# Polycythaemia in Pyridoxin Deficiency in the Rat

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Severe anaemia in pyridoxin deficiency has been reported in dogs (Fouts, Helmer, Lepkovsky & Jukes, 1938; Fouts, Helmer & Lepkovsky, 1940; Borson & Mettier, 1940; McKibbin, Schaefer, Frost & Elvehjem, 1942), pigs (Wintrobe, Follis, Miller, Stein, Alcayaga, Humphreys, Suksta & Cartwright, 1943), chicks (Luckey, Briggs, Elvehjem & Hart, 1945), ducks (Hegsted & Rao, 1945) and monkeys (McCall, Waisman, Elvehjem & Jones, 1946; Poppen, Greenberg & Rinehart, 1952). In the rat, Fouts & Lepkovsky (1942) observed only a slight reduction of haemoglobin concentration, and Kornberg, Tabor & Sebrell (1945) produced a moderately severe anaemia in a small proportion of deficient rats. More recently, Carpenter & Kodicek (1948) and Gubler, Cartwright & Wintrobe (1949) found no significant reduction in haemoglobin concentration, whereas Agnew (1949) observed that both haemoglobin concentration and colour index were significantly reduced in rats deficient in pyridoxin.

On the basis of haemoglobin values, it is generally believed that little or no anaemia occurs in pyridoxin deficiency in the rat, in marked contrast with other animals. In the course of studies of cutaneous and mucocutaneous changes in pyridoxin deficiency, which have been described elsewhere (Ramalingaswami & Sinclair, 1953), we followed the progressive changes in the blood of the rat at different stages of the deficiency and observed a significant and sustained elevation of the erythrocyte count in the deficient animals. This observation had been recorded earlier by Carpenter & Kodicek (1948) and Agnew (1949); the former also demonstrated microcytosis of the erythrocytes in this condition. A distinct increase in the erythrocyte count has been observed in the monkey during the late stages of pyridoxin deficiency and attributed by the authors to dehydration (Poppen *et al.* 1952). In the present study, which confirmed the occurrence of polycythaemia, observations have been made in an attempt to clarify its pathogenesis.

## EXPERIMENTAL

### *Animals*

Three experiments were made. In Exp. 1, twenty-four female albino rats, 11 weeks old, were divided into two groups of twelve deficient and twelve control animals, littermates being distributed equally between the groups. They were kept in individual cages with raised mesh floors and were pair-fed. In Exp. 2, weanling hooded rats, 24 days old, were divided into two groups of twenty-four deficient animals (twelve male and twelve female) and twelve controls (six male and six female). Food was

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allowed *ad lib.* In Exp. 3, adult hooded rats, 6 months old, were used. They were divided into two groups of twenty-three deficient (eleven male and twelve female) and twenty-three control animals (eleven male and twelve female). Food was allowed *ad lib.*

*Diet*

All rats were fed on the following vitamin-free basal diet, with vitamin supplements by mouth:

| Composition of the basal diet | (%) |
|---|---|
| Sucrose or raw rice starch | 63·0 |
| Casein (fat- and vitamin-free) | 21·0 |
| Salt mixture (Hegsted, Mills, Elvehjem & Hart, 1941) | 5·5 |
| Vegetable oil | 10·5 |
| **Composition of daily vitamin supplement** | **mg** |
| Thiamine hydrochloride | 0·1 |
| Riboflavin | 0·16 |
| Nicotinic acid | 1·0 |
| Calcium pantothenate | 0·2 |
| Biotin | 0·002 |
| Inositol | 2·0 |
| Choline chloride | 10·0 |
| *p*-aminobenzoic acid | 1·0 |
| Pteroylglutamic acid | 0·02 |
| Vitamin K ('Synkavit', Roche Products Ltd.) | 0·1 |
| **Composition of weekly vitamin supplement** | |
| Vitamin A | 1000 i.u. |
| Ergocalciferol | 100 i.u. |
| *a*-tocopheryl acetate | 5·0 mg |

In addition, the control animals received 0·1 mg pyridoxin hydrochloride daily by mouth.

*Methods of study*

The haematological studies included determination of erythrocyte and leucocyte counts, haemoglobin and haematocrit. Peripheral blood smears and bone-marrow smears were stained routinely with Jenner-Giemsa.

Pipettes certified by the National Physical Laboratory and counting chambers with improved Neubauer rulings were used for all counts. Haemoglobin was estimated as oxyhaemoglobin in an Evelyn Photoelectric Colorimeter. Haematocrit was determined on heparinized blood with Meyerstein tubes of uniform bore and spinning in a centrifuge for 30 min at 1800 *g*. From the values obtained by these methods, the corpuscular constants were calculated according to Wintrobe's formulas (Wintrobe, 1929).

Plasma proteins were estimated on heparinized blood by using the densitometric method of Jacobsen & Linderstrøm-Lang (1940), the density-protein relation being calculated for $d_{20}^{20}$ by the equation protein = 360 (density − 1·0070). Plasma volume was estimated with the dye T-1824 by a slight modification of the method described by Wang & Hegsted (1949).

The statistical significance of differences between means was tested at $P=0.02$ by 'Student's' (1908, 1925) $t$ test.

## RESULTS

The growth behaviour and clinical condition of the animals have been described in detail elsewhere (Ramalingaswami & Sinclair, 1953). It may be briefly mentioned here that the deficient animals showed retardation of growth and symmetrical skin lesions characteristic of pyridoxin deficiency whereas the control animals increased in weight and remained free from signs. The deficiency process as measured by growth and severity of cutaneous lesions was more acute in the weanling rats than in the older ones.

Table 1 shows the mean haematological values with relevant statistics for the rats in Exp. 1. The estimations were made on the tail blood of the same animals at different

Table 1. *Haematological results of repeated estimations on twenty-four adult female pyridoxin-deficient rats*

| | Days on diet | | | |
|---|---|---|---|---|
| | 70 | 112 | 140 | 159 |
| Erythrocyte count ($10^6$/cu.mm): | | | | |
| Deficient | 8·82 | 8·14 | 9·27 | 9·46 |
| Control | 8·69 | 8·10 | 8·16 | 8·39 |
| Difference† | +0·13±0·10 | +0·04±0·19 | +1·11*±0·18 | +1·07*±0·38 |
| Haemoglobin (g/100 ml.): | | | | |
| Deficient | 15·90 | 15·38 | 14·04 | 13·77 |
| Control | 16·62 | 15·88 | 15·22 | 15·54 |
| Difference† | −0·72±0·35 | −0·50±0·23 | −1·18*±0·33 | −1·77*±0·40 |
| Haematocrit (%): | | | | |
| Deficient | 45·2 | 44·8 | 44·3 | 43·8 |
| Control | 45·9 | 46·6 | 46·4 | 47·0 |
| Difference† | −0·7±1·2 | −1·8±1·8 | −2·1±1·5 | −3·2*±0·9 |
| Mean corpuscular volume (cu.$\mu$): | | | | |
| Deficient | 51·3 | 55·3 | 47·8 | 46·5 |
| Control | 52·7 | 57·5 | 57·0 | 56·4 |
| Difference† | −1·4±1·2 | −2·2±2·0 | −9·2*±1·8 | −9·9*±1·9 |
| Mean corpuscular haemoglobin ($\mu\mu$g): | | | | |
| Deficient | 18·1 | 19·0 | 15·2 | 14·6 |
| Control | 19·2 | 19·6 | 18·7 | 18·6 |
| Difference† | −1·1±0·5 | −0·6±0·6 | −3·5*±0·4 | −4·0*±0·5 |
| Mean corpuscular haemoglobin concentration (%): | | | | |
| Deficient | 35·4 | 34·6 | 31·7 | 31·5 |
| Control | 36·8 | 34·4 | 32·9 | 33·5 |
| Difference† | −1·4±1·2 | +0·2±1·4 | −1·2±1·0 | −2·0±0·9 |
| Leucocyte count ($10^3$/cu.mm): | | | | |
| Deficient | 12·75 | 16·43 | 9·45 | 12·30 |
| Control | 12·49 | 14·67 | 8·94 | 11·31 |
| Difference† | +0·26±1·55 | +1·76±2·51 | +0·51±0·92 | +0·99±0·74 |
| Pyridoxin in blood ($\mu$g/100 ml.): | | | | |
| Deficient | | 4·6 | | 2·8 |
| Control | | 30·8 | | 27·8 |
| Difference† | | −26·2*±3·2 | | −25·0*±4·2 |

*=Significant at $P\leqslant 0.02$.

† Value with its standard error. Standard error computed from variances of deficient-control pair differences.

stages of deficiency. It will be seen that a significant increase in the erythrocyte count of the deficient animals occurred between 112 and 140 days of deficient diet and persisted, increasing further in some animals, until the 159th day, by which time the deficient animals showed severe symmetrical skin lesions and became moribund. The haemoglobin and haematocrit values of the deficient group decreased gradually, becoming significantly below the control values on the 140th day for haemoglobin and the 159th day for haematocrit. Thus there was a marked reduction in the mean corpuscular volume (M.C.V.) and mean corpuscular haemoglobin (M.C.H.), whereas the mean corpuscular haemoglobin concentration (M.C.H.C.) was not significantly altered. The resulting blood picture was microcytic and normochromic. Examination of peripheral blood smears confirmed the presence of microcytosis with little or no anisocytosis. The amounts of pyridoxin in whole blood of all deficient and control animals in Exp. 1, determined by the microbiological method with *Saccharomyces carlsbergensis* (Atkin, Schultz, Williams & Frey, 1943), are also presented in Table 1. They indicate the severe state of depletion reached by the deficient animals.

Similar results were obtained on the weanling and adult hooded rats in Exps. 2 and 3. From each experiment, deficient animals selected at random, with their litter-mate controls, were killed at weekly intervals and their blood pictures at the time of killing were determined as in Exp. 1. The erythrocyte count was raised significantly in the deficient animals, but there was no significant alteration in the haemoglobin concentration or haematocrit. The resulting blood picture was again microcytic and normochromic. Since these results are closely similar to those of Exp. 1, they are not tabulated.

The plasma proteins were determined in Exps. 2 and 3, and the results are given in Table 2. No significant differences were found between deficient and control groups.

Table 2. *Mean plasma-protein values* (g/100 ml.) *of pyridoxin-deficient rats*

| Exp. no. | Days on diet | Pyridoxin-deficient Mean | Pyridoxin-deficient No. | Control Mean | Control No. | Average difference (deficient-control) | Mean standard deviation |
|---|---|---|---|---|---|---|---|
| 2 | 41 | 7·90 | 4 | 7·74 | 2 | 0·20 ± 0·22* | 0·61 |
| | 48 | 6·90 | 4 | 6·66 | 2 | | |
| | 55 | 7·50 | 4 | 7·61 | 2 | | |
| | 62 | 7·62 | 4 | 6·70 | 2 | | |
| | 69 | 7·90 | 4 | 8·10 | 2 | | |
| | 76 | 7·68 | 4 | 7·50 | 2 | | |
| 3 | 38 | 7·79 | 1 | 8·16 | 1 | −0·06 ± 0·06† | |
| | 45 | 7·32 | 1 | 7·30 | 1 | | |
| | 52 | 7·55 | 1 | 7·14 | 1 | | |
| | 59 | 8·20 | 1 | 8·39 | 1 | | |
| | 66 | — | — | 7·22 | 1 | | |
| | 73 | 7·19 | 1 | 7·33 | 1 | | |
| | 80 | 8·07 | 1 | 8·07 | 1 | | |
| | 87 | 8·78 | 1 | 8·58 | 1 | | |
| | 94 | 7·13 | 1 | 7·42 | 1 | | |
| | 104 | 6·71 | 6 | 6·73 | 6 | | |
| | 108 | 7·02 | 2 | 7·30 | 2 | | |
| | 115 | 6·98 | 2 | 7·12 | 2 | | |

* Standard error computed from mean S.D.

† Standard error computed from variance between means of contemporary deficient and control values.

The plasma volume was determined in eight deficient and eight control animals in Exp. 3 and showed no significant differences between the two groups. There were wide variations within the same group (Fig. 1). All the deficient animals showed polycythaemia but no cutaneous lesions. They had nevertheless reached a severe state of deficiency as indicated by their blood-pyridoxin levels which are shown in Fig. 1.

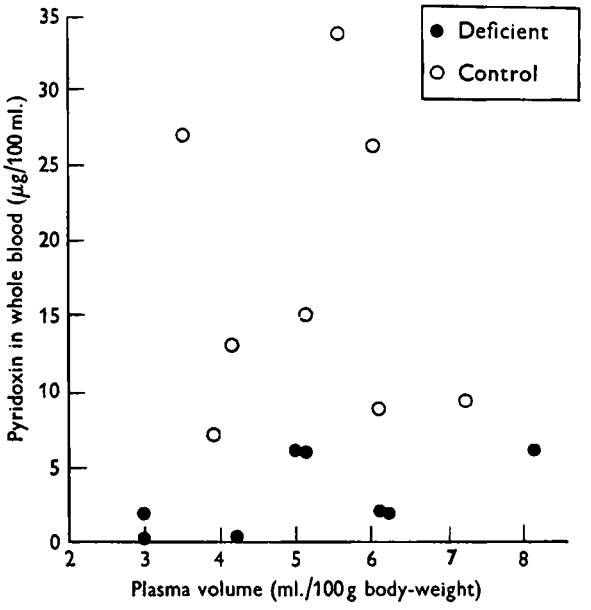


Fig. 1. Relation between plasma volume and blood pyridoxin in pyridoxin-deficient rats.

## DISCUSSION

The observations presented above show that a significant elevation of the erythrocyte count occurred in pyridoxin-deficient rats of both albino and hooded strains, whether pair-fed or fed *ad lib.* The polycythaemia began early in the deficiency before the appearance of characteristic cutaneous lesions and persisted, in some animals increasing in severity as the deficiency progressed, until they reached a moribund state and exhibited severe cutaneous lesions. The haemoglobin concentration and haematocrit either remained stationary or showed a tendency to decrease gradually as the deficiency progressed, eventually becoming significantly reduced in some animals.

That this polycythaemia was probably not due to uncomplicated haemoconcentration is suggested by the fact that there was no proportionate increase in haemoglobin concentration, haematocrit and leucocyte count. This is supported by the fact that the plasma-protein values of the deficient animals were not elevated significantly over those of the control animals, and final confirmation is obtained by the results of plasma-volume estimations, which revealed no significant differences between the deficient and control groups.

From these results it may be concluded that the polycythaemia was absolute and was due to increased production of erythrocytes. But the cells produced were micro-

cytic and it thus appears that a purely morphological abnormality of the erythrocytes is the fundamental haematological abnormality of pyridoxin deficiency in the rat. There is apparently no defect in the synthesis of haemoglobin until relatively late in the deficiency. If anaemia is defined as a condition in which there is a reduction either in the haemoglobin concentration or the erythrocyte count or both, then there is little or no anaemia in pyridoxin deficiency in the rat. Although the erythrocytes in this condition are microcytic, and consequently the amount of haemoglobin carried by each erythrocyte as measured by the M.C.H. is less than normal, the defect is compensated by an increase in the number of circulating erythrocytes, which helps to maintain adequate haemoglobin concentration in the blood.

It is not clear why this purely morphological abnormality of the erythrocyte occurs. A simple microcytic blood picture whose pathogenesis is obscure is found in a number of subacute and chronic inflammatory conditions in man (Wintrobe, 1951). There is no evidence to suggest that pyridoxin deficiency plays any part in its pathogenesis. Pyridoxin has been shown to be essential for porphyrin synthesis in pigs (Cartwright & Wintrobe, 1948). It is unlikely that the slight reduction in haemoglobin concentration found in the pyridoxin-deficient rat produces anoxia of sufficient intensity to stimulate the bone marrow to overproduction of erythrocytes. Further, there was little or no reduction in haemoglobin concentration during the early stages of the deficiency when polycythaemia was present. Even if anoxia was a significant factor, the persistent microcytosis of the erythrocytes remains to be explained. In chronic anoxic states, the tendency is for the development of macrocytosis. Thus in permanent residents at high altitudes, a distinct macrocytosis develops (Hurtado, Merino & Delgado, 1945; Ramalingaswami & Venkatachalam, 1950). During intra-uterine life, when a low oxygen saturation has been shown to exist, a high degree of macrocytosis of the erythrocyte associated with a low erythrocyte count is present (Wintrobe & Shumacker, 1946).

It is clear that microcytosis of the erythrocyte in the pyridoxin-deficient rat cannot be the result of defective haemoglobin formation. We are tempted to speculate that it may be due to a defect in the formation of the erythrocyte stroma in the absence of pyridoxin. It is well known that pyridoxin is intimately connected with the metabolism of proteins and of essential unsaturated fatty acids (Snell, 1953; Sherman, 1950; Sinclair, 1952, 1953). The stroma of the mammalian erythrocyte has been found to be a protein-lipid complex (Beech, Erickson, Bernstein, Williams & Macy, 1939; Erickson, Williams, Bernstein, Avrin, Jones & Macy, 1938; Tishkoff, Robscheit-Robbins & Whipple, 1953). The nutritional requirements for the formation of stroma are not known, but from the recently published pioneering work of Tishkoff *et al.* (1953) it appears that the stromal proteins are in a dynamic state undergoing characteristic changes during the development of anaemia. Pyridoxin may be essential for the synthesis of erythrocyte stroma and in its absence a microcytic erythrocyte, necessarily carrying less haemoglobin than normal, may be produced. The resulting anoxia would then lead to compensatory erythrocytosis.

SUMMARY

1. A significant and persistent increase in the erythrocyte count occurred in pyridoxin deficiency in the rat.

2. Plasma-volume and plasma-protein estimations indicated that the polycythaemia is not attributable to haemoconcentration.

3. The haemoglobin concentration and haematocrit were not significantly altered in the early stages of the deficiency and were only slightly reduced in the late stages. The resulting blood picture was microcytic and normochromic.

4. It is concluded that a purely morphological abnormality of the erythrocyte—microcytosis—is the fundamental haematological alteration in pyridoxin deficiency in the rat.

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