Relationship of Vitamin B_6 to the Metabolism of D-Amino Acids.* (17611)

KATHARINE L. ARMSTRONG, GLADYS FELDOTT, AND HENRY A. LARDY.
From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison

In the course of work upon D-amino acid oxidase (the enzyme which deaminates D-amino acids to their corresponding alpha-keto acids) it appeared possible that vitamin B_6 might influence the activity of this enzyme(1). This vitamin is known to be the coenzyme for transaminase enzymes, presumably acting as carrier for the amino group through the formation of a Schiff's base between pyridoxal phosphate and the amino acid(2,3,4). The transaminase reaction has been conclusively demonstrated only with L-amino acids. A similar mechanism appeared possible in the oxidative deamination of D-amino acids. This hypothesis was tested by assaying liver and kidney tissues from vitamin B_6 deficient and control rats for D-amino acid oxidase activity. Although preliminary experiments showed that the activity of the enzyme in deficient animals was lower than that of control animals fed *ad libitum*, pair feeding of the animals reduced the D-amino acid oxidase activity of the control almost to that of the depleted animals.

The recent work of Holden, Furman and Snell(5) has shown that vitamin B_6 can be substituted for D-alanine in the growth of *L. casei* and *S. faecalis*, and that this vitamin promotes the synthesis of D-alanine by these organisms. It was suggested that vitamin B_6 acts as a coenzyme in the synthesis of D-alanine. Their work stimulated a reinvestigation of the effect of vitamin B_6 on the utilization of certain D-amino acids by the rat. It has been demonstrated that the net nitrogen utilization of vitamin B_6 deficient animals which have been fed supplements of D-amino acids is only 30% of that of animals receiving simultaneous injection of pyridoxine. The reaction appears fairly specific, in that the utilization of nitrogen by B_6 deficient animals fed L-amino acids or nitrogen in the inorganic form is not affected to a similar extent.

Experimental. Male albino rats weighing 40-50 g were obtained at weaning from Sprague-Dawley and placed in individual wire cages. The basal ration employed for the production of a vitamin B_6 deficiency was that of Sarma, Snell, and Elvehjem(6), consisting of sucrose 73 g, blood fibrin 18 g, corn oil 5 g, salts IV 4 g, thiamin 0.2 mg, riboflavin 0.3 mg, nicotinic acid 2.5 mg, calcium pantothenate 2 mg, choline chloride 100 mg, 2-methyl, 1,4-naphthoquinone 1 mg, inositol, 10 mg, biotin 0.01 mg, and folic acid .3 mg per 100 g of diet. Two drops of haliver oil were administered weekly. Control animals received the same ration with 0.25 mg % of pyridoxine·HCl added. Water was supplied *ad libitum*. One group of control animals used for enzyme studies was fed *ad libitum, all* other control groups were pair-fed with the deficient animals. Growth of pair-fed controls closely paralleled that of the deficient animals. A B_6 deficiency was manifested by an almost immediate and continued excretion of xanthurenic acid by animals on the deficient ration.

D-amino acid oxidase activity of rat tissues was determined by the method of Axelrod, Sober and Elvehjem(7), which measures oxy-

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.
Supported in part by a grant from the Williams-Waterman Fund of the Research Corporation.

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TABLE I.
D Amino Acid Oxidase Activity of Vitamin B_6-Deficient and Control Rat Tissues.

		Series I—Controls fed *ad libitum* Q_{O_2}		Series II—Pair-fed controls Q_{O_2}	
Group		Liver	Kidney	Liver	Kidney
B_6-deficient	Avg	1.76 ± .29 (5)	8.24 ± .98 (4)	3.07 ± .69 (6)	12.3 ± 2.90 (6)
Control	Avg	2.42 ± .75 (6)	25.1 ± 6.82 (5)	3.05 ± .72 (5)	15.7 ± 6.22 (5)

Period on experimental rations varied from one to 12 weeks. Enzyme activity determined as described in text. Variation expressed as standard deviation from the mean. Figures in parentheses represent the number of determinations.

gen consumption with DL-alanine as substrate. Each Warburg flask contained 1.5 cc of Krebs-Ringer phosphate buffer, pH 7.4, 1 cc of tissue homogenate equivalent to 200 mg of fresh liver or 100 mg of fresh kidney tissue, 0.5 cc of 0.3 M DL-alanine, and 0.2 cc of 5 N NaOH in the center well. *In vitro* additions of pyridoxine, pyridoxamine, pyridoxal, and pyridoxal phosphate were made at a level of 10-50 μg per flask. Flasks were incubated at 37°C, readings of oxygen uptake were made at ten minute intervals for a period of one hour. All solutions and homogenates were made in glass redistilled water. Endogenous uptake in the absence of alanine was determined in each case and values deducted from those with added substrate. All data shown are averages from duplicate flasks which consistently checked closely. Oxygen uptake per hour was calculated from the two highest values for 10 minute periods. Activity is expressed as QO_2 values, which indicate μl O_2/hour/mg dry weight of tissue.

For the metabolism studies, all animals were maintained on the above rations for 4 weeks; at this time their weights ranged from 56 to 77 g in the deficient and 69 to 83 g in the control lots. The animals were then transferred to "low protein" rations, identical with those above except that the blood fibrin was reduced to 6% to the advantage of the sucrose, and fed these rations until the conclusion of the experiment.

After one week on the low protein rations, animals were placed in individual wire metabolism cages equipped with glass funnels. Urine was collected in bottles containing sulfuric acid and toluene; feces were collected separately on wire screens. Collections were made daily. Nitrogen in both excrements was determined by the Kjeldahl method. Several days were allowed the animals for adjustment to new conditions before the start of the experiment; all animals were in positive nitrogen balance when supplemented.

Animals receiving B_6 were injected intraperitoneally with 200 μg of pyridoxine·HCl in aqueous solution twice within an interval of 18 hours. Seven hours after the final injections of B_6, supplements of D-amino acids, L-amino acids, or di-ammonium citrate were given to animals as indicated in Table II. Amino acid supplements were composed of molecular equivalent weights of the crystalline L or D isomers of leucine, isoleucine, phenylalanine, and valine in aqueous solution, adjusted to pH 7.2, and administered by stomach tube to supply exactly 24 mg of nitrogen per animal. Di-ammonium citrate solutions were administered similarly to supply 24 mg of nitrogen per animal. Gavage supplements totalling 4-6 cc of the solution were given in two doses within a 24 hour period. Nitrogen excretion was followed until levels had reached the basal level established before supplementation. In each case this was reached by the third day; data are therefore given for the two days following supplementation.

Results. Data for D-amino acid oxidase determinations are given in Table I. Initial observations indicated that D-amino acid oxidase activity of the B_6 deficient animals was considerably lower than that of the control animals (Series I). When the experiment was repeated using pair-fed controls, there was no significant difference between the deficient and control groups (Series II). The effect of *in vitro* additions of pyridoxine, pyridoxamine, pyridoxal, or pyridoxal phosphate,

TABLE II.
Effect of Vitamin B_6 on the Utilization of Amino Acids and Inorganic Nitrogen.

		B_6 deficient animals				Control animals			
	Supplement	*Total N ingested mg	N excreted mg	% N utilized	Avg % utilized	*Total N ingested mg	N excreted mg	% N utilized	Avg % utilized
I	None	126.8	53.3	58	56 ± 2.6	140.9	50.2	66	66
		122.8	58.6	52					
		112.3	48.1	57					
II	B_6 (400 μg)	151.7	67.2	56	65 ± 7.8	138.1	43.2	68	68
		205.5	51.3	75					
		154.3	55.7	64					
III	D-amino acids	92.1	80.2	12	21 ± 9.0	162.2	60.0	63	54 ± 6.8
		131.6	83.0	37		210.0	91.8	56	
		122.8	87.8	29		139.2	78.3	44	
		161.4	128.1	21		163.2	74.5	54	
		108.4	95.7	12					
		151.1	124.6	18					
IV	D-amino acids + B_6 (400 μg)	144.0	66.5	54	60 ± 5.5	165.0	56.7	66	66
		219.9	76.4	65					
V	L-amino acids	203.0	99.0	51	39 ± 10.5	177.6	92.2	48	47 ± 9.9
		116.2	80.2	32		159.2	104.8	34	
		138.2	74.0	46		147.0	78.8	46	
		122.9	92.6	25†		163.0	68.5	58	
VI	L-amino acids + B_6 (400 μg)	198.8	95.6	52	52	176.6	98.3	44	44
VII	$(NH_4)_2$ citrate	139.2	91.2	36	33 ± 4.9	152.6	88.2	42	47 ± 10.3
		119.1	79.9	33		152.6	96.0	37	
		138.2	103.8	25†		167.9	64.8	61	

* Summation of dietary N and N from supplement. All animals received supplements as indicated to supply 24 mg N/animal. Values represent totals of two days following supplementation; each series of values represents a separate animal. Variation expressed as standard deviation from the mean.

† Severe deficiencies resulting from a longer period on the depletion diet may have affected total nitrogen utilization in these animals.

with and without ATP, upon the activity of tissues from B_6 deficient animals was tested frequently, and consistently failed to result in any increase in D-amino acid oxidase activity. The determinations were repeated on a third series of animals at a later time with the same results. The function of certain vitamins as coenzymes for enzyme systems has been demonstrated frequently by a lowered enzyme activity in animals deficient in that vitamin. Thus D-amino acid oxidase activity is decreased in rats deficient in riboflavin(7), and a B_6 deficiency has been shown to result in decreased transaminase activity(4). We have been unable to demonstrate a relationship between D-amino acid oxidase and vitamin B_6 by this technic.

Results of nitrogen balance studies upon animals given D-amino acids by gavage supplementation are shown in Table II. The data indicate that utilization of dietary nitrogen by animals deficient in vitamin B_6 is less than that of the control animals. Injection of the deficient animals with pyridoxine raises the nitrogen utilization to the same level as that of the control groups. Upon administration of D-amino acids, the B_6 deficient animal excretes a much geater amount of total nitrogen than does an animal with an adequate supply of the vitamin. Excretion of the excess nitrogen appears to be almost entirely in the urine of the first day's collection; however, nitrogen excretion is not again "normal" until the third day, necessitating use of two day excretion data. Administration of D-amino acids to the B_6 deficient animal depresses the level of nitrogen utilization to about one third of the normal value (Group III). Injection of the animals with pyridoxine prior to administration of the D-amino acids (Group IV) raises the level of nitrogen utilized to that of the untreated controls (Group I).

To determine whether a specific relationship between vitamin B_6 and D-amino acids is involved, the same amounts of nitrogen were administered as the L-isomers of the previously used amino acids or in the inorganic form as di-ammonium citrate. The administration of nitrogen in either of these forms decreases the net nitrogen utilization of the B_6 deficient animal, while that of the control groups is not thus affected. However, the effect is considerably less than that noted upon supplementation with D-amino acids. The net nitrogen utilization of the B_6 deficient animal is lowered to 21% by the administration of D-amino acids, while administration of L-amino acids or di-ammonium citrate decreases utilization only to 39% or 33% respectively. Exceptionally low values for nitrogen utilization by B_6 deficient animals given D-amino acids result from the excretion of a large percentage of dietary nitrogen in addition to that furnished by the D-amino acids. In the case of supplements with L-amino acids or inorganic nitrogen, the difference between deficient and control animals can be accounted for almost entirely by total excretion of the supplementary nitrogen. It has been demonstrated that ammonium nitrogen can be utilized by the rat fed only essential amino acids(8). However, no net utilization of ammonium nitrogen occurs in rats fed complete proteins. The administration of D-amino acids to the B_6 deficient animal appears to result in the excretion of large amounts of dietary nitrogen utilized under ordinary conditions. That these effects are related specifically to a B_6 deficiency and not to the nitrogen supplementation alone is shown by the observation that nitrogen utilization of the control animals is not adversely affected, and that the effect is completely overcome by simultaneous administration of pyridoxine to the B_6 deficient rats.

Discussion. The data demonstrate that the administration of D-amino acids, L-amino acids, or di-ammonium citrate decreases the net nitrogen utilization of the B_6 deficient animal, and that simultaneous injection of pyridoxine completely overcomes this effect. Lowered utilization is most pronounced following the administration of D-amino acids, the utilization of nitrogen after feeding L-amino acids or di-ammonium citrate being affected to a lesser degree. B_6 has been shown to be intimately related to protein metabolism and is known to be involved specifically in

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the transaminase reaction and amino acid decarboxylation; blocking these reactions by a deficiency of B_6 would be expected to decrease the utilization of L-amino acids and ammonium citrate. A mechanism by which B_6 may affect the utilization of D-amino acids has not been demonstrated. The data indicate that the administration of D-amino acids to the B_6 deficient animal causes the excretion of a large amount of dietary nitrogen which is otherwise utilized. The mechanism by which the toxicity of D-serine, administered by stomach tube, is reduced by simultaneous administration of pyridoxine(9) may be related to this effect. Since the only demonstrated reaction for the metabolism of D-amino acids is through the action of D-amino acid oxidase, it is possible that a deficiency of B_6 decreases the utilization of D-amino acids by its effect upon this enzyme,

Summary. Homogenized kidney from rats deficient in vitamin B_6 exhibited only one third as much D-amino acid oxidase activity as did normal rat kidney homogenates. However, when the food intake of control animals was restricted to that of the B_6 deficient group there was no significant difference between the two groups in their kidney D-amino acid oxidase activity.

A gavage supplement of D-amino acids (leucine, isoleucine, phenylalanine and valine) to vitamin B_6 deficient rats greatly depresses dietary nitrogen utilization. Simultaneous injection of, or an adequate dietary supply of, vitamin B_6 completely overcomes this effect.

Supplements of the L-isomers of the same amino acids or an equivalent amount of inorganic nitrogen effected a smaller depression of dietary nitrogen utilization, thus pointing to a specific relationship between D-amino acid metabolism and vitamin B_6.

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Received November 1, 1949. P.S.E.B.M., 1950, v73.

Adrenal Function and Blood Electrolytes. (17612)

J. M. ROGOFF, JOSEPH M. QUASHNOCK, E. NOLA NIXON, AND ALAN W. ROSENBERG.

From the Laboratory of Experimental Endocrinology and the Department of Physiological Chemistry School of Medicine, University of Pittsburgh.*

Prevailing concepts attribute special physiological significance to the levels of the blood sodium and potassium in relation to steroid hormone function of the adrenal cortex. They are based on the observation that a reduction of sodium and increase of potassium occurs in the blood serum of adrenalectomized animals, and that administration of desoxycorticosterone or its acetate can effect a reversal of that change. It is maintained that this "corticoid" is a specific hormone of the adrenal cortex whose function is to regulate the balance between those electrolytes in the blood. The probability that loss of something other than any of the adrenal steroids might be responsible for the disturbed electrolyte levels in the blood of adrenalectomized animals has not been given serious attention. In view of the fact that adrenalectomy deprives an animal of function of the adrenal medulla as well as of alleged functions of cortical steroids, we undertook this investigation to determine if the physiological secretion of epinephrine might exercise an influence upon the blood sodium and potassium. A possible relationship between epinephrine action and serum potassium is suggested, also, from the results of various pharmacological investigations.

The demonstration by Rogoff and Stewart(1,2) that sodium chloride is diminished

* Supported by the G. N. Stewart Memorial Fund.

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