.

5. Hepatic enzymes in order of their sensitivity to riboflavin deficiency are glycolate oxidase, D-amino acid oxidase, glycine oxidase, xanthine oxidase, L-amino acid oxidase, and DPNH dehydrogenase. Xanthine oxidase decrease was parallel to FAD in liver, but D-amino acid oxidase and glycolic acid oxidase decreases were parallel to FMN. Both the protein and coenzyme moieties of the last two enzymes disappear in depleted rats. The large decrease in these enzymes did not occur in weight control animals. Hence, dietary riboflavin had some special rôle in the formation or maintenance of these enzymes.

6. Rapid regeneration of FAD and of glycolic acid oxidase, D-amino acid oxidase, and xanthine oxidase in deficient liver has been demonstrated *in vivo*. FMN, L-amino acid oxidase, and glycine oxidase come back more slowly.

7. Regeneration of glycolate oxidase in liver slices has been shown to a limited extent. Under the same conditions D-amino acid oxidase did not regenerate.

8. Weight control animals on restricted food intake with ample riboflavin showed lower than normal activity for a number of enzymes in both liver and kidney.

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