CONTRIBUTION OF VITAMIN B₁₂ TO THE ACTIVATION OF APOENZYME FOR THE FORMATION OF PYRUVATE OXIDASE SYSTEM IN RATS¹

JUNZO HAYASHI*, TAKAO SUZUKI, AKIO MAEKAWA, TAKEICHI OSHIMA,
KIICHI IWAMOTO** AND YOSHIKAZU SAHASHI

*Laboratory of Biochemistry and Nutrition, Department of Agricultural Chemistry, Tokyo Agricultural University, Setagaya, Chitose, Tokyo; *Kyoritsu Women's University, Tokyo, and **Department of Agricultural Chemistry, Faculty of Agriculture, Mie University, Mie.*

(Received March 1, 1959)

The authors (1) have reported a study on the contribution of vitamin B₁₂ (B₁₂) to the formation of carboxylase in B₁₂-deficient rats. The increase in carboxylase activity was always observed in the group receiving vitamin B₁₂, whereas it was negative in the B₁₂-deficient group. These experiments were repeated and the addition of vitamin B₁₂ to the diet was confirmed to increase the formation of carboxylase (2).

Recently, further attempts were made to elucidate whether vitamin B₁₂ activates the pyruvate oxidase system in B₁₂-deficient rats, and the contribution of the vitamin to the activation of the apoenzyme in pyruvate oxidase system was confirmed (5, 6).

The first experiments were carried out in connection with the investigations by Gunsalus *et al.* (3), and the second attempt was made from the enzymic aspects under several additive conditions involving flavin-adenine dinucleotide, fumarate, *etc.*

EXPERIMENTAL AND RESULTS

1. Production of B₁₂-deficiency in Rats

Young rats weighing 35—45 g, born by female rats kept on B₁₂-deficient diets, were used in the experiments. The animals were divided at random into two groups, one of which was fed completely B₁₂-deficient diets. The other group receiving orally 0.2 μ g of the vitamin per head daily served as a control.

The B₁₂-deficient diet was composed of, in g, potato starch 55.7, purified casein 15.0, soy bean oil plus cod liver oil (10:1) 7.0, McCollum salt mixture 4.0, choline 0.2, cystine 0.1, vitamin mixture 1.0. The vitamin mixture consisted of, in mg, thiamine HCl 5, riboflavin 10, pyridoxine 5, niacin 20, calcium pantothenate 25, inositol 200, folic acid 0.25, *p*-aminobenzoic acid 2.5, and biotin

¹ Biochemical Studies on Vitamin B₁₂. XVI.

0.1. To this mixture, 99.73 g sucrose was added to make the total amount 100.00 g.

After feeding for 60 days, the body weight, red cell counts and B₁₂ content in liver were estimated. B₁₂-deficient animals indicated always lower values than those receiving the vitamin. The data are shown in Fig. 1 and Table I.

TABLE I
Vitamin B₁₂-Deficiency in Experimental Animals
The figures indicate average values.

	Group receiving 0.2 μ g B ₁₂ per head daily		B ₁₂ -deficient group	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Body weight (g)	165	185	144	158
Erythrocyte counts (cells μ l)	939×10^4	815×10^4	821×10^4	728×10^4
Diameter of cells (μ)	8.0	8.6	8.1	8.7
B ₁₂ content in blood (mg/ml)	1.1 ^a	1.4 ^b	0.76 ^a	1.0 ^b
B ₁₂ content in liver (μ g/100 g)	11.0 ^a	12.0 ^b	6.25 ^a	7.45 ^b

^a *L. leichmannii* assay

^b *Ochromonas* assay

2. Carboxylase Activity in B₁₂-Deficient Rats and in Those Receiving B₁₂

The animals treated as above were killed, and the thigh muscle and liver were rapidly homogenized after adding isotonic KCl solution. The evolution of CO₂ from pyruvic acid in the presence of the homogenate was estimated

FIG. 1 Growth Curve of B₁₂-Deficient Rats.
— Groups receiving orally 0.02 μ g B₁₂ per head daily
..... B₁₂-deficient groups.

using the Warburg apparatus according to Wright (4). The increase in carboxylase activity was always observed in the group receiving B₁₂, but was negative in the B₁₂-deficient group as shown in Table II.

TABLE II
Evolution of CO₂ from Pyruvate by Thigh Muscle Homogenate from B₁₂-deficient Rats and That from the Rats Receiving B₁₂

Group receiving orally 0.2 µg B ₁₂ per head daily		Control group fed on B ₁₂ -deficient diet.			
No. of animals	Q _{CO₂} (without adding B ₁₂)	No. of animals	Q _{CO₂} (without adding B ₁₂)	Q _{CO₂} (0.01 µg B ₁₂ added)	Q _{CO₂} (0.1 µg B ₁₂ added)
1	1.84	10	2.28		
2	2.76	11	1.49	1.31	1.36
3	1.92	12	0.83	0.90	1.05
4	2.10	13	1.75	1.65	1.46
5	3.06	14	2.08		
6	2.96	15	1.85	1.95	1.25
7	2.53	16	1.72	0.97	1.31
8	3.50	17	1.91		
9	2.14				
Average	2.53	Average	1.74	1.35	1.29

In parallel experiments, B₁₂ was added to the tissue homogenate of the B₁₂-deficient animals *in vitro*, but no increase in carboxylase activity was observed (Table II, III).

TABLE III
Evolution of CO₂ from Pyruvate by Whole Liver Homogenate from B₁₂-deficient Rats and That from the Rats Receiving B₁₂

Group receiving orally 0.2 µg B ₁₂ per head daily		Control group fed on B ₁₂ -deficient diet		
No. of animals	Q _{CO₂} (without adding B ₁₂)	No. of animals	Q _{CO₂} (without adding B ₁₂)	Q _{CO₂} (0.1 µg B ₁₂ added)
7	3.73	17	3.00	2.95
8	2.96	18	2.16	2.16
9	3.60	19	2.67	2.59
10	2.83	20	1.94	1.95
Average	3.28	Average	2.44	2.41

In the reaction system, 0.5 ml of a 25 per cent thigh muscle or liver homogenate and 0.3 ml of pyruvate solution containing, in moles, sodium pyruvate 0.0167, cytochrome c 9×10^{-6} , adenosine triphosphate (ATP) 2×10^{-3} , diphosphopyridine nucleotide (DPN) 2.5×10^{-4} , niacinamide 0.01, diphosphothiamine (DPT) 3×10^{-4} , MgCl₂ 0.01, and potassium phosphate buffer 0.01, were used.

3. Preparation of Washed Liver Homogenate

The animals were killed and the liver was rapidly homogenized by addi-

Liver
 ↓ cold 0.25 M sucrose solution (9 parts) was added under stirring
 Homogenate
 ↓ centrifuged at 600 × g for 10 min.
 Supernant
 ↓ 0.04 M MgCl₂ solution was added and centrifuged at 2200 × g for 10 min.
 Precipitate
 ↓ cold 0.04 M MgCl₂ solution was added and centrifuged at 2200 × g for 5 min.
 Precipitate
 ↓ cold 0.04 M MgCl₂ solution was added and centrifuged
 Precipitate
 ↓ cold 0.04 M MgCl₂ solution was added
 Liver homogenate

FIG. 2 *Preparation of Washed Liver Homogenate.*

tion of cold 0.25 M sucrose solution. Washed liver homogenate was prepared by the R.C. Wright technique as shown in Fig. 2.

4. Activity of Pyruvate Oxidase in Washed Liver Homogenate from B₁₂-Deficient Rats and from Those Receiving B₁₂

Activity of pyruvate oxidase in B₁₂-deficient rats was studied in washed liver homogenate by adding various cofactors as shown in Table IV. The uptake of O₂ was found to occur in the pyruvate oxidation system in rat liver homogenate. Experiments were carried out with

TABLE IV
Reaction System for Pyruvate Oxidation in Rat Liver Homogenate

	ml
Washed liver homogenate ^a	1
0.001 M cytochrome c (Ct c)	0.1
0.1 M niacinamide (NAA)	0.1
0.01 M adenosine triphosphate (ATP)	0.1
0.005 M diphosphopyridine nucleotide (DPN)	0.1
0.01 M diphosphothiamine (DPT)	0.1
0.01 M lipoic acid (LiA)	0.1
0.005 M CoA	0.1
1 μg/ml B ₁₂	0.1
0.01 M glutathione (GSH)	0.1
0.1 M pyruvate	0.2
M/15 phosphate buffer (pH 7.3)	0.3
20% KOH, 3 N H ₂ SO ₄ , water, etc. Total volume	3.0

^a The content of nitrogen was determined by micro-Kjeldahl method, yielding 3–4 mg/ml.

pyruvate oxidation system of varying compositions. The results are given in Tables V and VI.

Further, evolution of CO₂ from pyruvate by washed liver homogenate of B₁₂-deficient rats and of those receiving 0.2 μg B₁₂ per os per head daily was determined by the Warburg apparatus. The data are summarized in Table VII.

5. Effect of Endogenous Respiration on the Activity of Pyruvate Oxidation in Washed Liver Homogenate from B₁₂-Deficient Rats

Experiments were first carried out for the effect of 0.1 ml of 0.001 M flavin-adenine dinucleotide (FAD) and 0.1 ml of 0.02 M fumarate on O₂ uptake by

TABLE V
Uptake of O₂ for Pyruvate Oxidation by Washed Liver Homogenate
in the Gunsalus Reaction System

No. of exp.	Composition of cofactors added	Q _{O₂} (N) in				B ₁₂ -deficient
		Group receiving B ₁₂		B ₁₂ -deficient group		B ₁₂ received
		μl, mg N	per cent	μl, mg N	per cent	per cent
1	Ct c + NAA + ATP + DPN + DPT	23	100	14	100	60.8
	do + B ₁₂	22	95.6	16	114.2	72.7
2	Ct c + NAA + ATP + DPN + DPT + LiA	41	100.0	23	100.0	56.1
	do + B ₁₂	38	92.6	24	104.3	63.1
	do + GSH	36	87.8	20	86.9	55.5
	do + B ₁₂ + GSH	57	139.0	36	156.6	62.1
3	Ct c + NAA + ATP + DPN + DPT + LiA + CoA	34	100.0	16	100.0	44.1
	do + B ₁₂	41	120.5	14	87.5	34.1
	do + B ₁₂ + GSH	51	147.0	22	137.8	43.1

TABLE VI
Uptake of O₂ for Pyruvate Oxidation by Washed Liver Homogenate
in the Gunsalus Reaction System

No. of exp.	Composition of cofactors added	Q _{O₂} (N) in		B ₁₂ -deficient
		Group receiving B ₁₂	B ₁₂ -deficient group	B ₁₂ received
		μl/mg N	μl/mg N	per cent
1	None (negative control) ^a	11	4	36.3
		19	10	52.6
2	Ct c + NAA + ATP + B ₁₂ + GSH	20	12	60.0
		16	7	43.7
3	do + DPT	27	14	51.8
		26	27	103.8
4	do + LiA	18	5	27.8
		23	10	43.4
5	do + DPN	37	27	72.9
		19	14	73.9
6	do + CoA	28	24	85.7
		15	10	66.6
7	do + DPN + DPT	47	30	63.7
8	do + DPN + CoA + DPT	32	17	53.1
9	do + CoA + LiA + DPT	44	25	56.8
10	do + DPN + LiA + DPT	81	68	83.9
		38	24	60.5
		37	20	54.1
11	do + DPN + CoA + LiA	54	42	77.8
12	do + DPN + CoA + LiA + DPT	57	27	47.3

^a Uptake of O₂ for pyruvate oxidation by washed liver homogenate in phosphate buffer solution.

TABLE VII
*Evolution of CO₂ from Pyruvate by Washed Liver Homogenate
 in the Gunsalus Reaction System*

No. of exp.	Composition of cofactors added	Q _{CO₂} (N) in				B ₁₂ -deficient
		Group receiving B ₁₂		B ₁₂ -deficient group		B ₁₂ received
		μl/mg N	per cent	μl/mg N	per cent	per cent
1	Ct c + NAA + ATP	24	100			
	+ DPN + DPT + LiA					
	do + B ₁₂	23	96			
2	do			30	100	
				27	90	
3	Ct c + NAA + ATP	42	100			
	+ DPN + DPT + LiA					
	do + GSH	44	104			
4	do			11	100	
				15	136	
5	Ct c + NAA + ATP	31	100			
	+ DPN + DPT + LiA					
	do + B ₁₂ + GSH	48	155			
6	do			20	100	
				28	140	
7	Ct c + NAA + ATP	67		48		72
	+ DPN + DPT + LiA					
	+ B ₁₂ + GSH	27		15		52
8	Ct c + NAA + ATP					
	+ DPN + DPT + CoA	32		16		50
	+ B ₁₂ + GSH					
9	Ct c + NAA + ATP					
	+ DPN + CoA + LiA	23		15		65
	+ B ₁₂ + GSH					
10	Ct c + NAA + ATP					
	+ CoA + LiA + DPT	26		16		62
	+ B ₁₂ + GSH					
11	Ct c + NAA + ATP					
	+ CoA + LiA + DPT	29		19		65
	+ DPN + B ₁₂ + GSH					

the washed liver homogenate. Tests were performed with the washed liver homogenate treated by the same method as described in Fig. 2. The difference between the activities of washed liver homogenate and of untreated whole homogenate was marked as shown in Fig. 3, and the effect of fumarate and FAD on pyruvate-oxidase activity is clearly seen in Fig. 4.

6. Apoenzyme Activity of Pyruvate Oxidase in B₁₂-Deficient Rats

Uptake of O₂ and evolution of CO₂ were tested in completely washed liver homogenate in the reaction system with the addition of FAD and fumarate. The apoenzyme activity of pyruvate oxidase system was higher in the group receiving B₁₂ than in the B₁₂-deficient group as shown in Tables VIII and IX.

TABLE VIII
Uptake of O₂ for Pyruvate Oxidation by Washed Liver Homogenate with
the Gunsalus Reaction System with Addition of FAD and Fumarate

No. of exp.	Composition of cofactors added to a basal reaction system ^a	QO ₂ (N) uptake in				B ₁₂ -deficient
		Group receiving B ₁₂		B ₁₂ -deficient group		B ₁₂ received
		$\mu\text{l/mg N}$	per cent	$\mu\text{l/mg N}$	per cent	per cent
I	DPN+CoA+DPT+LiA	64.1		52.7		82.2
	DPN+CoA+DPT+LiA	55.0	100	48.3	100	
	DPN	45.4	82.5	38.1	81.0	
	CoA	43.8	79.6	34.2	70.8	
	DPT	40.8	74.2	42.7	88.4	
	LiA	36.4	66.2	30.6	63.4	
	0	40.0	72.7	29.5	61.1	
II	DPN+CoA+DPT+LiA	62.5		40.0		64.0
	DPN+CoA+DPT+LiA	60.2	100	32.0	100	
	DPN+CoA+DPT	58.9	97.8	33.7	105	
	CoA+DPT+LiA	56.0	93.0	27.5	85.6	
	DPT+LiA+DPN	55.5	92.2	30.6	95.6	
	LiA+DPN+CoA	53.6	89.6	26.8	83.8	
	0	43.7	72.6	20.6	64.0	

^a Basal reaction system contained 0.1 ml of 0.001 M cytochrome c, 0.1 ml of 0.1 M niacinamide, 0.1 ml of 0.01 M ATP, 0.1 ml of 0.001 M FAD, 0.1 ml of 0.01 M GSH, 0.1 ml of 1 $\mu\text{g/ml}$ B₁₂, 0.1 ml of 0.02 M fumarate, 0.3 ml of M/15 phosphate buffer (pH 7.3), 1 ml of homogenate, 0.2 ml of 0.1 M pyruvate, and 0.4 ml of 20% KOH in 3.0 ml of water.

Average body weights were: Experiment I, group receiving B₁₂, 197 g; B₁₂-deficient group, 180 g. Experiment II, group receiving B₁₂, 220 g; B₁₂-deficient group, 190 g.

TABLE IX
Evolution of CO₂ from Pyruvate by Washed Liver Homogenate in the
Gunsalus Reaction System with Addition of FAD and Fumarate

No. of exp.	Group receiving B ₁₂		B ₁₂ -deficient group		B ₁₂ -deficient
	Body weight	QCO ₂ (N)	Body weight	QCO ₂ (N)	B ₁₂ received
	g	$\mu\text{l/mgN}$	g	$\mu\text{l/mgN}$	per cent
1	197	54.5	180	39.0	71.5
2	186	44.3	175	29.0	65.4
3	220	60.9	190	29.5	48.4
4	230	50.6	175	41.8	82.6
Average		52.6		34.6	65.7

The reaction was carried out at 37.5° by the complete reaction system with addition of FAD and fumarate as described above.

SUMMARY

1. The apoenzyme activity of pyruvate oxidase system in the tissue of B₁₂-deficient rats was studied *in vitro* under various additions of B₁₂, cytochrome c, niacinamide, DPN, DPT, lipoic acid, CoA, ATP, FAD, glutathione as well as fumaric acid. The decrease of the apoenzyme activity in B₁₂-efficient rats

FIG. 3 Difference in Activities for Pyruvate Oxidation between Washed Liver Homogenate and Untreated Whole Homogenate.

- Washed liver homogenate
- △..... Untreated whole homogenate
- Washed liver homogenate, with pyruvate added
- △—— Untreated whole homogenate, with pyruvate added.

was always recognized in the uptake of O₂ and the evolution of CO₂ after adding pyruvate.

2. The animals receiving 0.2 μg of B₁₂ per os daily before experiments showed always an increased apoenzyme activity of pyruvate oxidase system.

3. The results seem to lead to the conclusion that vitamin B₁₂ contributes to the activation of the apoenzyme in pyruvate oxidase system in rats.

ACKNOWLEDGMENT

This work was supported in part by the Vitamin B Research Committee and a Research Grant from the Ministry of Education to which the authors' thanks are due. The authors also wish to express their hearty thanks to Messrs. K. Ito, S. Kobayashi, F. Mitsui and M. Kusubayashi of this laboratory for their assistance throughout the experiment.

REFERENCES

1. Iwamoto, K., Hayashi, J., and Sahashi, Y., *J. Vitaminol.*, **3**, 279 (1957).
2. Sahashi, Y., Iwamoto, K., and Hayashi, J., *Vitamins*, **9**, 443 (1955).
3. Gunsalus, I. C., A Symposium on the Mechanism of Enzyme Action. p. 552 (1955).
4. Wright, R. C., and Scott, E. M., *J. Biol. Chem.*, **206**, 725 (1954).
5. Sahashi, Y., Hayashi, J., Suzuki, T., Maekawa, A., Oshima, T., and Iwamoto, K., *Vitamins*, **16**, 80 (1959).
6. Sahashi, Y., Hayashi, J., Suzuki, T., Maekawa, A., Oshima, T., and Iwamoto, K., *ibid.*, **16**, 171 (1959).

FIG. 4 Effect of FAD and Fumarate on the Activity of Pyruvate Oxidation in Washed Liver Homogenate of B₁₂-deficient Rats.

- Completely washed liver homogenate (endogenous)
- △..... Effect of FAD and fumarate on washed liver homogenate
- Completely washed liver homogenate with pyruvate
- ×—— Effect of FAD and fumarate on washed liver homogenate with pyruvate