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Studies on the Blood Pyridoxine of Vitamin B_6 Deficient Monkeys.*

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Past experience has shown that in general the vitamin content of the blood is influenced by the quantity in the diet. In the case of vitamin C, thiamin and pantothenic acid, it is possible to use the blood level as a criterion of the adequacy of the intake. When this work was commenced, no reports on the concentration of the pyridoxine in blood of any species were to be found in the literature. Since then Ritchey *et al.*[1] using an assay with Neurospora have reported a value of 42 μg per 100 ml for pooled mouse blood. Subsequently Ray and co-workers[2] using a modification of the yeast method of Atkins and associates,[3] presented data on the blood pyridoxine content of control and cobalt deficient sheep. Employing a modification of the yeast method of Atkins and associates, our laboratory has carried out a rather extensive study of the blood pyridoxine in monkeys and humans and has studied its distribution in plasma and erythrocytes.

The present paper deals with the blood pyridoxine levels in monkeys and its alteration during deficiency of this vitamin. To date observations have been made on 14 deficient monkeys. A few of our early results were presented along with a description of the early manifestations of pyridoxine deficiency at the Federation meetings in 1946.[4] Since then our study has been greatly extended.

Experimental. Young rhesus monkeys weighing between 1.8 and 3.0 kg were used in these experiments. The methods employed in handling and feeding the animals have been described in an earlier publication.[5] The basal diet was a modified M-3 diet[6] and contained powdered sucrose 73, vitamin-free casein (GBI or Labco) 18, Hawk and Oser salt mixture 4, and corn oil 2. It was compressed into 2 g tablets on a Stokes tablet machine following granulation and the addition of 1% calcium stearate as a lubricant. The tablets were fed *ad libitum*. A vitamin tablet containing the following was fed daily; Nicotinic acid 5 mg, riboflavin 1 mg, thiamin chloride 0.5 mg, calcium pantothenate 3 mg, choline dihydrogen citrate 100 mg, para-aminobenzoic acid 100 mg, inositol 100 mg, ascorbic acid 25 mg, plus sufficient powdered sugar to make a tablet weighing 1.5 g. In addition the monkeys received by mouth twice weekly 5 drops vitamin A and D concentrate (100,000 I. U. vitamin A and 10,000 I. U. vitamin D per g), 385 μg† pteroylglutamic

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1 Ritchey, M. G., Wicks, L. F., and Tatum, E. L., *J. Biol. Chem.*, 1947, **171**, 51.

2 Ray, S. N., Weir, W. C., Pope, A. L., and Phillips, P. H., *J. Nutrition*, 1947, **34**, 595.

3 Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Indust. and Engr. Chem. Anal. Ed.*, 1943, **15**, 141.

4 Greenberg, L. D., and Rinehart, J. F., *Fed. Proc.*, 1946, **5**, 222.

5 Rinehart, J. F., Greenberg, L. D., and Ginzton, L. L., *Blood*, 1948, in press.

6 Waisman, H. A., and McCall, K. B., *Arch. Biochem.*, 1944, **4**, 265.

† Two of the monkeys were started on one-half this intake of pteroylglutamic acid. This is equivalent to a daily dose of 55 μg and was originally reported to be adequate by Totter and associates.[10] Dr. Totter had later informed us that this intake was not adequate, so we increased it to 110 μg per day and we have found this intake to be adequate.

10 Totter, J. R., Shukers, C. F., Kolson, J., Minus, V., and Day, P. L., *J. Biol. Chem.*, 1944, **152**, 147.

acid, 10 micrograms biotin and 5 drops of mixed natural tocopherols (Nopco) once a week. Control monkeys were also provided with 3.5 mg of pyridoxine hydrochloride twice a week.

During the course of these studies careful records of the semi-weekly weights and of the daily food consumption were kept. Generally, blood was taken by venipuncture at weekly or bi-weekly intervals and used for total blood counts, for serum iron and pyridoxine analyses. At autopsy, portions of several tissues were removed for vitamin assay. The monkeys were usually placed on the diet with complete supplements for 2 to 4 weeks before withdrawal of the pyridoxine, in order to perform control tests so that each animal might serve as his own control. In addition to studies of the pyridoxine levels of the blood, periodic examinations of the metabolism of tryptophane and of carbohydrate were undertaken. Since this paper is to be confined to the studies on the blood pyridoxine levels of the monkeys, it will suffice at present to merely mention the fact that in the pyridoxine deficient monkey, as in the pyridoxine deficient dog,[7] rat,[8] and pig[9] there is a derangement of tryptophane metabolism, resulting in the excretion of xanthurenic acid.

Neurospora assay was found to lack sufficient sensitivity for our work, so we finally settled on the method of Atkins *et al.*[3] with slight modifications. The procedure for the extraction of the vitamin B_6 consisted in adding one volume of blood or plasma to 18 volumes of 0.055N H_2SO_4 (1 ml of 10N H_2SO_4 to 179 cc of H_2O) and autoclaving for 1 hour at 20 lb pressure. After cooling, the pH of the mixture was adjusted to 5.2 and the volume was made up to 25 to 30 times that of the blood used. The precipitated proteins were removed by centrifugation and the supernatant fluid was decanted. Since assay tubes containing the extracts developed turbidity following sterilization as a result of additional precipitation of protein or protein cleavage products, it was found necessary to reheat the extracts either in the autoclave for a period of 5-10 minutes at 15 lb pressure or in flowing steam for 10 minutes and to recentrifuge them. In this manner development of turbidity in the assay tubes could be avoided. The extract was now ready for testing. If the extracts were to be stored they were given a short period of sterilization and kept in the refrigerator until it was convenient to assay them. Because of the destructive effects of light, precautions were taken to keep the exposure to light at a minimum during the preparation and handling of the extracts.

As an alternate method of extraction of the vitamin we have employed takadiastase digestion in a manner similar to that described by Luckey and coworkers[11] for the extraction of pteroylglutamic acid. This has yielded, with few exceptions, substantially the same values as acid extraction. Treatment of the two sets of values by a standard statistical method showed that there was no statistically reliable difference between the data obtained with the two extraction procedures.

The growth of the test micro-organism, *Saccharomyces carlsbergensis* No. 4228, the preparation of the inoculum and the assay procedure were essentially similar to that described in the original method. A minor modification consisted in the incubation of cultures and assay tubes at room temperature (ca 25-26°) instead of at 30°. The extracts were generally assayed at levels of 2, 3 and 4 ml and growth was measured turbidimetrically with the Evelyn colorimeter using filter 620 after 16 and 18 hours of continuous agitation on a Kahn shaker. Since it was found that little was to be gained by the second set of readings, this was discontinued in many of our later analyses. Owing to the fact that extracts of blood contain some color, it was found advisable to take blank readings on the

[7] Axelrod, H. E., Morgan, A. F., and Lepkovsky, S., *J. Biol. Chem.*, 1945, **160**, 155.

[8] Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J., *J. Biol. Chem.*, 1943, **149**, 195.

[9] Cartwright, G. E., Wintrobe, M. M., Jones, P., Lawritsen, M., and Humphreys, S., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 35.

[11] Luckey, T. D., Briggs, G. M., and Elvehjem, C. A., *J. Biol. Chem.*, 1944, **152**, 157.

assay tubes immediately following inoculation with the yeast.

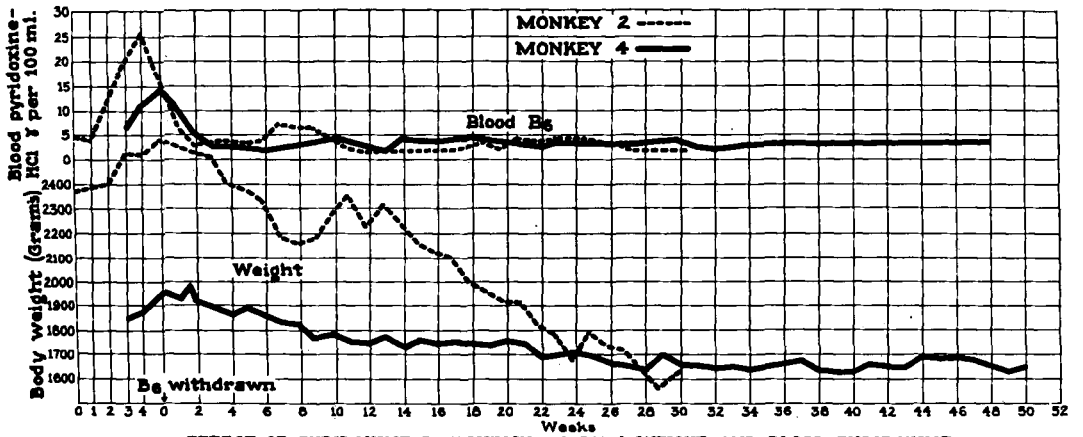
Results. Following control observations on the experimental animals, pyridoxine was withdrawn from the diet and within 2 to 4 weeks after withholding the vitamin the monkeys ate less food and began to lose weight slowly and progressively. In most cases this weight loss continued throughout the experiment. Aside from decreased food consumption, loss in weight and some loss in vigor, the animals showed little change in outward appearance until approximately 6 to 9 months after pyridoxine deprivation when they became unkempt, sluggish in their movements and showed some signs of being hyperirritable. In addition they generally developed edema around the eyes. Similar observations have been made by McCall *et al.*[12] in the monkey. Most of the animals have shown changes in their hair and these have varied considerably from animal to animal. These alterations have consisted of thinning of the hair in some monkeys, of patches of baldness in others, of extensive loss of hair in others, while others have exhibited little change in the appearance of the coat except slight greying. The majority of the monkeys studied have usually developed fissuring and cracking of the epidermis of the hands after some 3 to 6 months of the dietary regime. A constant finding has been the development of a slowly progressive anemia. Details of this will be published later.

During the course of the experiment the pyridoxine content of the blood was followed. It was found that when monkeys were first brought into the laboratory their blood levels were usually below 5 μg per 100 cc of whole blood, but after having been on the diet with complete supplements for 2 to 3 weeks the levels increased to values ranging from 5 to 25 μg per 100 cc. Following withdrawal of the vitamin it was a matter of but a week or two until the pyridoxine of the blood had decreased to values below 5 μg, and 3 to 4 weeks following the time of withdrawal of the vitamin the levels had decreased to the neighborhood of 2 to 3 μg. As the deficiency progressed the values remained in the same range or declined still further except for a few unexplainable fluctuations. Frequently, in long-standing deficiencies we have observed values of the order of 1 μg. Two typical cases which are representative of the alterations occurring in the weight curve and in the pyridoxine content of the blood deficiency are represented graphically in Fig. I. The pyridoxine level of the blood is affected very early and it exhibits a fall before there is any appreciable loss in weight. Fig. 2 and 3 are examples of the dramatic response in the weight, food consumption and pyridoxine levels of the blood of two long-standing pyridoxine deficient monkeys following the administration of the vitamin. Monkey 3 (Fig. 2) received 3.5 mg of pyridoxine hydrochloride twice a week while monkey 1 (Fig. 3) received a supplement of 1 mg daily. The increase in weight following the administration of the vitamin was phenomenal. In addition to the functions represented in the graphs there was a return to normal of all the observed abnormalities such as blood picture, etc. Fig. 2 also shows the fall in the pyridoxine level of the blood following the withholding of the vitamin from the diet for a second time. In this instance also the fall is rapid.

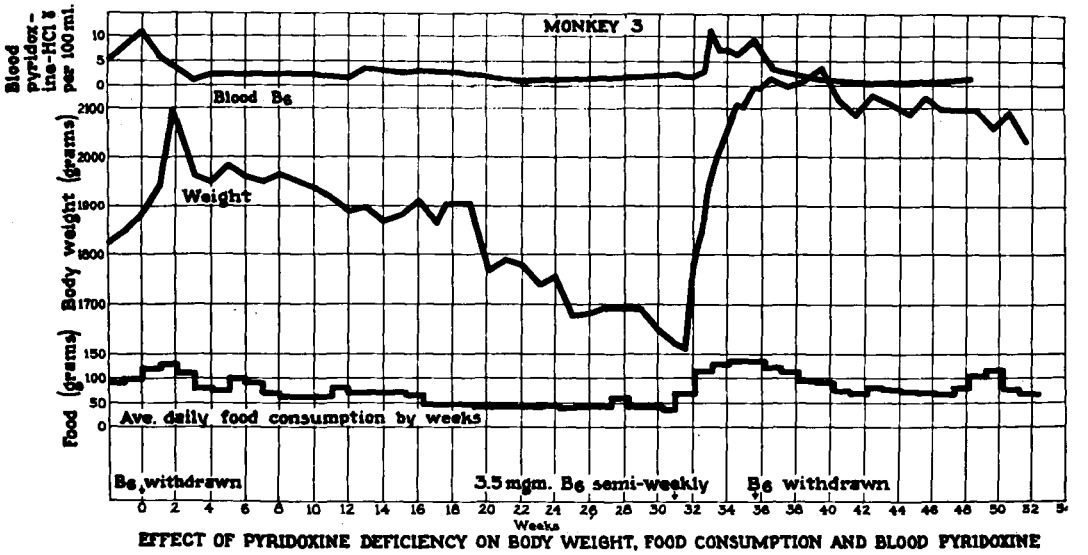
For purposes of comparison the pyridoxine levels of 2 control monkeys are given in Fig. 4. Although there is considerable fluctuation in the blood pyridoxine values, it can be seen that in no case have the values fallen below 5 μg per 100 ml. In a majority of the cases the values have been above 10 μg. In the cases of some rapidly growing monkeys which we have been studying recently we found it necessary to double the pyridoxine intake in order to bring their blood pyridoxine levels above 5 μg.

The blood pyridoxine levels of 2 monkeys (A.A. deficient 3360 and 3397) which had been followed before and after deprivation of ascorbic acid are also represented in Fig. 4. The diet and supplements were the same as used for control animals with exception that

[12] McCall, K. B., Waisman, H. A., Elvehjem, C. A., and Jones, E. S., *J. Nutrition*, 1946, **31**, 685.



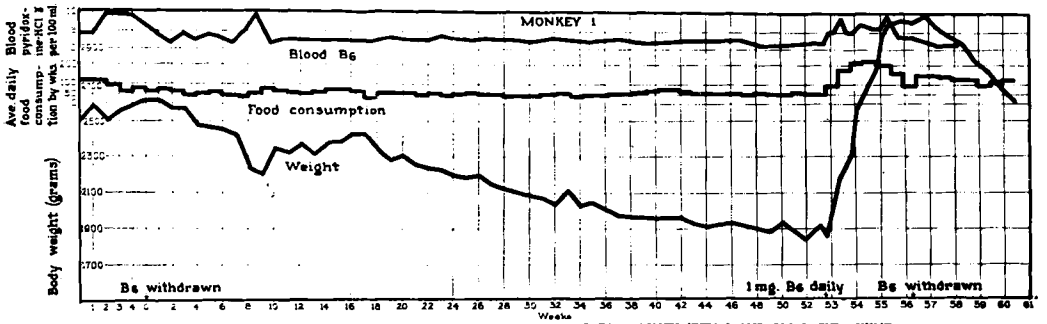
EFFECT OF PYRIDOXINE DEFICIENCY ON BODY WEIGHT AND BLOOD PYRIDOXINE
FIG. 1.



EFFECT OF PYRIDOXINE DEFICIENCY ON BODY WEIGHT, FOOD CONSUMPTION AND BLOOD PYRIDOXINE
FIG. 2.

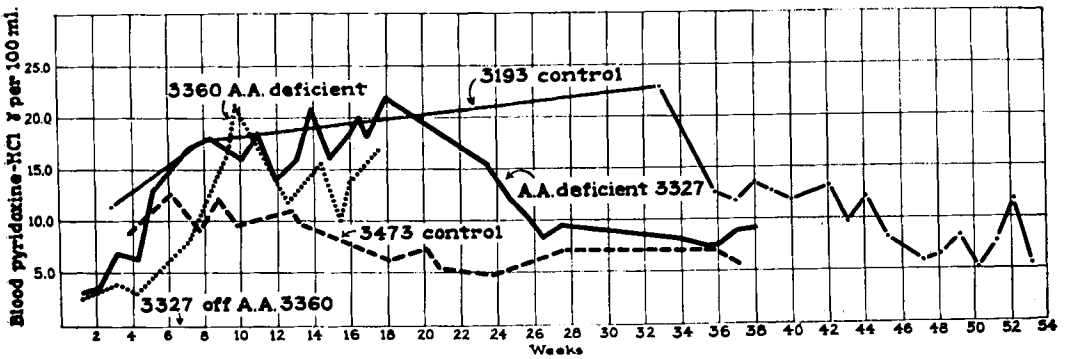
after a period of 44 days ascorbic acid was withdrawn in connection with some other work. The curves are of the same general type as is found in our long-standing control animal (3193). It is significant that when these animals were first brought to the laboratory and placed upon the experimental diet with complete supplements, their blood pyridoxine levels were in the range of approximately 2.5 to 3.0 μg per 100 ml. During the course of 4 to 6 weeks on the diet the pyridoxine values had risen considerably above 5 μg to a range of between 8 and 18 μg. Similar observations have been made with several other monkeys.

In addition to the studies on the pyridoxine of whole blood, the distribution of the vitamin in plasma and red cells has been investigated in several instances. The results which have been obtained to date are summarized in Table I and these show that the vitamin is present in both plasma and erythrocytes. The data in the last 2 columns show that the actual blood pyridoxine concentration is in good agreement with the values calculated from the packed cell-volume, and the plasma



EFFECT OF PYRIDOXINE DEFICIENCY ON BODY WEIGHT, FOOD CONSUMPTION AND BLOOD PYRIDOXINE

FIG. 3.



BLOOD PYRIDOXINE CURVES

FIG. 4.

and erythrocyte concentrations of the vitamin. In monkeys on a good intake the plasma generally shows a higher concentration than the erythrocytes, but in deficient monkeys the values approach nearly equal distribution in plasma and red cells. Alterations in intake appear to influence the plasma concentration more rapidly than the erythrocyte concentration of the vitamin. The distribution of pyridoxine in the blood differs from that of thiamin, since it has been shown that approximately 90 per cent of the latter is located in the red cells. On the other hand there is a great similarity in the distribution of pyridoxine and pantothenic acid in the blood, for Pearson[13] has shown that in all the animals studied, with the exception of man, the plasma had a greater concentration of pantothenic acid than the red cells.

Discussion. The blood pyridoxine values observed are somewhat similar to those found for thiamin in the monkey.[5] The former may reach values somewhat higher than is found with thiamin, when the intakes of the 2 vitamins are of about the same order.

The average value of 42 μg per 100 ml of whole blood obtained upon mice by Ritchey *et al.*[1] is approximately twice as high as the highest values observed in our experiments. On the other hand, the results obtained by Ray and co-workers[2] on sheep are more nearly in the range of values observed by us in the monkey. They obtained an average value of 11.8 μg of pyridoxine per 100 ml of whole blood on control sheep. However, the vitamin B_6 intake of the sheep was not reported. The values on 2 control monkeys have ranged between 5.0 and 20.7 μg and have averaged 11.2 μg per 100 ml for 35 serial determinations. The average of 36 assays on the 2 monkeys used in the ascorbic acid deficiency experiment is 13.6 μg and the range is 6.1 to 21.9 μg per 100 ml of whole blood.

At present we have no adequate explanation for the wide fluctuation in the vitamin

[13] Pearson, P. B., *J. Biol. Chem.*, 1941, **140**, 423.

TABLE I.
Distribution of Pyridoxine in Blood (μg pyridoxine-hydrochloride per 100 ml).

Monkey and specimen No.	Plasma pyridoxine (1)	Red cell pyridoxine (2)	Packed-cell vol. (3)	Whole blood pyridoxine	
				observed	calculated*
3327	8.9	21.1	44	13.7	14.3
6 —a	8.4	4.6	44	5.6	6.7
—b	2.5	3.9	42	3.3	3.1
8 —a	18.2	4.7	41	11.6	12.6
—b	15.3	5.7	41	9.9	11.4
—c	10.1	4.0	42	6.2	7.5
3473	5.5	5.7	47	5.4	5.6
3193 —a	10.6	4.4	42	9.4	8.0
—b	8.3	4.4	40	7.8	6.7
—c	8.0	4.0	37	6.8	6.5
—d	2.6	3.8	37	3.4	3.0
3 D†	1.9	—	—	1.9	—
4 D	2.2	1.8	41	2.3	2.0
1 D	1.2	1.8	42	1.6	1.4
11 D	2.4	3.9	44	2.9	3.1

* Calculated from columns 1, 2, and 3.
† D—Pyridoxine Deficient.

B_6 values of the animals receiving pyridoxine, except to say that it does not appear to be a question of technic, since duplicate assays yield values in close agreement. In view of the fact that Ray *et al.*[2] have shown that the blood pyridoxine levels are lowered in cobalt deficient sheep, it is possible that this fact may have some bearing on the lower values obtained in the 2 monkeys (No. 3327 and No. 3193) after a lapse of some 6 to 8 months. However, this seems rather unlikely since so far only ruminants have been shown to be susceptible to cobalt deficiency. Since the vitamin B_6 intake remained constant, the decrease in the blood pyridoxine concentration would most likely be attributable to the smaller intake of the vitamin on a weight basis resulting from the great increase in size of the animal.

Summary. Suitable modifications for the extraction and assay of blood pyridoxine are described. The vitamin B_6 levels of 14 rhesus monkeys were followed before and after withdrawal of the vitamin from the diet. The vitamin B_6 levels fell during the first 2 weeks of deprivation. This usually brought the levels down to values of 2-3 μg per 100 ml of whole blood or lower, where they remained throughout the rest of the experiment for periods exceeding 1 year. Control animals on a daily intake of equivalent to 1 mg of pyridoxine hydrochloric acid had values ranging from 5.0 to 20.8 μg, averaging 11.2. Some of the changes observed in deficient animals are discussed.