Liver Pyridine Nucleotide Synthesis in the Vitamin B_6-Deficient Rat.* (27230)

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The concentration of pyridine nucleotides in the liver of rats or mice increases greatly after injection of excessive amounts (2 mg/100 g body weight or more) of nicotinamide or nicotinic acid(1,2), but returns to preinjection levels approximately 24 hours after the injection(1). The physiological significance of the increase is unknown. It has been suggested(2,3), however, that the magnitude of the increase in liver pyridine nucleotides indicates the ability to form purines and ATP since this increase is accompanied by an increase in the adenine and organic phosphate ester content of the liver.

Purine synthesis requires glycine and formate(3). Any interference with the supply or transfer of these compounds could reduce adenine synthesis and possibly pyridine nucleotide synthesis after injection of nicotinamide or nicotinic acid. Vit. B_6 deficiency could conceivably impair purine synthesis by interfering with the production of glycine and formate and with the transfer of formate. Glycine formation from serine or from glyoxylic acid may be reduced in Vit. B_6 deficiency(4,5,6). Vit. B_6 deficiency has been reported to reduce concentrations of folic acid and citrovorum factor in rat liver(7).

Although Ling *et al.*(8) reported that Vit. B_6-deficient and Vit. B_6-supplemented rats injected with nicotinic acid showed similar increases in erythrocyte pyridine nucleotides, no observations have been made of the effect of Vit. B_6 deficiency on the synthesis of liver pyridine nucleotides after injection of nicotinic acid.

The experiments reported here tested the effect of Vit. B_6 deficiency on the synthesis of liver pyridine nucleotides at 1, 4, and 10 hours after injection of nicotinic acid or nicotinamide. After these experiments had been completed, Tulpule(9) reported lower concentrations of liver pyridine nucleotides in Vit. B_6-deficient rats than in control rats 10 hours after injection of nicotinamide.

Methods. Male rats of the Long-Evans strain, raised in our laboratory, were used in all the experiments. Their dams were fed a Vit. B_6-deficient diet† 1 week before weaning of the young to prevent them from obtaining Vit. B_6 from the dam's food. At weaning (3 wks. of age), the young rats, weighing from 45-60 g, were divided into deficient and control groups, caged separately, and fed a purified casein-sucrose diet.‡ Vit. B_6 deficiency was created by omission of pyridoxine from the supplement. The control groups received 50 μg of pyridoxine daily. Both pair-fed and *ad lib.*-fed control groups were used. The food intake of each rat in the pair-fed control group was restricted to the food intake of his littermate in the deficient group.

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† Composition, per 100 g diet: "vitamin-free" casein (Nutritional Biochemicals Corp.), 36.0; fat (Primex), 8.0; salts (U.S.P. 14, '50), 6.0; Vit. A, D, and E in cottonseed oil, 3.2; B Vit. in sucrose, 2.0; Powdered sucrose, 44.8. The following amounts of Vit. were supplied/100 g diet: Vit. A, 3200 I.U.; Vit. D_2, 282 I.U.; DL-alpha-tocopherol acetate, 16 mg; thiamine HCl, 0.9 mg; riboflavin, 0.9 mg; calcium D-pantothenate, 7.0 mg; niacin, 3.4 mg; D-biotin, 0.2 mg; folic acid, 0.2 mg; Vit. B_{12}, 4 μg; menadione, 1.0 mg.

‡ Composition, per 100 g diet: "vitamin-free" casein (Nutritional Biochemicals Corp.), 20.0; cottonseed oil, 5.0; salts (U.S.P. Salts 14, Nutritional Biochemicals Corp.), 4.0; choline bitartrate, 0.18; powdered sucrose, 70.82. Two ml of a 20% ethanol solution of B Vit. and menadione were fed 3 times weekly to provide the following intakes/rat/day: thiamine HCl, 40 μg; riboflavin, 40 μg; niacin, 170 μg; D-biotin 8.5 μg; folic acid, 8.5 μg; calcium D-pantothenate, 510 μg; Vit. B_{12}, 0.20 μg; menadione, 50 μg; pyridoxine (when supplied), 50 μg. Two drops of a solution of Vit. A, D, and E in cottonseed oil were fed 3 times weekly to provide 32 I.U. Vit. A, 3.0 I.U. Vit. D_2, and 162 μg DL-alpha-tocopherol acetate/rat/day.

The rats in the *ad lib.*-fed control group were usually littermates of the rats in the deficient group.

When Vit. B_6 deficiency was established, *i.e.*, when the rats ceased to gain (3-5 weeks after weaning), the deficient rats and their controls were injected intraperitoneally with solutions of nicotinic acid (5 mg/100 g body weight) or nicotinamide (50 mg/100 g body weight). Injection of this level of nicotinamide has been shown to produce a maximum increase in liver pyridine nucleotides(1). A lower level of nicotinic acid was used since Langan *et al.*(10) reported that concentrations of nicotinic acid greater than 5 mg/100 g body weight inhibited pyridine nucleotide synthesis. The solutions of nicotinic acid and nicotinamide, dissolved in 0.9% saline, were adjusted to pH 7 with NaOH.

The rats were killed by decapitation at 1, 4 or 10 hours after injection. Oxidized pyridine nucleotides were extracted by homogenization with 20 volumes of ice-cold 5% trichloroacetic acid and then measured by the alkali fluorescence method of Ciotti and Kaplan(11). Fluorescence was measured on a Farrand or Coleman photofluorometer equipped with thiamine filters (primary filter, 365 mμ; secondary filter, 430 mμ). Fluorescence readings were converted to μg of DPN§ by reference to a standard curve. Pyridine nucleotide concentrations were calculated as μg/g fresh liver since no significant differences were found between the deficient and control groups in percentages of water and fat in the liver. In all of the groups, the average value for liver water was 70-71%, and average value for liver fat was 4-5%.

Results. Pyridine nucleotide synthesis 10 hours after injection of nicotinamide or nicotinic acid. Initially, the concentrations of pyridine nucleotides were measured 10 hours after injection of nicotinamide or nicotinic acid. This time interval was selected since preliminary studies found, in agreement with the results of Kaplan *et al.*(1), that the highest levels of pyridine nucleotides in normal rats occurred from 8 to 12 hours after injection of nicotinamide. It was thought that any difference between the deficient and control rats in their ability to form pyridine nucleotides might be more evident at the time when the highest values would be expected.

After the injection of nicotinamide, the concentration of liver pyridine nucleotides was significantly less in the Vit. B_6-deficient group (2510 $\pm$ 149 μg/g) than in either control group (2930 $\pm$ 209 μg/g in the pair-fed group and 3040 $\pm$ 170 μg/g in the *ad lib.*-fed group). Pyridine nucleotide concentration in the Vit. B_6-deficient group injected with saline was also significantly lower (458 $\pm$ 16 μg/g) than the concentrations of the control groups injected with saline (542 $\pm$ 36 μg/g for the pair-fed group and 595 $\pm$ 39 μg/g for the *ad lib.*-fed group). If the net increase in pyridine nucleotides is estimated by subtracting the values for the saline-injected groups from the values for the nicotinamide-injected groups, this estimate also indicates that the level of pyridine nucleotides attained in the Vit. B_6-deficient group injected with nicotinamide was less than the levels reached in the control groups.

After the injection of nicotinic acid, the concentration of liver pyridine nucleotides was again significantly less in the Vit. B_6-deficient group (635 $\pm$ 19 μg/g) than in the pair-fed control group (1420 $\pm$ 49 μg/g) or in the *ad lib.*-fed control group (770 $\pm$ 27 μg/g). The estimated net increase in the Vit. B_6-deficient group was significantly less than in the pair-fed control group but did not differ significantly from the *ad lib.*-fed control group.

The difference in response of the control groups injected with nicotinic acid emphasizes the importance of differences in food intakes and feeding patterns in the reactions of control groups considered to represent the "normal" state(12). The feeding pattern of the pair-fed control group differed from the *ad lib.*-fed groups in that the pair-fed rats, fed once daily, ate all of their food within 15 minutes of feeding, in contrast to the other groups which had constant access to food. The difference between the control groups did not result from inanition, however, since the

§ DPN: diphosphopyridine nucleotide, Sigma Grade, Sigma Chemical Co.

TABLE I. Concentrations of Liver Pyridine Nucleotides in Vit. B_6-Deficient and Pair-Fed Control Rats at 1 and 4 Hr after Injection of Nicotinamide or Nicotinic Acid.

Treatment	Compound inj.	DPN, μg/g rat liver Hr after inj. 1	4
$-B_6$	Nicotinamide	640 ± 11* (10)†	1100 ± 53 (10)
$+B_6$, pair-fed	"	728 ± 55 (5)	1160 ± 38 (10)
$-B_6$	Nicotinic acid	724 ± 34 (9)	397 ± 69 (7)
$+B_6$, pair-fed	"	897 ± 62 (5)	655 ± 25 (7)
$-B_6$	Saline	—	369 ± 20 (6)
$+B_6$, pair-fed	"	—	430 ± 28 (6)

* Mean and stand. error of mean.
† Figure in parentheses is No. of rats/group.

pair-fed rats were fed just before injection of either nicotinic acid or nicotinamide.

Pyridine nucleotide synthesis 1 hour after injection of nicotinamide or nicotinic acid. The smaller increases in liver pyridine nucleotides in the deficient rats could reflect either a less rapid synthesis of pyridine nucleotides after injection or a more rapid loss of the nucleotides formed. To test the former possibility, Vit. B_6-deficient and pair-fed control rats were injected with nicotinic acid or nicotinamide, and liver pyridine nucleotides were determined at 1 and 4 hours after injection. The pair-fed groups were fed a short time before injection. The results are expressed in Table I.

One hour after the injection of nicotinic acid, the pyridine nucleotide concentration in the Vit. B_6-deficient group was significantly less than in the controls (724 ± 34 μg g *vs* 897 ± 62 μg/g). At 4 hours after injection of nicotinic acid, pyridine nucleotide concentration was again significantly less in the Vit. B_6-deficient group (397 ± 69 μg/g *vs* 655 ± 25 μg/g). Pyridine nucleotide concentrations in the deficient and control groups 4 hours after injection of saline were respectively, 369 ± 20 μg/g and 430 ± 28 μg/g. This difference was not significant. Thus, the pyridine nucleotide concentration of the deficient rats injected with nicotinic acid seemed to have returned to normal levels after 4 hours, although the levels of the control group were still significantly elevated.

The deficient and control groups injected with nicotinamide had similar pyridine nucleotide levels.

Discussion. The lower pyridine nucleotide values in the Vit. B_6-deficient rats at 1 and 4 hours after injection of nicotinic acid suggest that the deficient rats did not use nicotinic acid as efficiently for pyridine nucleotide synthesis as the controls. No differences appeared in utilization of nicotinamide, however, until 10 hours after injection.

The somewhat lower pyridine nucleotide levels in the deficient rats injected with saline suggest also that Vit. B_6 deficiency interferes with the utilization of dietary sources of nicotinic acid. This observation agrees with the results of Tulpule(9) and of Terroine and Adrian(13), who found that Vit. B_6 deficiency caused a 15% decrease in the concentration of nicotinic acid in the liver.

The lower values in the Vit. B_6-deficient groups may have resulted either from a decreased synthesis or an increased breakdown of the newly formed pyridine nucleotides. Possibly, the injection of glycine or adenine, together with nicotinic acid, might increase the synthesis of liver pyridine nucleotides in the Vit. B_6-deficient animals.

There is no obvious explanation for the finding that Vit. B_6 deficiency appeared to affect the utilization of nicotinic acid for pyridine nucleotide formation to a greater extent than the utilization of nicotinamide. Possibly the cellular absorption or excretion of nicotinic acid was more affected by Vit. B_6 deficiency than was the absorption or excretion of nicotinamide. Nicotinic acid is incorporated into pyridine nucleotides more rapidly than is an equivalent amount of nicotinamide(10), although, over a longer period, nicotinamide produces higher tissue concentrations of pyridine nucleotides(1).

Summary. Vit. B_6-deficient and pair-fed or *ad lib.*-fed control rats were injected with nicotinic acid or nicotinamide to determine the effect of Vit. B_6 deficiency on the ability of the rat to synthesize liver pyridine nucleotides. At 10 hours after injection of either

compound, liver pyridine nucleotide concentration was significantly lower in the Vit. B_6-deficient rats than in the control rats. Liver pyridine nucleotide values were also significantly lower in the deficient rats at 1 and 4 hours after injection of nicotinic acid but not after injection of nicotinamide.

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Chemotherapy of Experimental *Leishmania enriettii* Infection. (27231)

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L. enriettii, first described by Muniz and Medina, produces a muco-cutaneous leishmaniasis of guinea pig which is not transferable to other species(1). Muniz(1), Adler (2), Paraense(3) and Torres *et al.*(4) have described the biological and pathological characteristics of the organism.

Extending our studies(5) on treatment of the cutaneous leishmaniasis with *L. brasiliensis* responsible for the clinical condition known as *Leishmaniasis tegumentaria diffusa* (6), we have examined the effect of drug treatment on *L. enriettii* infection produced experimentally in the laboratory.

Materials and methods. The strain forthcoming from Dr. Julio Muniz, Instituto Oswaldo Cruz, was obtained by us in Jan. 1960 from Professor Enrique Tejera. For experimental infection, we injected guinea pigs intracutaneously with a suspension of infected tissue into the nasal region. In 115 guinea pigs, the lesions developed within 20 and 40 days after inoculation, except in 5 animals in which symptoms appeared after 10 days and in one, with delayed reaction (90 days). The foreleg also appeared a sensitive site for inoculation; 15 out of 17 pigs so infected developed wide lesions within 21 days, the remaining 2, within 52 days. Daily subcutaneous treatment, 6 times weekly, was given during the period indicated to the animals showing well developed lesions, except for Sodium-stibogluconate (Pentostam), which was injected intraperitoneally, twice daily. The animals were observed grossly and microscopically twice a week.

Results. The effect of therapy on regression of lesion and number of leishmania is contained in Table I. Fig. 1 and 2 illustrate the effects of drug action. It appears that Fuadin, given up to 18 to 21 times in doses of 300 mg/kg, remained ineffective, as did tartar emetic given 35-51 times in 15 mg/kg doses. No effect followed administration of maximal hydroxy-stilbamidine doses which could be administered repeatedly (25 mg/kg). Pentostam given 58 times in 25-50 mg/kg (i.p.) doses did not interfere with the progress of the infection. The only drug tested which showed a prompt effect in tol-