Experimental Production of Pyridoxine Deficiency Anemia in Rats

By Shu Chu Shen, Peter Y. C. Wong and Massao Oguro

A microcytic hypochromic anemia induced by a diet deficient in pyridoxine is well documented in swine, monkeys, dogs, cats, ducks, and chicks. In man, pyridoxine deficiency anemia was first described in 1956 by Harris et al.; subsequently an increasing number of cases have been reported by others. In 1953, Synderman et al. produced pyridoxine deficiency in two infants with cerebral defects by means of a pyridoxine-deficient diet; one of them developed microcytic anemia after 160 days. A similar anemia in the rat has not been clearly demonstrated. Since rats are sensitive to pyridoxine deficiency and on diets deficient in this substance develop regularly the other classical signs of pyridoxine deficiency, it seems improbable that they were resistant to the anemia found in other species. The present study demonstrated that a well-defined microcytic hypochromic anemia, responding promptly to pyridoxine administration, can be produced consistently in the rat by a pyridoxine-deficient diet.

Method

Sixty male Wistar rats, 250 to 380 Gm., were fed a diet composed of 18 per cent vitamin-free casein, 68 per cent glucose, 10 per cent vegetable oil (cotton seed oil), 4 per cent salt mixture (USP No. 2). To 100 pounds of a vitamin-free diet preparation was added 1 Kg. of Vitamin Diet Fortification Mixture triturated in dextrose containing a complete supplement of vitamins with the exception of pyridoxine. The resultant diet contained about 222 mg. of iron per Kg. of diet and the vitamins listed here in mg. per Kg. of diet:

- Vitamin A concentrate (200,000 units per Gm.) 99.00
- Vitamin D concentrate (400,000 units per Gm.) 5.50
- Alpha tocopherol 110.00
- Ascorbic acid 990.00
- Inositol 110.00
- Choline chloride 1650.00
- Menadione 49.50
- P-aminobenzoic acid 110.00
- Niacin 99.00
- Riboflavin 22.00
- Thiamine hydrochloride 22.00
- Calcium pentothenate 66.20
- Biotin .44
- Folic acid 1.98
- Vitamin B₁₂ 0.29

This diet was fed for about a year. At each interval of approximately 10 weeks, 10 animals...
were selected at random from a pool of rats during the course of the pyridoxine-deficient diet. From each of these animals 4 ml. of blood was drawn by cardiac puncture for the determination of red cell counts, hematocrit, reticulocyte and both serum iron and iron-binding capacity. The first stage (without tryptophane) and second stage (with d-l-tryptophane, 50 mg. by gastric catheter) of the tryptophane loading test for measuring 24-hour urinary excretion of xanthurenic acid were carried out approximately every 4 weeks after the inception of the experimental diet (the third stage being omitted because the rats were on the pyridoxine-deficient diet); the test was discontinued after it had become definitely positive for each animal. The rats were weighed every 2 weeks and were watched closely for signs of pyridoxine deficiency. Each group of 10 rats selected for hematologic studies was removed from the experimental group in order that no rat would be used twice for blood studies.

An additional group of 20 male Wistar rats, 350-450 Gm., were fed on the pyridoxine-deficient diet. At the end of 50 weeks, a blood sample was taken from each of the surviving rats, and the existence of definite anemia was proven. Two weeks later, 2 mg. of pyridoxine hydrochloride was administered intramuscularly 3 times a week for 2 weeks. Immediately before and after the initial injection, daily reticulocyte counts were made. Hemoglobin, red cell counts, and hematocrit were determined by micro-method on tail blood samples before and subsequent to the injection and once a week for 2 or 3 weeks after the initial injection. Three weeks after the first dose of pyridoxine, the serum iron and iron binding capacity were determined on blood samples obtained by cardiac puncture. The rats were kept on the pyridoxine-deficient diet during this period.

Bone marrow aspirations were done on six severely anemic rats immediately before, 7 days and 2 to 3 weeks after the first injection of pyridoxine. On three of these rats, additional aspirations were taken on the 2nd and 4th day of the course of treatment. The smears were made from the aspirated marrow; and were stained with a "double" Giemsa and Wright stain. The myeloid:erythroid ratio was determined, and Prussian blue stains for iron deposits were made.

RESULTS

The tryptophane-load test was positive in all rats after 11-13 weeks on the pyridoxine-deficient diet. The rats excreted over 1.3 mg. of xanthurenic acid in the urine in 24 hours after the administration of 50 mg. d-l-tryptophane, in contrast to less than 0.3 mg. in the first stage before the tryptophane loading. All but one of these rats on the pyridoxine-deficient diet showed an increase in body weight for the first 10 weeks but declined thereafter. At the end of 50 weeks, the average weight loss of the rats was about 30 per cent. Ninety per cent of these rats developed acrodynia around the nose and mouth and a few also developed it around the eye. Over-half had loss of equilibrium and wasting of the musculature; almost all rats had coarse, dry hair with generalized scaliness. Some had hematuria and a few had epistaxis. Many of the animals in the later stages of the experiment had infections, especially in the tail. Of the 20 rats on pyridoxine-deficient diets at the end of 50 weeks, there were 14 living—a mortality rate of 30 per cent. Two of these later died accidentally during experimentation. In the surviving rats, these signs and symptoms disappeared rapidly upon the administration of pyridoxine.

Table 1 presents some of the hematologic findings, serum iron level and iron binding capacity measured in the rats selected at random at each in-

*The pyridoxine hydrochloride ampules were kindly furnished by Merck & Co., Inc., Rahway, New Jersey.
Pyridoxine Deficiency Anemia in Rats

Table 1.—Average Value of Pertinent Hematologic Changes Including Serum Iron and Iron Binding Capacity of the Blood in Rats during Pyridoxine-deficient Diet

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Pyridoxine Deficient Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Red Blood Cells Million</td>
<td>7.6</td>
</tr>
<tr>
<td>Hemoglobin Gm. Per 100 ml.</td>
<td>13.9</td>
</tr>
<tr>
<td>Hematocrit Percent</td>
<td>46.1</td>
</tr>
<tr>
<td>M.C.V. Cu Micr</td>
<td>58.</td>
</tr>
<tr>
<td>M.C.H. Cu Micr</td>
<td>30</td>
</tr>
<tr>
<td>Reticulocytes Percent</td>
<td>3.8</td>
</tr>
<tr>
<td>Serum Iron ug Percent</td>
<td>158</td>
</tr>
<tr>
<td>Iron Binding Capacity ug Percent</td>
<td>194</td>
</tr>
</tbody>
</table>

*10 rats per interval of approximately 10 weeks were used.

interval from a pool of animals during the course of the pyridoxine-deficient diet. At the end of about 50 weeks, the average red cell count was only slightly changed, although the range was wide, 7.8 to 5.0 million per cu. mm. The average hemoglobin level and hematocrit were markedly reduced, to 8.1 Gm. per cent and 33 per cent respectively; a significant decline in mean corpuscular volume (to 45.8 cu. µ) and mean corpuscular hemoglobin concentration volume (to 24.6 per cent) was also seen. Peripheral blood smears showed some target cells in many of the rats, but no marked anisocytosis at the end of 50 weeks. There was no significant change in reticulocyte counts. As table 1 shows, hematologic changes were not evident in most of the cases until after 30 to 40 weeks of the experimental diet period had elapsed. At the end of about 50 weeks, all the rats demonstrated a moderately severe microcytic anemia. The serum iron level was increased slightly to the upper end of the normal range, but the free iron-binding capacity decreased from an average 194 µg. per cent to 130 µg. per cent.

Table 2 presents the hematologic response to the administration of pyridoxine in 12 of these rats suffering from pyridoxine deficiency anemia. Reticulocyte counts reached a peak on the 4th to 9th day after the first in-
<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Before Pyridoxine Therapy</th>
<th>2-3 Weeks After First Injection of Pyridoxine</th>
<th>Retic Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight</td>
<td>R.B.C.</td>
<td>M.G.V.</td>
</tr>
<tr>
<td>1</td>
<td>220</td>
<td>8.1</td>
<td>8.2</td>
</tr>
<tr>
<td>2</td>
<td>350</td>
<td>6.7</td>
<td>8.7</td>
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<tr>
<td>3</td>
<td>423</td>
<td>6.5</td>
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<tr>
<td>4</td>
<td>345</td>
<td>8.4</td>
<td>8.7</td>
</tr>
<tr>
<td>5</td>
<td>403</td>
<td>5.6</td>
<td>6.1</td>
</tr>
<tr>
<td>6</td>
<td>331</td>
<td>7.0</td>
<td>6.9</td>
</tr>
<tr>
<td>7</td>
<td>300</td>
<td>8.1</td>
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<td>8</td>
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<td>9</td>
<td>420</td>
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<tr>
<td>11</td>
<td>377</td>
<td>8.7</td>
<td>7.2</td>
</tr>
<tr>
<td>12</td>
<td>330</td>
<td>10.0</td>
<td>9.1</td>
</tr>
</tbody>
</table>

*I.B.C. = free iron binding capacity.*
Table 3.—The M:E Ratio of the Bone Marrow of Pyridoxine-deficient Anemia Rats on Pyridoxine Therapy

<table>
<thead>
<tr>
<th>RAT NO.</th>
<th>BEFORE THERAPY</th>
<th>DAYS AFTER INSTITUTION OF PYRIDOXINE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>1</td>
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<td>4.4</td>
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<tr>
<td>2</td>
<td>5.1</td>
<td>—</td>
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<tr>
<td>3</td>
<td>4.4</td>
<td>—</td>
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<tr>
<td>4</td>
<td>3.5</td>
<td>3.2</td>
</tr>
<tr>
<td>6</td>
<td>5.0</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>4.6</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Hemoglobin, hematocrit and red cell counts were generally restored to normal level less than 2 weeks after the start of pyridoxine therapy. Serum iron and iron binding capacity were somewhat decreased in this period. All but two rats gained weight in the first three weeks.

The myeloid:erythroid ratio in the marrow of a part of the group of treated rats, as shown in table 3, exceeded 4:1 (normal, 2.5:1) before therapy. On the 7th day after the first pyridoxine injection, the ratio decreased markedly to less than 2:1, returning to normal after the passage of 20 to 30 days. Prussian blue stains of the marrow failed to reveal any abnormal accumulation of iron.

Figures 1 and 2 are graphic representations of the progressive changes occurring in the deficient rats during response to pyridoxine therapy.

DISCUSSION

The presence of anemia in pyridoxine-deficient rats has not been heretofore clearly demonstrated. Thus in the past two decades, findings concerning the presence of anemia with pyridoxine deficiency in rats have been somewhat inconsistent, some researchers reporting some evidence of the anemia, others none at all. Dining and Day observed an increase in red cell count with reduction of hemoglobin in rats after 15 weeks on a pyridoxine-deficient diet. Pike and Brown reported that both pregnant and non-pregnant rats fed with a deoxypyridoxine-supplement diet showed a decrease in hemoglobin and hematocrit, although in the pregnant rats it occurred earlier and was quantitatively greater. However, such anemia may not necessarily be the result of pyridoxine deficiency because various complications such as infections, hematuria, epistaxis could have been at least partly responsible. Impaired blood regeneration after hemorrhage in pyridoxine-deficient animals with or without anemia was clearly shown by Kornberg et al. and Harkens et al. The former also noticed that 18 per cent of these rats after 17 weeks on pyridoxine-deficient diets had some degree of anemia,
and the latter also demonstrated the absence of polycythemic response to cobalt.

The failure of others to produce a clear and consistent anemia in pyridoxine-deficient rats might well be caused by the comparatively short term of the experiment, the longest lasting for only 18 weeks. In our study, not until after 40 weeks on a pyridoxine-deficient diet did all the rats develop a moderate or severe anemia.

The remarkable efficiency of erythropoietic response to hemorrhage (frequently observed in normal rats), might delay the development of severe anemia in pyridoxine deficiency. Other species, lacking such regenerative power, develop severe anemia earlier on a pyridoxine-deficient diet. The increased M:E ratio and the microcytic hypochromic anemia without hyperferremia in these deficient rats indicates the possibility of failure or erythropoiesis, which may be brought about by impaired synthesis of porphobilinogen. As shown by Schulman et al. and Shemin et al., pyridoxal phosphate is involved in the first step of this synthesis as an activator of the amino acid glycine which, condensed with succinyl CoA, forms alpha-amino-beta-keto-adipic acid. Two molecules of this acid upon decarboxylation yield delta-amino levulinic acid, which, by action of dehydrogenase, forms porphobilinogen, the precursor of protoporphyrin.

In these anemic rats the serum iron level was, in contrast with other species, only slightly increased. This fact is difficult to explain because of the multiplicity of factors existing simultaneously in these deficient rats which tend to elevate or depress the serum iron level. Among these factors are decreased protein anabolism, infection, and impairment of iron utilization.
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Fig. 2.—Rat No. 4. Hematologic response to the administration of pyridoxine.

for the synthesis of heme. Iron absorption as determined by Fe$^{59}$ technic did not reveal an increase, but rather a slight decrease in these rats.$^{21}$ There was also no evidence of iron accumulation in the tissues of these rats.

Free iron binding capacity seemed to be reduced, possibly because of decreased protein formation and heme synthesis. The frequent occurrence of infections and the high mortality in this series of animals may result both from anemia and inability to form antibodies.$^{22}$

The development of anemia and the disturbance in tryptophane metabolism were not related to each other, the increase in urinary excretion of xanthurenic acid after tryptophane loading appearing long before the onset of anemia. This does not seem to invalidate the view that tryptophane metabolism and heme synthesis are unrelated biochemically.

It is interesting that the positive tryptophane load test and the beginning of the decline of body weight occurred almost at the same time. It suggests that the tissue pyridoxine was not depleted until after 10 weeks on a pyridoxine-deficient diet.

**Summary**

Rats fed a diet deficient in pyridoxine all exhibited a severe microcytic hypochromic anemia after 40 to 50 weeks. This anemia responded promptly to pyridoxine administration. The myeloid:erythroid ratio in the bone marrow of the severely anemic rats was definitely increased, suggestive of hypoplasia of the erythroid series, after prolonged deprivation of pyridoxine. The ratio was markedly decreased shortly after the inception of pyridoxine treatment, indicating active erythropoiesis induced by therapy; the ratio subsequently re-
turned toward normal when hemoglobin level improved. There was no
evidence of accumulation of iron in the bone marrow. The serum iron level
increased only slightly, to high-normal values in the anemic rats, but fell to
low-normal level after the administration of pyridoxine.

SUMMARIO IN INTERLINGUA

Rattos mantenite con un dieta carente in pyridoxina exhibiva omnes un
sever microcytic anemia hypochromic post inter 40 e 50 septimanas. Iste anemia
respondeva promptemente al administration de pyridoxina. Le proportion
myeloide:erythroide in le medulla ossee del severmente anemic rattos eseva
deфинитemente elevate, suggestionante hypoplasia del serie erythroide post
prolongate privation de pyridoxina. Le proportion eseva marcatemente
reduce brevemente post le inception del tractamento a pyridoxina, lo que
indicava que un active erythropoiese eseva induce per le therapia. Sub-
sequentemente le proportion retornava verso le norma quando le nivello de
hemoglobina se meliorava. Esseva trovate nulle evidentia de un accumulation
de ferro in le medulla ossee. Le nivello de ferro seral montava solo levemente,
usque a valores alto-normal in le rattos anemic, sed post le administration de
pyridoxina illo declinava usque a nivellos basso-normal.

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