Vitamin B_6 in Blood, Urine, and Liver of Monkeys [1]

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ABSTRACT Blood and urinary vitamin B_6 and urinary 4-pyridoxic acid values are reported for 3 rhesus monkeys before and after a 5-day period of pyridoxol·HCl supplementation. Blood and liver vitamin B_6 values are given for a group of free-ranging howler monkeys. Microbiological procedures employing *Saccharomyces carlsbergensis* ATCC 9080, *Streptococcus faecium* Ø 51 NCDO 1229 and *Lactobacillus casei* ATCC 7469 were used for the vitamin B_6 analyses and a microprocedure of the lactone method was used for 4-pyridoxic acid determinations. Large increments of vitamin B_6 in blood and urine and increased excretion of 4-pyridoxic acid were observed in the rhesus monkeys following supplementation. Of significance was the prolonged elevation of values several days after supplementation had ceased. Blood values for the howler monkeys were higher than those for the rhesus. Differential assay of liver hydrolysates from howler monkeys revealed that most of the vitamin was present in the pyridoxamine form. A lesser amount was in the pyridoxal form and the amount of pyridoxol was negligible.

Previous reports on vitamin B_6 values for monkey blood include those of Greenberg and Rinehart (1), Marsh et al. (2), and Marquez.[3] Marquez also determined the vitamin B_6 content of monkey urine. All these studies were done on the rhesus monkey and the method of determination was microbiological assay employing the yeast *Saccharomyces carlsbergensis*. No information on vitamin B_6 values for the howler monkey is available. Data of any kind for this species are limited since efforts to maintain it in captivity have been largely unsuccessful.

Vitamin B_6 values are reported here for 2 species of monkey, the rhesus (*Macaca mulatta*) and the howler (*Alouatta caraya*). Blood, urine and liver samples were made available through collaboration with the Oregon Regional Primate Research Center. The effect of a period of vitamin B_6 supplementation was studied in rhesus monkeys. These animals were maintained in the controlled environment of the Primate Center and for this study blood and urinary vitamin B_6 and urinary 4-pyridoxic acid determinations were made before and after a period of pyridoxol·HCl supplementation. The howler monkeys were free-ranging in their native habitat of Argentina. Hence, for this species, vitamin B_6 determinations were made on blood and liver samples that had been obtained in the field and constituted part of a study to be reported elsewhere on the possible relationship between vitamin B_6 deficiency and the presence of coronary lesions (3).

For the studies reported here the same routine microbiological procedure of Atkin et al. (4) employing *S. carlsbergensis* ATCC 9080 was used. In addition, 2 other organisms, *Streptococcus faecium* Ø 51 NCDO 1229 and *Lactobacillus casei* ATCC 7469, provided a differential type assay based on that of Rabinowitz and Snell (5) and modified by Gregory (6). *S. carlsbergensis* responds to all 3 forms of the vitamin (pyridoxal, pyridoxol, and pyridoxamine). *S. faecium* responds to pyridoxal and pyridoxamine, and *L. casei* responds to pyridoxal. To our knowledge vitamin B_6 values for monkey blood, urine and liver based on the latter two vitamin

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[2] Chairman, Division of Cardiovascular Physiology, Oregon Regional Primate Research Center, Beaverton, Oregon.

[3] Marquez, L. R. 1955 Studies on the metabolism of vitamin B_6. Ph.D. Thesis, University of Wisconsin, Madison.

B_6-dependent assay organisms have not been reported.

The problems encountered in applying microbiological procedures to the determination of vitamin B_6 in blood have been discussed by Storvick and Peters (7) and by Haskell and Wallnöfer (8). Blood appears to contain growth-promoting, or inhibitory substances, or both, for the various organisms that invalidate use of the differential technique. Moreover, inhibition has been mentioned by Rabinowitz and Snell (5, 9) and by Short and Fairbairn (10) as a problem in *S. carlsbergensis* assays for the vitamin B_6 content of urine.

EXPERIMENTAL

Rhesus monkey study

Vitamin B_6 supplementation procedure. This experiment was designed to demonstrate the effect of an extended period of pyridoxol·HCl [4] supplementation on the vitamin B_6 content of blood and urine of 3 adult rhesus monkeys. These animals had been conditioned to restraining chairs and were fed a pelleted commercial diet [5] plus small amounts of apple and banana. The individual monkeys consumed approximately 200 g daily of the commercial diet, which by analysis with *S. carlsbergensis* contained approximately 7 μg of vitamin B_6/g of diet. Therefore, the estimated vitamin B_6 intake during the unsupplemented period was about 1.4 mg/day.

To establish base levels for the vitamin in blood and urine, control samples were obtained before the period of supplementation. For the 3 days preceding supplementation 24-hour collections of urine were made and peripheral blood samples were drawn 48 hours before supplementation.

During the period of supplementation the monkeys were fed, in addition to their regular diet, 20 mg of pyridoxol·HCl daily for 5 consecutive days. Blood was not taken and urine was not collected during this 5-day period.

Beginning 24 hours after the last supplement was given, daily urine collections were made for 3 consecutive days. Peripheral blood samples were drawn 48 hours after supplementation had ceased.

Preparation of the samples for analysis. Blood samples were oxalated and kept frozen until hydrolyzed before microbiological assay. The samples were protected from light as much as possible. Hydrolysis was done with 0.055 N HCl in the ratio of 1 ml of blood plus 20 ml of acid by autoclaving for 5 hours at 121°. After hydrolysis, the samples were adjusted to pH 7 with KOH and then back to pH 5.2 with HCl before adjustment of the volume and filtration through Whatman no. 50 filter paper. The filtrates were refrigerated overnight, autoclaved for 5 minutes at 121°, and refiltered to remove any precipitated protein. If not assayed immediately the samples were layered with benzene and refrigerated.

Urine collections were made under toluene in plastic bottles surrounded by ice and protected from light as much as possible. They were stored frozen until hydrolyzed before analysis. The urine samples were prepared for microbiological assay according to the method of Sauberlich.[6] A 10 ml aliquot of urine was autoclaved with 50 ml of 0.1 N HCl for 30 minutes at 121°. The sample was cooled, adjusted to pH 5.2 with KOH, and diluted to 100 ml before filtration through Whatman no. 1 filter paper. For the determination of urinary 4-pyridoxic acid the microprocedure of Woodring et al. (11) was used.

Howler monkey study

The monkeys were trapped on the islands opposite Bella Vista, Corrientes and transported to Buenos Aires. During captivity, water and food were provided ad libitum. Within 72 hours of their capture, blood and liver samples were obtained under thiopental sodium anesthesia.

Preparation of the samples for analysis. Blood samples were oxalated and frozen for air shipment to the Oregon Regional Primate Research Center. They remained frozen until hydrolyzed and assayed as described for the rhesus monkey samples.

[4] Hexa-betalin, pyridoxine·HCl, 100 mg/ml, Eli Lilly and Company, Indianapolis, Indiana.
[5] Purina Monkey Chow, Ralston Purina Company, St. Louis.
[6] Sauberlich, H. E., U.S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado, personal communication.

Samples weighing 3 to 4 g from each of 6 livers were received frozen for determination of the vitamin B_6 content. Approximately 1 g of each sample was homogenized in a portion of the 100 ml of 0.055 N HCl used for hydrolysis. The homogenates were hydrolyzed for 5 hours at 121°. The sample preparation was continued in the same manner as for blood, except that it was unnecessary to heat and refilter the hydrolysate following refrigeration overnight since no additional protein precipitation took place upon heating.

RESULTS AND DISCUSSION

Rhesus monkeys. Response to a vitamin B_6 supplement. The limitations of the 3 organisms, utilizing the differential technique with regard to the assay of blood, have been pointed out (7, 8). Therefore, it must be recognized that vitamin B_6 values for blood, particularly for samples drawn before supplementation, are probably not valid due to the very small amount of vitamin B_6 present in relationship to interfering substances. All 3 assay organisms, however, were able to measure the response of the blood to vitamin B_6 supplementation, since large increments of the vitamin were found in blood following supplementation (table 1). The results showed that a high proportion of the vitamin was still in the form of pyridoxol. A significant feature was the time of sampling at which these high values were obtained, namely, 48 hours after supplementation had ceased. In studies reported by Marquez [3] on humans there was a rapid return to normal levels of vitamin B_6 in blood when a single 100 mg test dose of pyridoxol was given. On the other hand Marsh et al. (2) studied both humans and rhesus monkeys receiving pyridoxine supplements over extended periods of time. Elevation of blood vitamin B_6 continued for some time after supplementation had ceased. These investigators suggested that return to initial levels following supplementation depends to some extent upon the duration of the period of increased intake. The prolonged high levels of vitamin B_6 in blood found in our study may be due to the sustained 5-day period of supplementation as opposed to a single test dose. They may suggest that the laboratory diet, although adequate for prevention of clinical vitamin B_6 deficiency symptoms, did not provide for tissue saturation.

The literature indicates that the requirement for vitamin B_6 in all species studied including the monkey is still controversial. It varies with a number of factors, including the type of diet and the maturity of the animals, and must be reported in terms of the criteria of adequacy used. The requirement for rhesus monkeys indicated by Rinehart and Greenberg (12) of 50 μg/kg of body weight is frequently cited if optimum growth and freedom from obvious clinical lesions are the criteria employed. Based on the same parameters a somewhat higher requirement, namely 1 to 2 mg/day, has been suggested by Marquez [3] and by Emerson et al. (13). When biochemical parameters such as maximum enzyme levels are used as the

TABLE 1

Vitamin B_6 in blood of rhesus monkeys before and after supplementation with pyridoxol·HCl (calculated from pyridoxal·HCl standard curves)

Monkey no.	Weight	Day sample drawn [1]	*S. carlsbergensis*	*S. faecium*	*L. casei*
	kg		*μg/100 ml*	*μg/100 ml*	*μg/100 ml*
339	3.4	2	1.77	22.72	37.84
		10	392.21	153.24	148.56
1385	3.8	2	1.63	19.22	34.25
		10	198.98	156.52	157.68
1481	6.9	2	5.13	19.98	40.38
		10	109.20	76.91	104.48

[1] Day 2 represents 48 hours before vitamin B_6 supplementation was initiated. Day 10 represents 48 hours after vitamin B_6 supplementation was discontinued.

indexes of adequacy, however, the intake needed to achieve such levels may be even higher. For example, Marsh et al. (2) have shown that 4 mg/day are necessary to maintain maximum transaminase levels in the blood of rhesus monkeys. Whether or not maximum enzyme levels are necessarily optimal has not yet been established.

In the study reported here, the only criteria of adequacy were apparently normal growth and the absence of obvious clinical lesions. However, the blood values as measured by *S. carlsbergensis* for the period before supplementation approximate those found by Marquez [3] for monkeys on what she considered a less than optimum intake of 0.5 mg/day. It may be that the 1.4 mg/day provided by the laboratory diet at the Oregon Regional Primate Research Center was still only marginal for these monkeys. Certainly the continued elevation of blood levels after supplementation had ceased, together with the considerably delayed urinary excretion of the vitamin, indicates that the monkeys had the capacity to retain larger amounts of the vitamin than were provided by the regular laboratory diet. Whether this represents an adaptation by the organism to larger amounts of the vitamin or whether it can be related to the achievement of tissue saturation remains speculative.

Table 2 shows the urinary vitamin B_6 and 4-pyridoxic acid levels before and after supplementation. The assay values obtained by *S. carlsbergensis* on the control samples were somewhat lower than those obtained by *S. faecium*. This type of problem has been reported in assays on human urine by Rabinowitz and Snell (5, 9). They suggested that it was due to the presence of materials toxic to the yeast which suppressed its response to vitamin B_6. Nevertheless, *S. carlsbergensis* has been the organism of choice in all studies to determine vitamin B_6 require-

TABLE 2

Vitamin B_6 and 4-pyridoxic acid in urine of rhesus monkeys

Monkey no.	Day	Pyridoxal·HCl supplement	Urine volume	Vitamin B_6 (calculated from pyridoxal·HCl standard curves)			4-Pyridoxic acid
				S. carlsbergensis	*S. faecium*	*L. casei*	
		mg/day	*ml/24 hr*	*mg/24 hr*	*mg/24 hr*	*mg/24 hr*	*mg/24 hr*
339	1	0	480	0.024	0.036	0.017	0.358
	2	0	340	0.071	0.103	0.044	0.270
	3	0	100	0.023	0.030	0.015	0.330
				(0.039) [1]	(0.056)	(0.025)	(0.319)
	4–8	20 (no collections made)					
	9	0	90	4.01	2.56	1.62	8.60
	10	0	378	19.05	9.50	6.67	9.15
	11	0	210	13.60	3.23	1.99	8.50
				(12.22)	(5.10)	(3.43)	(8.75)
1385	1	0	400	0.069	0.106	0.044	0.220
	2	0	130	0.009	0.017	0.006	0.175
	3	0	374	0.017	0.025	0.007	0.210
				(0.032)	(0.049)	(0.019)	(0.202)
	4–8	20 (no collections made)					
	9	0	210	13.08	5.96	3.67	5.00
	10	0	268	16.42	7.10	4.86	6.10
	11	0	326	11.90	9.42	6.51	4.70
				(13.80)	(7.49)	(5.01)	(5.27)
1481	1	0	405	0.025	0.043	0.019	0.310
	2	0	216	0.068	0.132	0.067	0.330
	3	0	175	0.058	0.104	0.057	0.295
				(0.050)	(0.093)	(0.048)	(0.312)
	4–8	20 (no collections made)					
	9	0	755	19.25	9.51	6.44	7.85
	10	0	392	6.86	4.59	3.06	5.55
	11	0	273	8.97	5.07	3.58	7.55
				(11.69)	(6.39)	(4.36)	(6.98)

[1] Numbers in parentheses indicate averages for 3 days.

ments. In this study the urine values obtained by the yeast for the control samples approximate those reported by Marquez [3] for rhesus monkeys on a pyridoxol·HCl intake of 0.5 mg/day. With regard to urinary vitamin B_6 values following supplementation, all 3 organisms measured large increments of vitamin B_6. The *S. carlsbergensis* assays revealed that there was considerable excretion of the vitamin as pyridoxol. It seems likely that this pyridoxol represents excess intake and probably was not involved coenzymatically. However, there was also a marked increase in the excretion of 4-pyridoxic acid in the urine following supplementation, indicating considerable conversion of some of the extradietary pyridoxol to the metabolite. These results show that a prolonged elevation of urinary vitamin B_6 occurs with a sustained period of pyridoxol·HCl supplementation and corroborate the observations for blood. Somewhat analogous findings on the delayed elimination of pyridoxol supplements in humans and some interesting hypotheses to explain these phenomena have been made by Johansson et al. (14).

It is hoped that in future studies samples might be analyzed at close intervals both during and after supplementation, and that sampling might be continued long enough to allow the blood and urinary vitamin B_6 levels to return to those of the presupplementation state.

Howler monkeys. Vitamin B_6 content of blood and liver. The vitamin B_6 content of the blood of 19 free-ranging howler monkeys is shown in table 3. With one exception, the values obtained by the *S. carlsbergensis* assay of the blood ranged between 4 and 10 μg/100 ml. These values are higher than those found in the study on rhesus monkeys reported here, but are similar to values found for rhesus monkeys by Greenberg and Rinehart (1).

The livers of 6 of these monkeys were assayed for vitamin B_6 (table 4). Significant was the fact that the differential assay technique could be applied to liver hydrolysates. It is possible that the inhibitory or stimulatory substances, or both, that interfere with such assays for blood do not exist in liver. A more logical explanation, however, may be based on the observation that the vitamin B_6 content of liver is much higher than that of blood. This higher concentration requires considerable dilution before assay which may result in diluting out the interfering substances. The differential technique reveals that most of the vitamin in liver hydrolysates is present in the pyridoxamine form. A lesser amount is in the pyridoxal form and the amount of pyridoxol is negligible.

TABLE 3

Vitamin B_6 in blood of howler monkeys (calculated from pyridoxal·HCl standard curves)

Monkey no.	*S. carlsbergensis*	*S. faecium*	*L. casei*
	μg/100 ml	*μg/100 ml*	*μg/100 ml*
32	5.37	38.50	39.38
33	1.74	45.54	39.16
34	7.13	37.40	42.68
35	4.05	36.08	34.98
36	6.25	38.94	37.18
37	9.90	50.82	42.90
38	8.10	46.42	32.56
39	5.32	48.18	41.14
40	8.71	52.14	44.44
41	7.70	41.58	39.38
42	7.30	46.64	41.36
43	6.42	45.32	47.74
44	6.69	45.98	45.76
45	8.32	49.06	51.48
46	8.40	50.16	39.38
48	7.96	41.36	42.68
49	5.94	45.76	41.80
50	6.12	46.86	39.60
51	6.64	49.94	42.68

TABLE 4

Vitamin B_6 in liver of howler monkeys

Liver no.	Pyridoxal and pyridoxamine content mixture standard [1]		Pyridoxal content pyridoxal·HCl standard
	S. carlsbergensis	*S. faecium*	*L. casei*
	values expressed as μg/g of wet wt		
33	9.25	8.55	2.62
40	13.44	12.96	3.59
45	13.36	15.64	4.12
46	14.68	15.76	4.44
50	13.72	15.84	4.17
51	14.24	16.52	3.83

[1] The composition of this standard (30% pyridoxal·HCl and 70% pyridoxamine·2HCl) was determined by application of differential calculations to a preliminary assay and denotes approximate ratio of pyridoxal to pyridoxamine in the liver hydrolysates.

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