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Heme Biosynthesis in Copper Deficient Swine* (32848)

G. R. LEE,[1] G. E. CARTWRIGHT, AND M. M. WINTROBE
University of Utah College of Medicine, Salt Lake City, Utah 84112

Severe anemia is a prominent manifestation of copper deficiency in swine (1). Although this fact has been known for more than 20 years, the pathogenesis of the anemia remains unknown. Morphologically, the anemia is characterized by hypochromia and microcytosis of the erythrocytes and erythroid hyperplasia of the bone marrow. These features strongly suggest a defect in hemoglobin synthesis. It is therefore reasonable to propose that copper is essential for the biosynthesis or metabolism of one of the three main components of the hemoglobin molecule: protoporphyrin, iron, or globin. The present investigations deal with the first of these, the biosynthesis of protoporphyrin, and with the combination of protoporphyrin and iron to form heme.

Materials and Methods. The 57 pigs used in this study were of mixed breed. The copper deficient diet consisted of canned condensed milk supplemented with iron. Normal control animals received copper sulfate in addition. The details of the diet preparation have been described elsewhere (1). In some experiments, pigs with other experimentally induced anemias were used as additional controls: iron deficiency was produced by eliminating iron supplements from the control diet; the pyridoxine deficient diet and the method used for producing hemorrhagic anemia have been described by Deiss, *et al.* (2).

Free erythrocyte porphyrins were determined by the method of Schwartz and Wikoff (3) as modified by Wranne (4). The synthesis of porphobilinogen (PBG) and porphyrins from delta-aminolevulinic acid (ALA) was studied by a method reported elsewhere (5). Routine hematologic determinations were performed as described by Cartwright (6).

The heme synthetase activity of circulating reticulocytes was measured by a modification of a method reported previously (7). Hemolysates were incubated with protoporphyrin (0.001 *M*), cysteine (0.01 *M*), and $^{59}Fe^{2+}$

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[1] Markle Scholar in Academic Medicine.

TABLE I. Synthesis of Porphobilinogen and Porphyrins from Delta-aminolevulinic Acid by Erythrocytes.[a]

	Control	Cu deficient	B_6 deficient	Fe deficient
Number of animals	10	12	7	5
Volume of packed red cells (ml/100 ml)	41 ± 0.6	21.5 ± 1.0	24.1 ± 1.6	20.1 ± 1.6
Reticulocytes (%)	6.7 ± 1.2	7.4 ± 1.2	6.7 ± 1.6	11.2 ± 1.5
Synthesis: (mμM/hour per ml of erythrocytes)				
Porphobilinogen	344 ±70	803 ±129	765 ±92	289 ±34
Uroporphyrinogen	12.9 ± 1.3	20.7 ± 1.8	26.3 ± 4.6	22.8 ± 1.8
Coproporphyrinogen	10.4 ± 1.0	20.0 ± 1.8	25.7 ± 4.6	21.9 ± 1.8
Protoporphyrin	6.9 ± 2.2	19.4 ± 1.8	24.8 ± 4.6	21.7 ± 1.8

[a] Values are means ± SE.

(0.001 *M*, 50 μC) in tham-chloride buffer (0.4 *M*, pH 8.0), under nitrogen at 37°C for 1 hour. Hemin was extracted with 0.5% strontium chloride in 3:1 acetone–glacial acetic acid, crystallized by boiling off the acetone, and recrystallized twice from pyridine, chloroform, and methanol. Radioactivity was assayed by crystal scintillation counting, and hemin was determined spectrophotometrically as the cyanohemochromogen (8). Heme synthesis was calculated from the determined value for specific activity and the known concentrations of iron and hemoglobin heme in the incubation mixture. Values were corrected for the rate of nonenzymatic heme synthesis, which was measured simultaneously in a flask containing an equivalent amount of hemoglobin, but no reticulocytes. Duplicate determinations differed by less than 10%.

The ALA synthesis in circulating reticulocytes was evaluated by two procedures. In the first, the rate at which glycine was incorporated into heme was compared with the rate of ALA incorporation into heme. Hemolysates from reticulocyte enriched blood were incubated with glycine-2-^{14}C or ALA-^{14}C (500 μM, 10 μC), Fe^{2+} (0.1 μM) and cysteine (1 μM) in phosphate buffer (6.67 *M*, pH 7.2) at 37°, under nitrogen for 2 hours. Hemin was extracted and crystallized as described above, and radioactivity was assayed in a low background, gas-flow Geiger counter.

The second procedure made use of the observation that under certain conditions glycine decarboxylation in the porcine erythrocyte is primarily related to ALA synthesis (9). The "stromal" fraction of an erythrocyte hemolysate was incubated with glycine (0.2 *M*), glycine-1-^{14}C (5 μC), transaconitate (0.02 *M*), alpha-keto glutarate (KGA) (0.025 *M*), and potassium phosphate buffer (0.08 *M*) at pH 7.0 for 2 hours at 37°C. A duplicate flask without KGA served as a "blank." Other details of the assay procedure have been reported (9). Total glycine decarboxylation was calculated from the amount of $^{14}CO_2$ liberated in the assay flask. KGA-dependent glycine decarboxylation represented the difference between the $^{14}CO_2$ liberated in the assay flask and that liberated in the "blank."

Results. Free erythrocyte porphyrins. In 13 normal control animals, the free erythrocyte protoporphyrin content was 74 ± 5 μg/100 ml of erythrocytes (mean ± SE) and the free erythrocyte coproporphyrin 0.7 ± 0.1 μg/100 ml. In 23 copper deficient animals with a volume of packed red cells of less than 25 ml/100 ml, the free erythrocyte protoporphyrin was 70 ± 4 μg/100 ml and the free erythrocyte coproporphyrin was 0.9 ± 0.2 μg/100 ml. The copper deficient animals were not significantly different from control animals with respect to these measurements.

Synthesis of porphobilinogen and porphyrins from ALA. The rate of synthesis of all porphyrin intermediates was greater in copper deficient pigs than in normal controls (Table I). Two additional control groups consisted of animals with anemia due to defi-

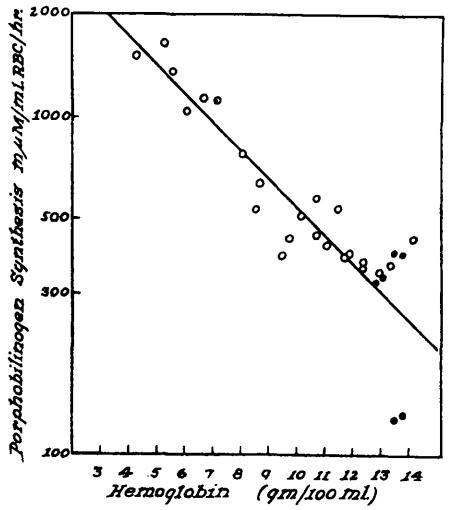

FIG. 1. The relation between blood hemoglobin concentration and the rate of porphobilinogen synthesis (log scale) in copper deficient pigs (○); and normal controls (●).

ciencies of iron and of vitamin B_6. The rate of synthesis of porphyrin intermediates was increased in both of these groups with one exception, namely, porphobilinogen synthesis in the iron deficient pigs.

In copper deficient pigs, the increased synthesis of heme intermediates correlated with the degree of anemia. In one litter of 7 pigs, 5 of which were copper deficient and 2 of which served as controls, biweekly determinations were performed as anemia developed on the deficient diet. The relation between the logarithm of the rate of porphobilinogen synthesis (ALA dehydratase activity) and the blood hemoglobin concentration is depicted in Fig. 1. The correlation was highly significant ($r = 0.93$). A similar correlation was observed between hemoglobin concentration and the synthetic rate of the other porphyrin intermediates, uroporphyrinogen ($r = -0.81$), coproporphyrinogen ($r = -0.62$) and protoporphyrin ($r = -0.61$). These correlation coefficients all differed significantly from zero ($p < 0.001$)

Heme synthetase. Values for the rate of synthesis of heme from protoporphyrin and iron are given in Table II. Since heme synthesis is more easily and accurately measured when the reticulocyte count is high, an additional control group was used which consisted of 6 pigs with hemorrhagic anemia. In three of these animals, the rate at which blood was removed was such that the reticulocyte count was about 20%, and heme synthetase activity was similar to that found in the other anemic pigs. In the other three, greater amounts of blood were removed, resulting in a reticulocyte count of 33–43%. Heme synthetase activity was then markedly increased in relation to the other groups. The severe hemorrhagic stress in these 3 animals produced an outflow of very large, polychromatophilic erythrocytes, presumably "younger" than those seen in pigs in the other experimental groups. Possibly the presence of these young cells accounted for the increased synthetic activity in this group.

ALA synthesis. The rate at which glycine was incorporated into heme was compared with the rate of ALA incorporation in 3 control and 3 copper deficient animals (Table III). The ALA incorporation rate was markedly increased in blood from copper deficient

TABLE II. Heme Synthetase Activity in Reticulocytes.[a]

				Hemorrhagic anemia	
	Controls	Cu deficient	Fe deficient	Mild	Severe
Number of animals	14	11	6	3	3
Volume of packed red cells (ml/100 ml)	42 ± 0.7	21 ± 1.1	17 ± 1.0	34 (33–34)	21 (18–24)
Reticulocytes (%)	5.2 ± 0.9	7.6 ± 0.9	10.7 ± 1.3	19 (18.3–19.5)	38 (32.7–42.5)
Heme synthetase (mμ*M*/hour per 10^9 reticulocytes)	0.9 ± 0.2	2.8 ± 0.4	2.7 ± 0.5	2.2 (1.9–2.7)	6.8 (5.6–8.2)

[a] Values are means ± SE. In the phlebotomized groups, the range is given in parentheses.

TABLE III. Heme Synthesis from Glycine Compared with that from Delta-aminolevulinic Acid.

	Pig no.	VPRC (ml/100 ml)	Retics. (%)	Radioactivity in heme[a] From Glycine-^{14}C	From ALA-^{14}C
Controls	1837	42	3.7	257	36,708
	1838	46	3.6	205	44,079
	1860	45.5	3.5	313	10,600
Copper deficient	1840	25	4.3	267	68,125
	1842	33	6.1	1088	108,578
	1862	18	4.3	793	103,500

[a] Counts/min per 10^9 reticulocytes.

TABLE IV. Aminolevulinic Acid Synthetase Activity in Reticulocytes as Estimated by Glycine Decarboxylation.[a]

	Control	Copper deficient
Number of animals	5	9
Reticulocytes (%)	2.9 ± 0.4	2.7 ± 0.7
Glycine decarboxylation:[b]		
Total	211 ± 32	360 ± 88
KGA-dependent[c]	81 ± 18	165 ± 20

[a] Values are means ± SE.
[b] $m\mu M/10^8$ reticulocytes/2 hours.
[c] Alpha-ketoglutaric acid-dependent.

animals as compared with controls. Incorporation of glycine into heme proceeded at a rate that was either the same or increased in deficient as compared with control animals. In both groups, ALA was incorporated much more rapidly than was glycine; therefore, the glycine incorporation rate reflected chiefly the rate of ALA synthesis.

ALA synthetase was measured by the glycine decarboxylation method in 5 control and 9 copper deficient animals (Table IV). Total glycine decarboxylation was increased in copper deficient as compared with control animals. KGA-dependent glycine decarboxylation, a more specific measure of ALA synthetase activity, was also increased in the deficient pigs.

Discussion. Cooper and Gordon (10) reported that a negative correlation existed between the logarithm of splenic ALA dehydratase activity and the volume of packed red cells (VPRC) in rats. In their experiments, the VPRC was varied *in vivo* by hypertransfusion and by phlebotomy. In our studies, which employed a different experimental model and a different tissue as enzyme source, a similar phenomenon was observed. As anemia developed in copper deficient animals, there was a progressive, logarithmic increase in erythrocyte ALA dehydratase activity (Fig. 1). In addition, similar increases in the rate of synthesis of uroporphyrin, coproporhyrin, and protoporphyrin were observed. Since these changes were also observed in deficiencies of iron and vitamin B_6, this phenomenon is not specific for copper deficiency. Cooper and Gordon proposed that the activity of heme biosynthetic enzymes might be controlled by erythropoietin, although they could demonstrate no effect of erythropoietin *in vitro*.

The present studies contribute to the understanding of the anemia of copper deficiency only in a negative way. None of the heme biosynthetic enzymes were reduced in activity in copper deficiency. Thus, copper does not appear to play a role in heme or porphyrin biosynthesis in erythropoietic tissues

of swine. Observations which suggested a different conclusion were reported by Iodice and co-workers and by Anderson and Tove. Initial studies of ALA dehydratase by Iodice *et al.* suggested that copper was an essential constituent of the enzyme (11). Subsequently, however, these workers found that all of the copper could be removed without loss of enzyme activity (12). Anderson and Tove reported that the rate of incorporation of glycine into heme was reduced in erythrocytes from copper deficient chicks, and that the rate increased upon the addition of copper *in vitro* (13). Subsequently, these investigators were unable to reproduce this observation consistently (14).

If the assumption that the morphological characteristics of the anemia of copper deficiency indicate an abnormality in hemoglobin synthesis is correct, a further definition of the problem becomes possible. Since no abnormality was found in the heme biosynthetic pathway, then copper must play a role either in globin synthesis or in iron metabolism.

Summary. The steps in the heme biosynthetic pathway were evaluated in normal and in copper deficient swine. As anemia developed, the activity of heme biosynthetic enzymes increased. These data suggest that the anemia of copper deficiency is not the result of defective heme biosynthesis and, therefore, that copper is not a cofactor in any of the reactions studied. Since the morphologic characteristics of the anemia of copper deficiency suggest defective hemoglobin synthesis, it is concluded that copper is essential either for the normal metabolism of iron or for the synthesis of globin.

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