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The Influence of Riboflavin Deficiency on Blood Pressure (III)**PART III EXPERIMENTAL & PATHOLOGICAL STUDY ON RAT ESPECIALLY FED
ON THE HIGH CONCENTRATION SALT**

By

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CONTENTS

CHAPTER I.	INTRODUCTION
CHAPTER II.	EXPERIMENTAL METHODS
	1) Experimental Subjects
	2) Experimental Diets
	3) Experimental Periods
	4) Reading of Blood Pressure Levels
	5) Determination of Mono-Amine Oxidase Activity in Liver
	6) Histological Investigation
CHAPTER III.	RESULTS
	1) Blood Pressure Level
	2) Mono-Amine Oxidase Activity in Liver
	3) Ratio of Liver Weight to Body Weight
	4) Histological Changes of Liver
CHAPTER IV.	DISCUSSION
CHAPTER V.	SUMMARY

INTRODUCTION

In Part I and II (111–112), the author described the results of the epidemiologic and clinical investigation on the people living in pure rice planting area, situated in the basin of the Kitakami river. The high rate of hypertension and the high mortality from apoplexy in the area might be due to the dietary imbalance for many years such as the overeating of “polished rice”, “salt” and furthermore taking “poor vitamin diet” especially on B₂ poor diets and B₂ deficiency might cause liver lesion. Now, in this paper, as to the pathogenesis of hypertension from experimental and pathological point of view, the author wish to describe the influence of B₂ deficiency on the blood pressure of the rat fed on the high concentration of salt, liver monoamine oxidase activity and the histological changes in the liver.

EXPERIMENTAL METHODS

1. *Experimental Subjects:*

Alltogether 26 cases of about 6 weeks old albino rats (16 males and 10 females) were employed to be performed the experiments. After the preliminary periods (for 10 days), all cases of them were divided into 3 divisions such as Div. I, Div. II and III, and furthermore each division of them were divided into 2 groups A group and B group, were given the prepared experimental diet as being described below. But, 2 cases in Div. III had been dead before the experiments entirely ended.

2. *Experimental Diet:*

The contents of the experimental diet were shown in Table I. All cases of Div. I were fed on the diet containing 1.0% of salt, all of Div. II were given 2.5% of salt, and Div. III were given 5.0% of Salt. And besides, all A groups of every

division were respectively supplemented 1.0mg of riboflavin per 100g of the diet, and all B group of every division were respectively given B₂ deficient diet. In addition, all cases of 26 cases were given 3 drops of cod liver oil, but, no drinking water was supplied to them. After a period of time all cases of Div. III were given drinking water so as to recover the loss of their appetite and to keep their lives to go on for about 2 weeks after started 5th week. On 15th week, all cases of all divisions were also given continuously 1.0% of salt solution for drinking water.

3. Experimental Periods:

These rats were fed for 17 weeks on the experimental diets as mentioned above for 17 weeks.

After the experimental feeding, the reading of the systolic blood pressure levels were performed throughout 3 days, and during 1 week these rats were killed one by one by being cut off the cervical artery. On the occasion of the slaughter, every body weight were measured and immediately after the slaughter, the livers were extirpated to be measured the weight and to be presented for the experimental and pathological investigation—at first these extirpated livers were weighed one by

one to be calculated the ratio of liver weight to body weight, and secondly a part of the liver was cut off to be determined mono-amine oxidase activity and last of all, the rest of it was fixed in 10% of formaldehyde solution for the purpose of histological investigation.

4. Reading of Blood Pressure Level:

According to the method practiced by J. R. Williams and his co-worker (113), the reading and interpreting of the systolic blood pressure levels in rats were held.

5. Determination of Mono-Amine Oxidase Activity in Liver:

1.0g of the fresh liver section cut off immediately after the slaughter was employed to be determined mono-amine oxidase activity. The liver section was homogenized with M/15 phosphate buffer at PH 7.4 in Potter-Elvehjem's Homogenizer. According to the method described by Blashko et al (114), the determination was practiced. First of all cyanic acid was added to them in order to inhibit the activity of some other oxidases except mono-amine oxidase, and secondly tyramine, as the substrate for the mono-amine oxidase, was added to them. Each one case of both Div. I and II

TABLE I CONTENTS OF EXPERIMENTAL DIETS

DIV.	GROUP	NO.	SEX	POLISHED RICE %	CASEIN %	SOY BEAN CAKE %	CaCO ₃ %	B ₁ mg %	NaCl %	B ₂ mg %
I	A	1	M	88.5	5.0	5.0	0.5	0.1	1.0	1.0
		2	M							
		3	F							
	B	4	M	88.5	5.0	5.0	0.5	0.1	1.0	0
		5	M							
		6	F							
		7	M							
		8	M							
II	A	9	F	87.0	5.0	5.0	0.5	0.1	2.5	1.0
		10	F							
		11	F							
		12	F							
		13	M							
	B	14	F	87.0	5.0	5.0	0.5	0.1	2.5	0
		15	F							
		16	F							
		17	F							
		18	F							
III	A	19	M	84.5	5.0	5.0	0.5	0.1	5.0	1.0
		20	F							
		21	M							
		22	F							
	B	23	M	84.5	5.0	5.0	0.5	0.1	5.0	0
		24	M							
		25	M							
		26	M							

respectively could not be determined on account of the imperfection of the manometer.

6. Histological Investigation:

In order to investigate the changes of the liver pathologically, the author made up both paraffin section and frozen section, and stained the former in hematoxylin and eosin, and the latter in Sudan III. The microscopical photograph of the section stained in Sudan III was taken immediately after the staining.

RESULTS

1. Blood Pressure Level:

The blood pressure levels of these rats were obtained as shown in Table II and in Fig. 1. In Div. I, the average of the blood pressure levels in A group is 87 mmHg, that in B group is 91 mmHg. In Div. II, the average in A group is 89 mmHg, in B group is 98 mmHg. In Div. III, the average in A group is 95 mmHg, and that in B group is 105 mmHg. The averages of every A group of all divisions are lower than those of every B group, and that of Div. I is lower than that of Div. II and in comparison of A group with B group respectively the highest average is Div. III. There-

fore, it can be considered that the blood pressure levels in every B group given the lack of B₂ supplementation more elevate than those in every A group supplemented B₂, and that the blood pressure levels elvate with the concentration of salt in the experimental diet.

2. Mono-Amine Oxidase Activity:

The mono-amine oxidase activity in the liver extirpated immediately after being killed is shown in Table III and Fig. 2. The activity is indicated by the volume (μ l) of oxygen that the liver section at 100mg wastes to oxidize the substrate tyramine for 20 min. In Div. I, the average of 3 cases of A group is 31, and the average in 5 cases of B group is 25.2. In Div. II, the average of the mono-amine oxidase activity in 5 cases of A group is 27.4, and that in 4 cases of B group is 24. The average of that in 3 cases of A group is 35, in Div. III, 2 cases of B group is 27.5. According to these experimental results, the activity in the liver of the rat fed on the B₂ supplemented diet is higher in every division than the rat given the B₂ deficient diet. However, considering the relationship between the activity and the concentration of salt, it can not be felt that the activity may be ascribed to

TABLE II SYSTOLIC BLOOD PRESSURE LEVELS

DIVISION	GROUP	NO.	SYSTOLIC BLOOD PRESSURE LEVEL	AVERAGE
			mm Hg	
I	A	1	90	87
		2	80	
		3	90	
	B	4	85	91
		5	90	
		6	90	
		7	100	
		8	90	
II	A	9	95	89
		10	85	
		11	90	
		12	80	
		13	95	
	B	14	100	98
		15	105	
		16	90	
		17	95	
		18	100	
III	A	19	95	95
		20	95	
		21	90	
		22	100	
	B	23	105	105
		24	105	
		25	100	
		26	110

Fig. 1 Blood pressure levels
— B₂ supplemented group
..... B₂ deficient group

the concentration of salt in the sphere of this experiment.

3. Ratio of Liver Weight to Body Weight:

For the purpose of checking the liver swelling, the author measured the liver weight and the body weight to calculate 1000 times ratio of liver weight to body weight as shown in Table IV, and Fig. 3.

The mean ratio in Div. I's A group is 29, and B group is 33; while the ratio of A group is 28, in Div. II, that of B group is 35; in Div. III, A group is 27 and B group is 29. From these facts, it can be felt that the liver swelling in the rats on B₂ deficient diet is more highly found in every division than that of the rats supplemented B₂. Therefore, the liver swelling in B₂ deficient group can be clearly seen.

4. Histological changes of the liver:

As the histological changes of the liver in this experiment, the author mainly observed the parenchymal cloudy swelling and atrophy, the proliferation of Kuffer's cells, the congestion, and fatty granules of both liver cells and Kuffer's cells.

The author divided the grade of these histological changes into 6 classes as follows; 1). having no changes at all (*), 2). having changes of low degree (***), 3). having changes of middle grade (****), 4). having changes of high degree (*****), 5). having the slight low changes than the low degree (**), 6). having that of the degree between the low degree and the middle grade (****).

These histological changes of the livers are demonstrated in Table V, and in Fig. 4, 5.

1) Cloudy Swelling of Liver Cell:

Judging from the experimental results, demonstrated in Table V, it can be considered that the degree of cloudy swelling in B₂ deficient group is higher in every division than that in B₂ supplemented group. Therefore, it can be also considered that riboflavin deficiency may cause cloudy swelling of liver, and furthermore, these facts as well as the results mentioned in the section on "Ratio of Liver Weight to Body Weight" can be felt as suggesting the swelling of the liver.

2) Atrophy of Liver Cell:

TABLE III MONO-AMINE OXIDASE ACTIVITY IN LIVER

DIVISION	GROUP	NO.	MONO-AMINE OXIDASE ACTIVITY *	AVERAGE *
			ul	ul
I	A	1	41	31
		2	29	
		3	23	
	B	4	34	25.2
		5	20	
		6	33	
		7	14	
		8	25	
II	A	9	25	27.4
		10	45	
		11	28	
		12	15	
		13	24	
	B	14	28	24
		15	20	
		16	--	
		17	24	
		18	24	
III	A	19	44	35
		20	27	
		21	--	
		22	34	
	B	23	29	27.5
		24	26	
		25	--	
		26	--	

* Oxygen volume that was wasted by 100 mg of liver section for 20 min. to oxidize substrate.

FIG. II. MONO-AMINE OXIDASE ACTIVITY

Fig. 2
— B₂ supplemented group
..... B₂ deficient group

It can be seen in Table V that almost all cases have both cloudy swelling and atrophy simultaneously. In the most typical one, as D in Fig. IV shows, cloudy swelling and atrophy can also be seen simultaneously here and there in the same microscopical views. Now, considering only regarding atrophy of the liver, it can be seen there that the degree of atrophy in B₂ deficient group is more serious in Div. I and II than that in B₂ supplemented group, however in Div. III, the difference can not be seen between B₂ supplemented group and B₂ deficient group.

3) Proliferation of Kupffer's Cell:

As shown in Table V, the degree in B₂ deficient group is little higher comparing to B₂ supplemented groups in Div. I and Div. II. But, it is felt that there is no such difference between B₂ deficient and B₂ supplemented group in Div. III.

4) Congestion:

Regarding the congestion of liver the remarkable difference of the degree between B₂ supplemented and B₂ deficient group, judging from Table V, is not found. However, it can be felt that the degree in Div. II is higher than that in Div. I,

TABLE IV. RATIO OF LIVER WEIGHT TO BODY WEIGHT

DIV.	GROUP	NO.	BODY Wt.	LIVER Wt.	LIVER Wt. X 1000 BODY Wt.	AVERAGE
I	A	1	318 ^g	9.0 ^g	28	29
		2	260	8.2	32	
		3	236	6.5	28	
	B	4	284	8.9	31	33
		5	235	8.1	34	
		6	210	8.3	40	
		7	320	8.5	26	
		8	238	7.7	32	
II	A	9	177	5.6	32	28
		10	310	7.5	24	
		11	302	7.7	25	
		12	325	9.2	28	
		13	343	10.0	29	
	B	14	230	7.1	39	35
		15	252	8.1	32	
		16	163	5.6	34	
		17	200	7.1	36	
		18	238	7.7	32	
III	A	19	283	7.0	25	27
		20	212	6.4	30	
		21	313	7.9	25	
		22	210	6.1	29	
	B	23	235	6.9	29	29
		24	174	4.8	28	
		25	---	---	---	
		26	---	---	---

Fig. 3 1000 times ratio of liver weight to body weight
 — B₂ supplemented group
 B₂ deficient group

and furthermore, the degree in Div. III is the highest comparing with other divisions. Therefore, it may be considered that there is no relation between B₂ and congestion of liver, while the degree of congestion increases with the concentration of salt.

5) Fatty Granules:

The author investigated the distribution of fatty granules in both liver cells and Kupffer's cells, the degrees of the distribution in them are presented in Table V. The distribution of fatty granules in every B group increases more in Kupffer's cells than in liver cells, while that in A group of every division has no difference between liver cells and Kupffer's cells. So, it is felt that B₂ deficiency may increase the distribution of fatty granules in Kupffer's cells or decrease it in liver cells.

DISCUSSION

In Part I and II (111-112), as to the etiolo-

gical cause of hypertension, the author described that riboflavin deficiency may be one of the important dietary factors from the results of the epidemiologic and clinical investigation on the people living in the pure rice planting area situated in the basin of the Kitakami river.

In this part, the results as mentioned above, regarding the pathogenesis of hypertension, could be obtained from the experimental and pathological investigation on the rats fed on B₂ deficient diet. Now, the author will discuss these facts from the pathogenetic point of view as to the cause of hypertension.

Regarding the influence of riboflavin deficiency, Calder (21) reported that riboflavin deficiency caused hypertension in rats, though Merrit (28) reported that the deficiency had no effect on blood pressure. According to the author's experimental results, it could be acknowledged that there were difference of blood pressure levels between B₂ deficient rats

TABLE V HISTOLOGICAL CHANGES OF LIVER

DIV.	GR.	NO.	LIVER CELL CLOUDY SWELLING	ATROPHY	PROLIFERATION OF KUPFFER'S CELL	CONGESTION	FATTY GRANULES LIVER CELL	KUPFFER'S CELL
I	A	1	*	*	**	*****	***	***
		2	*	***	**	***	***	***
		3	***	*	**	*****	***	***
	B	4	*****	***	***	*****	**	***
		5	*****	***	**	*****	**	***
		6	***	**	***	*****	*	***
		7	***	***	***	*****	*	***
		8	***	***	***	*****	*	***
II	A	9	*****	**	**	***	***	***
		10	***	***	***	***	*****	***
		11	*	*	***	*****	***	*****
		12	**	**	***	*****	***	***
		13	***	***	***	*****	*****	***
	B	14	***	*****	***	***	***	*****
		15	*****	***	**	***	**	***
		16	*****	***	*****	*****	***	*****
		17	*****	***	***	*****	**	*****
		18	*****	***	***	*****	*	*****
III	A	19	***	***	***	*****	**	**
		20	*****	***	*****	*****	*****	***
		21	***	*****	**	*****	**	***
		22	*****	***	***	*****	*****	*****
	B	23	*****	***	***	*****	***	*****
		24	*****	*****	***	*****	***	***
		25	*****	***	***	*****	***	*****
		26	---	---	---	---	---	---

* no changes at all.
 *** low grade.
 ***** middle grade.
 ***** high grade.

** slightly low than the low grade.
 **** between the low grade and the middle grade.

and B₂ supplemented rats which were fed on the high concentration of salt, and furthermore that B₂ might inhibit the elvation of blood pressure by salt.

There are numerous studies regarding NaCl as the pressor substance. For example, Nakazawa (3), Fukuda (4), Ohji (101), Sapirstein (24), Okabe (25-27), Lenel (98), Stamler & Katz (99), Holly (100), Inoue (102), Knowlton (104), Chapman (115), Corcoran (116), Grollman (117), and Selye (118-119), etc., respectively reported that NaCl was one of the dietary factors to cause hypertension. That

is to say, some of them reported that high concentration of NaCl were found in hypertensive individuals, and that high concentration of salt cause hypertension in animals, and other hand, some of them explained that NaCl restrictions in diet is effectual to treat hypertensive patients. In the author's experiment too, the blood pressure levels of rats increased with the concentrations of NaCl in the experimental diets. On the other hand, on the occasion of feeding on the same concentrations of salt, the blood pressure levels of rats fed on

Fig. 4 Histological changes of liver (1)
stained by Hematoxylin and Eosin

A: No. 1, B₂ supplemented case in Div. I.

B: No. 5, B₂ deficient case in Div. I.

C: No. 14, B₂ deficient case in Div. II.

D: No. 25, B₂ deficient case in Div. III.

A; Liver cells show neither cloudy swelling nor atrophy.

B and C; Liver cells shows atrophy, and there can be seen congestion in them.

D; Liver cells show both cloudy swelling and atrophy in the same microscopical view.

B₂ deficient diets were higher than that fed on B₂ supplemented diets.

As to these facts especially the relationship of riboflavin to NaCl, there is note worthy report, according to K. H. Linder (120), explaining that B₂ administration brings the decrease both Na and Cl in blood while that increases the excretion of urine Na and Cl. Judging from his explanation and from the author's results, it may be justified that B₂ supplementation can protect the blood pressure level from the elvation by salt.

Kasai (70-71) reported in his histological study on B₂ complex deficient rats that the changes of the liver cells were marked by cloudy swelling and atrophy, and that the fatty granules more increased in Kupffer's cells than in liver cells with the severity of B₂ complex deficiency. In the author's pathological investigation too, that on B₂ deficient rats, such histological changes of the liver were recognized, though they were not so severe as that which Kasai had found. On the other hand, there are a number of studies regarding to the influence of

Fig. 5 Histological changes of liver (2)
stained by Sudan III (10×10)

- A: No. 1, B₂ supplemented case in Div. I
- B: No. 4, B₂ deficient case in Div. I
- C: No. 17, B₂ deficient case in Div. II
- D: No. 25, B₂ deficient case in Div. III

A; It is impossible to distinguish the difference between the distribution of fatty granules in liver cells and that in Kupffer's cells.
B, C, and D; It can be seen that the fatty granules in Kupffer's cells increase more than those in liver cells.

riboflavin on the liver function. And besides, in Part I and II (111-112), these relationship between riboflavin and liver function has been clarified by the author. Therefore, it can be concluded that the histological changes of the liver by riboflavin deficiency decrease the liver function. As the results, firstly the liver function to resolve the pressor amine analogs such as tyramine, 1-adrenalin, and 1-noradrenalin, etc., may be lowered, and secondly hypertension may be caused by the pressor amine analogs. Koyanagi and Ohta (121) acknowledged that B₂ deficiency lowered the mono-amine oxidase activity, and the discussed that B₂ deficiency might cause hypertension. In the author's experimental results mentioned above, it was recognized that the blood pressure levels in B₂ deficient rats showed clearly high levels, that the mono-amine oxidase activity decreased in B₂ deficient rats, and that the histological changes of the liver were found in them.

Now, the author feels that the cause of the elevating the blood pressure level in riboflavin deficient rats could be clarified by the pathological study on liver and enzymeologic investigation to determine the mono-amine oxidase activity.

SUMMARY

As the results of the epidemiologic investigation in Part I and II (111-112) upon the people living in a pure rice planting area situated in the basin of the Kitakami river, being felt that riboflavin deficiency was one of the important factors as to the etiological cause of hypertension, the investigation regarding the influence of B₂ on blood pressure of rats fed on 3 classified concentrations of salt (i. e. 1.0%, 2.5%, and 5.0% of salt) was held experimentally and pathologically in this Part.

The results obtained from these investigations as follows:

1. The blood pressure levels of B₂ deficient rats were higher in the same concentration of salt in the experimental diet than those in B₂ supplemented rats, and the blood pressure levels increased with the concentration of salt.

2. The activity of mono-amine oxidase to resolve the pressor amine analogs in the liver of the rats fed on the lack of B₂ supplementation was lower in the same concentration of salt than that of B₂ supplemented rats. However, the relationship between the activity and the concentration of salt

in the sphere of the experiment could not be found.

3. The ratio of liver weight to body weight in the B₂ deficient rats was higher than that in B₂ supplemented rats. Therefore, it can be felt that the liver swelling may be caused by B₂ deficiency.

4. Cloudy swelling of the liver in B₂ deficient rats was found more severely in every concentration of salt than that B₂ supplemented rats. The degree of the atrophy in B₂ deficient rats was found more highly than that in B₂ supplemented rats in 1.0% and 2.5% of salt. However, in 5% of salt, the difference of the degree between B₂ deficient and B₂ supplemented rats could not be found.

5. In 1.0% and 2.5% of salt, it was felt that there might be any sign of difference of the proliferation of Kupffer's cells between B₂ deficient rats and B₂ supplemented rats, the degree in B₂ deficient rats was a little higher than that in B₂ supplemented rats. But, in 5% of salt, there were no differences had shown between B₂ supplemented and B₂ deficient rats.

6. The difference of the congestion of the liver between B₂ supplemented and B₂ deficient rats was not greatly found, however, the degree of the congestion increased with the concentration of salt.

7. The fatty granules in Kupffer's cells were more increased in B₂ deficient rats than that in the liver cells. Although, in B₂ supplemented rats, the granules in Kupffer's cells did not increase in comparison with that in the liver cells.

Judging from these experimental results, it can be felt that B₂ deficiency in the rats fed on high concentration of salt firstly causes liver lesion that lowers the activity of mono-amine oxidase and secondly elevates blood pressure level, it also, B₂ deficiency help to elevate the blood pressure level by salt.

Therefore, from the pathogenetic point of view, it can be considered that B₂ deficiency may be one of the etiological factors of hypertension.

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