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UTILIZATION OF VITAMIN B_{12} IN PANTOTHENIC ACID DEFICIENCY IN THE RAT

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Vitamin B_{12} has been reported to be interrelated to a number of vitamins, such as folic acid,[1,2] ascorbic acid,[1,3] choline[4] and panthothenic acid. *Evans et al*[5] found that chicks deficient in vitamin B_{12} have high liver pantothenic acid contents. *Yacowitz el al*[6] reported an interrelation between vitamin B_{12} and pantothenic acid by demonstrating sparing effect of vitamin B_{12} on the growth of chicks in pantothenic acid deficiency and vice versa. They also found higher vitamin B_{12} contents in the liver of pantothenic acid deficient chicks, and postulated that the lower B_{12} levels in pantothenic acid treated chicks are the result of the better growth and more rapid exhaustion of vitamin B_{12} stores. In a series of liver B_{12} determinations in normal rats, however, it was found that the new born and young rats have lower liver B_{12} stores than the adult. This contradicts their hypothesis, and the retarded growth does nor seem to result in elevated liver B_{12} levels. Some unknown abnormality in the metabolism of vitamin B_{12} was thought to exist in pantothenic acid deficiency. To study such an abnormality may also help elucidate the interrelation between these two vitamins. Vitamin B_{12} has been claimed[7] to be related to the blood levels of sulf hydryl group. The possibility seems to exist that pantothenic acid is also related to this vitamin through Co-A which contains pantothenic acid and active SH group. Since those papers published on this relationship were based on chick's studies, and such sparing effect of vitamin B_{12} as reported by *Yacowitz et al*[6] was not confirmed by *Welch* and *Couch*,[8] it appeared desirable to investigate further into this problem using rats instead of chicks, The present experiments were, therefore, designed to study the effect of vitami B_{12} on pantothenic acid deficiency, and the pattern of B_{12} metabolism in pantothenic acid deficent rats.

Preparation of pantothenic acid deficient rats

The following procedure was used to produce pantothenic acid deficiency in young rats. Pregnant females were removed from the breeding stock cages and put into individual cages 3 to 4 days before they were ready to deliver young. They were kept on the stock diet until the delivery of their litters. Within several

hours after delivery the females were put on the pantothenic acid-deficient diet. To prevent the young from falling through the mesh of the false bottom, these were removed from the cages and the mothers were provided with bedding material (wood chips) until the young were 5 to 6 days old. At this time the metal screen bottoms were returned to the cages.

Experience had proven that mortality of mothers and young was so high 14 to 17 days after the initiation of lactation, that a brief recovery period at this time was imperative. Hence, when the young were 15 days old 2 mg. of calcium pantothenate was added to 100g. of the mother's ration, all of which was consumed within 3 to 4 days.

When the young were 28 days old the mothers were removed from the cage. The young animals were kept on the deficient diet and weighed weekly. At the end of approximately 4 weeks the weight remained stationary and the animal were ready to testing.

For the production of pantothenic acid deficiency in young adult rats, normal weanling rats were fed pantothenic deficient diet for 7 to 14 weeks until deficiency manifested itself in gross appearance. The control animals were fed the same ration and received Ca pantothenate by subcutaneous injection, 100γ three times a week.

The composition of pantothenic acid deficient diet: Alcohol extracted casein 24%, sucrose 64%, salt #IV 4%, and primex* 8%. To each kilogram of ration the following vitamins were added; thiamine 2mg, pyridoxine 2mg, riboflavin 5mg, PABA 5mg, niacin 10mg, inositol 200mg, choline chloride 500mg, vit. A 12,510 I.U. and vit. D 1,772 I.U. in percomorph oil, vit. K 2.1mg and alpha tocopherol 23mg. The fat-soluble vitamins were dissolved in Mazola oil in quantities to yield the above amounts in 2cc of solution. The water-soluble vitamins were dissolved in 50% alcohol in amounts to yield the desired dosage in 20cc of solution.

METHOD

Determination of vitamin B_{12} activity in liver and plasma: Animals were killed by total bleeding under ether anesthesia. Blood was heparinized and the separated plasma was used for plasma B_{12} determination. The liver was removed immediately at necropsy and a piece of liver weighing about 1gm was cut from the left lobe and frozen. The liver piece was subsequently homogenized with distilled water in the cold room to yield 5% homogenate. To 2ml of the homogenate was added 50γ NaCN, and the mixture was diluted with acetate buffer to 10cc to give a final concentration of 0.03M of the buffer. The diluted homo-

Hegsted, Mills, Elvehjem and Hart: J.B.C. 138, 459, 1941.
* Hydrogenated cottonseed oil, Swift and Co.

genate was then autoclaved at 120°C for 20 minutes. The cooled and separated supernatant was neutralized with a half volume of 0.15M Na_2HPO_4. The neutralized liver extract was added to the *Skeggs'* media[9] at the levels of 0.025, 0.05, 0.1 and 0.3ml per tube. The amount of medium used per tube was 10ml and the growth of Lactobacillus leichmannii 4797 was measured turbidimetrically after 40 hours of incubation. Plasma B_{12} determination: Plasma was directly added to 5ml medium at the levels of 0.025, 0.05 and 0.075ml, and the growth was measured titrimetrically after 70 hours incubation.

Measurement of vitamin B_{12} absorption: Co^{60}-labeled radioactive vitamin B_{12} (B_{12}*) with a specific activity of approximately 800 μc per mg (90cpm per mγ under our conditions) was used. Aqueous solution of B_{12}*, 30 or 50 mγ in 1 ml distilled water, was force-fed to rats by a stomach tube, and the animals were transferred to individual metabolism cages for the collection of specimens. Feces and urine were collected separately in sample bottles of 100cc capacity for 6 days in two divided collections. Six days after administration of B_{12}*, the animals were sacrificed and, liver, kidney and digestive tract were removed for radioactivity measurement.

Measurement of excretion of vitamin B_{12}: 20mγ of B_{12}* were injected subcutaneously to rats, and the same procedure as above was employed for specimen collections, i. e., feces, urine and organs, which were measured for radioactivity.

Determination of radioactivity in specimens: γ-scintillation counter with a flat crystal of tallium activated Na iodide installed in a well typed sample holder was used for measurement. The amount of sample was made up to 50ml to yield the same geometry. The urine and washings of the cage were collected together and condensed down to the volume on a water-bath. The feces was soaked in water and homogenized with concentrated sulfuric acid. The organs were digested with 6 ml of 30% KOH and brought up to the volume. The same amount of KOH was used as a background for K^{40} correction.

EXPERIMENTAL AND RESULTS

A. Vitamin B_{12} levels of the liver and plasma.

Experiment 1 (Table I) – Three litters of pantothenic acid deficient weanlings of both sexes were distributed into two groups, experimental (group A) and control (B). The control group was fed pantothenic acid deficient diet but received Ca pantothenate by injection. The data indicate that the pantothenic acid deficient rats had definitely higher B_{12} content, $0.188 \pm 0.02\gamma$ per gram of wet liver weight as compared to $0.112 \pm 0.012\gamma$ of the control ($p < 0.05$). However, the difference in the bodyweight between the two groups was not big enough to explain the difference of B_{12} levels on the basis of growth and thereby exhaustion of B_{12} stores in the liver.

Experiment 2 (Table II)- Thirty day old young male rats were divided into three groups and fed pantothenic acid deficient diet for 7 weeks, during which time the control group (A) received Ca pantothenate by injection. At the end of 7 weeks, groups A and B were sacrificed for determintaion of vitamin B_{12} levels of the liver and plasma. From this time the third group (C), which had been

TABLE I

Liver vitamin B_{12} contents of pantothenic acid deficient young rats.

Group	Diet	Treatment	No. of Rats	Av. Body Weight (gm)	Liver B_{12} Level (γ/gm)
A	Pant.Acid Deficient	None	10	41.3	0.188±0.020
B	Pant. Acid Injection	Ca Pant.* Injection	7	59.7	0.112±0.012

* Ca pantothenate 100 γ 3 times a week.
All animals were of the deficient diet for 21-23 days after weaning.

depleted for pantothenic acid for seven weeks, received Ca pantothenate by injection (100γ three times a week) for two weeks, and was sacrificed for the same determinations. The results of this experiment given in Table II clearly indicate that pantothenic acid deficient animals (B) had higher liver B_{12} content than those receiving Ca pantothenate (A). Group C receiving pantothenic acid after the manifestation of deficiency showed an average liver B_{12} level which falls between groups A and B. However, there was no difference in plasma B_{12} levels

TABLE II

Effect of Ca pantothenate on liver and plasma B_{12} levels of pantothenic acid deficient rats.

Group	Treatment	No. of Rats	Av. Body Wt. (gm)	Liver B_{12} (γ/gm)	Plasma B_{12} (mγ/ml)
A	Ca Pant. for 7 Weeks	5	278	0.053±0.010	0.94±0.05
B	None	6	156	0.152±0.030	0.96±0.05
C	Ca Pant. for 2 Weeks after 7 Week Depletion	5	164	0.093±0.028	1.18±0.01

between A and B. This indicates a phenomenon in which plasma B_{12} level does not reflect the liver B_{12} content. Group C showed a significantly higher plasma B_{12} level than the other twe groups ($p < 0.05$), which is also suggestive of an effect of pantothenic acid administration to deficient animals on their plasma B_{12} level.

B. Effect of vitamin B_{12} on survival rate in pantothenic acid deficiency

Experiment 3 (Table III)– The effect of vitamin B_{12} on pantothenic acid requirement was investigated by giving two different doses of B_{12} combined with two different suboptimal doses of pantothenic acid, and measuring survival rate. Eight litters of pantothenic acid deficient weanling rats were divided evenly into 5 groups on the basis of body weight and litters, and fed pantothenic acid deficient ration for 8 weeks. Group 1 served as control receiving saline, groups 2 and 3 received 2γ Ca pantothenate combined with 1 mγ and 100 mγ of B_{12} per injection respectively, and groups 4 and 5 received 20γ Ca pant. combined with 1 mγ and 100 mγ B_{12} respectively. One injection dose consisted of 0.2 ml of aqueous solution containing the indicated amounts of Ca pant. and B_{12}, and three injections were given. The mortality and survival rate were given together with the measured liver B_{12} contents in Table III.

TABLE III

Effect of feeding different levels of calcium pantothenate and vitamin B_{12} on mortality of weanling rats.

Group	Treatment (Each Inj. Dose*)	Number of Rats Survived					Liver B_{12} Content (γ/gm)
		Beginning	2 Wks	4 Wks	6 Wks	8 Wks	
1	None (Control)	8	7 (12.5)	3 (63)	2 (75)	2 (75)	0.082±0.008
2	Ca Pant. 2 γ B_{12} 1 mγ	8	8	4 (40)	3 (63)	3 (63)	0.197±0.132
3	Ca Pant. 2 γ B_{12} 100 mγ	8	8	6 (25)	5 (37)	3 (63)	0.270±0.040
4	Ca Pant. 20 γ B_{12} 1 mγ	9	7 (22)	7 (22)	4 (56)	4 (56)	0.046±0.009
5	Ca Pant. 20 γ B_{12} 100 mγ	8	8	8 (0)	8 (0)	8 (0)	0.146±0.016

( ) Denotes mortality percentage.
* Three injections per week.

As will be seen from the number of surviving animals in the table, none of group 5 receiving 20γ Ca pant. and 100 mγ B_{12} died during the 8 week experimental pariod, whereas the rest of the groups had lost more than half of the animals. The pantothenic acid deficiency signs were also least manifest in group 5. The difference between groups 4 and 5 is statistically significant and is important in that 20γ Ca pant. alone was not enough to keep all the animals alive, but the addition of 100mγ B_{12} brought up the survival rate to 100%. At 2γ level of Ca pant., supplementation of a large dose of B_{12} didnot render any effect on survival rate. This dose of Ca pant. is apparently below the suboptimal dose.

The liver B_{12} contents were also determined at the end of 8 weeks. The mean

value of liver B_{12} contents of group 4 was lower than that of group 1 (statistically significant), which is the same finding as in experiments 1 and 2. Group 2 had a big variation in values, In the groups in which 100 mγ B_{12} were injected three times a week, a similar trend was also observed, namely, group 3 had higher liver contents than group 5, The difference in the treatment among these groups was only the amount of Ca pant. administrated. Ca pant., therefore, appeared to help lower the accumulation of injected B_{12} in the liver.

C. Absorption of orally administered vitamin B_{12}

Experiment 4 (table IV) – The absorption pattern of B_{12}* in pantothenic acid deficient and control rats was studied. In the first study (A), 50 mγ B_{12}* were administered orally to a group of pantothenic acid deficient adult male rats as well as to a control group, and the fecal excretion and organ radioactivity were measured. In the second study (B), an oral does of 30 mγ was used to young adult male rats of similar groups in order to ascertain whether oral dosage makes

TABLE IV

Absorption of orally administered radioactive vitamin B_{12}.

Study	Oral Dose of B_{12}	Group	No. of Rats	Av. Body Wt. (gm)	Amount of B_{12}* Absorbed (mγ)	B_{12}* Recovered (mγ)			
						Liver		Kidney	Urine
						Total	mγ/10gm	Total	
A	50 mγ	Deficient*	5	262	31.9±1.7	4.48± 0.29	5.98± 0.61	4.46± 0.16	1.15± 0.07
		Control**	4	309	29.7±1.7	3.66± 0.12	3.85± 0.16	3.48± 0.40	0.85± 0.09
B	30 mγ	Deficient*	6	156	11.7±1.3	1.50± 0.17	2.78± 0.40	1.94± 0.11	0.79
		Control**	5	278	13.1±1.0	1.70± 0.31	1.55± 0.30	1.83± 0.21	0.64

* Deficient in pantothenic acid.

** Received Ca pant. 100 mγ three times a week by injection.

difference in absorption. As shown in the table, there was no difference between two groups in absorption rate either at the level of 50 mγ or 30 mγ. However, the deficient rats had higher radioactivity in the liver on the basis of wet gram in both studies, the mean values being 5.98 ± 0.61 mγ in deficient group against 3.85 ± 0.16 mγ in control in Study A, and 2.78 ± 0.40 mγ against 1.55 ± mγ in Study B. Although there was a considerable difference in body weight between the groups in Study B, this difference was rather small in Study A. Therefore, the difference in the measured radioactivity in the liver is not simply a reflection of the body weight.

D. Excretion and retention of injected radioactive vitamin B_{12}

Experiment 5 (Table V) – Excretion and retention of the injected radioactive B_{12} were studied in order to visualize the dynamic pattern of the distribution and metabolism of vitamin B_{12}* introduced into the pantothenic acid deficient rats. Two separate studies using 18 rats were carried out in this experiment. As shown in table V, if 20 mγ of B_{12}* which is considered within the physiological range of intestinal absorption were given parenterally, about 4 mγ were

TABLE V

Excretion and retention of injected radioactive vitamin B_{12}.

Study	Group	No. of Rats	Av. Body Wt. (gm)	Excretion of Radioactivity @ (mγ)			B_{12}* Recovered (mγ)			
							Liver		Kidney	Digest. Tract
				Urine	Feces	Total in 5 Days	Total	mγ/10gm		
A	Deficient	6	266	2.17	1.66	3.83±0.30	2.80± 0.14	3.03± 0.24	3.06	2.83
	Control	6	344	2.24	1.89	4.13±0.39	2.38± 0.14	2.30± 0.29	2.91	3.00
B	Deficient	3	248	1.75	1.77	3.52±0.07	2.88± 0.31	3.78± 0.27	2.34	2.36
	Control	3	310	2.00	2.02	4.4±0.10	2.59± 0.10	2.85± 0.19	2.12	2.12

Deficient–pantothenic acid deficient.
Control–received Ca pant. 100mγ 3 times a week by injection.
@ Calculated as Radioactive B_{12} in mγ.

eliminated in the urine and feces in 5 days in both groups. The radioactivity retained in the liver, however, was always higher in the deficient groups than in the controls if calculated on the basis of gram liver.

DISCUSSION

It has been repeatedly demonstrated in this study that the liver B_{12} levels in pantothenic acid deficient rats are much higher than those in the control rats. Plasma B_{12} levels in deficient animals, however, didnot differ from the control, indicating that the plasma B_{12} levels are not proportional to the liver B_{12} contents in deficient animals. Vitamin B_{12} transportation from tissue to blood seems to be impaired in pantothenic acid deficiency. It has been generally accepted that plasma B_{12} levels reflect tissue B_{12} levels in animals and humans. There has not as yet been demonstrated any abnormal equilibration of B_{12} between the tissue and the plasma, although such an abnormality was suspected to exist in some of the metabolic conditions.

It is also clearly demonstrated from the third experiment that if rats are kept on diet containing suboptimal amounts of pantothenic acid, administration of a large dose of B_{12} has a sparing effect and helps animals to survive. This finding seems contradictory in that pantothenic acid deficient rats have high B_{12} contents in the liver but still require B_{12}. However, if the utilization of liver B_{12} is blocked, or the liver B_{12} is not in normal way available to the metabolic systems of rats, administration of a large quantity of B_{12} could give a beneficial effect by introducing utilizable B_{12} into the body. In other words, it appears feasible to postulate that in pantothenic acid deficiency the utilization of liver and plasma B_{12} is blocked, and this blockage is manifested in impaired proportionality of liver and plasma B_{12} and accumulation of B_{12} in the liver. Such derangement of B_{12} equilibration in the body seems to render limitation of interpretation of plasma B_{12} levels. Without tissue B_{12} determination, utilization of vitamin B_{12} is not fully understood.

From the data obtained in the absorption and retention study with radioactive vitamin B_{12}, it was proved that the deficient rats have normal capacity of absorption, but retains more B_{12} in the liver on the basis of gram liver. This also seems to explain the accumulation and resultant higher concentration of B_{12} in the liver. This postulate is compatible with the finding that administration of pantothenic acid to deficient rats brought up plasma B_{12} levels and lowered liver B_{12} concentration as demonstrated in experiment 2.

The proposed mechanism does not nullify any interrelationship in the intermediary metabolism between vitamin B_{12} and panthothenic acid. There will be more than one mechanisms which result in elevated liver B_{12} levels in pantothenic acid deficiency. Retarded growth and less consumption of tissue B_{12} may partly account for the altered metabolism, although this does not seem to be the main mechanism. Vitamin B_{12} may also be related to pantothenic acid in the intermediary metabolism. Since one of the claimed multitudinous roles of vitamin B_{12} is to reduce –S–S– to –SH state,[10] and SH is the active part of Co–A molecule, it is likely that B_{12} is concerned with the metabolic processes where Co–A or pantothenic acid as a part of Co–A is involved. In fact, in absence of B_{12} Co–A fails to be reduced to –SH form, thereby inhibiting the normal pathway of fat metabolism.[11] Another supporting evidence comes from the work of *Gräsbeck*[12] who demonstrated lowered acetylation of sulfonamide in vitamin B_{12} deficient rats. The impaired utilization of vitamin B_{12} in pantothenic acid deficiency demonstrated in the present experiments confirms the close interrelation between these two vitamins.

CONCLUSION

The metabolic patter and the sparing effect of vitamin B_{12} were studied in

pantothenic acid deficiency in rat and the following results were obtained.

(1) The liver of pantothenic acid deficient rat had higher vitamin B_{12} content than the control, but the plasma B_{12} level was not elevated in the deficient rat.

(2) Administration of a large dose of vitamin B_{12} to rats receiving a sub-optimal dose of pantothenic acid had sparing effects on the survival rate.

(3) Absorption and excretion of radioactive vitamin B_{12} was not impared in pantothenic acid deficiency.

(4) Radioactive vitamin B_{12} introduced either by intestinal absorption or by injection was retained more in the liver in pantothenic acid deficient rats than the normal.

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