HEME SYNTHESIS IN VITAMIN B₆ AND PANTOTHENIC ACID DEFICIENCIES*

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Shemin and coworkers (1-4) showed that the α-carbon and nitrogen atoms of glycine were precursors of specific atoms of the heme molecule. This was substantiated by Muir and Neuberger (5, 6). Other carbon atoms were shown to come from acetate (5-7) or succinate (8). The nature of the intermediate precursors synthesized from these metabolites remained unknown until the structure for urinary porphobilinogen¹ was elucidated (9-13). Falk et al. (14) found PBN to be incorporated into porphyrins and heme in avian red cells. Subsequently, Shemin and Russell (15) and Neuberger and Scott (16) independently synthesized δ-aminolevulinic acid and found that it was a precursor of porphyrins and heme. An enzyme was soon shown to be present in nature, which readily converts ALA to PBN (17-21) and has been named δ-aminolevulinic acid dehydrase by Gibson et al. (22). Shemin and Russell (15) proposed that ALA is biosynthesized from glycine and an asymmetric succinyl derivative, perhaps succinyl coenzyme A, by the condensation of these substances to form α-amino, β-keto adipic acid, which is subsequently decarboxylated.

Vitamin B₆ and pantothenic acid are among a variety of nutritional substances which, when fed in inadequate amounts, may result in anemia. Anemia from vitamin B₆ deficiency has been reported in such species as dog (23), pig (24), rat (25), duck (26), and chick (27). Cartwright and Wintrobe (28) suggested that in swine the fundamental disturbance is a failure to synthesize protoporphyrin, since low erythrocyte protoporphyrin levels were found in this deficiency. An anemia from pantothenic acid deficiency has been described by several groups of investigators (29-31). However, in rats it appears that special conditions such as the simultaneous deficiency of additional food essentials (32) may be necessary for anemia to appear.

The purpose of these studies was to see whether the rates of incorpora-

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¹ The following abbreviations are used: porphobilinogen, PBN; δ-aminolevulinic acid, ALA; pyridoxal-5-phosphate, B₆-PO₄.
tion of different labeled precursors into heme were affected by vitamin B₆ and pantothenic acid deficiencies. The incorporation of radioactive glycine (33), succinate (8), ALA (15, 16), and PBN (14) into heme has been studied conveniently with avian red cells by incubating the cells with the labeled substrate and isolating the radioactive heme as crystalline hemin. The rate of incorporation of the precursors was determined by the specific activity of the hemin.

It was found that blood samples from vitamin B₆- and pantothenic acid-deficient ducklings incorporated glycine and succinate into heme at reduced rates, whereas ALA incorporation was essentially normal. The addition of pyridoxal-5-phosphate in vitro restored glycine and succinate incorporation, but had no effect on ALA incorporation. Added coenzyme A was without effect when added to blood from pantothenic acid-deficient ducks, but the injection of calcium pantothenate 1 hour before the blood specimens were drawn restored the rate of glycine incorporation into heme. Some of these results have been reported in preliminary communications (34, 35).

**EXPERIMENTAL**

δ-Aminolevulinic acid-2,3-C¹⁴ was prepared by the method of Neuberger and Scott (36). The succinic anhydride used for preparing radioactive ALA was prepared from succinic acid-2-Cl⁴ (37). Hemoglobin levels were determined by the method of Schultze and Elvehjem (38).

For the determination of tissue porphyrin, the entire liver or small intestine was homogenized with several volumes of a 4:1 mixture of ethyl acetate and acetic acid and filtered. The residue was reextracted until the filtrates were colorless. The combined filtrates were washed with water to remove the acetic acid. The washings were adjusted to pH 3.1 and extracted with ethyl acetate to recover small amounts of porphyrin. The ethyl acetate fractions were combined and extracted several times with small volumes of 10 per cent (w/v) HCl. The acid extract was adjusted to pH 3.1 with concentrated NH₄OH and the porphyrin was extracted into ethyl acetate. A final 10 per cent HCl extract was made and diluted appropriately for optical density measurements in the Beckman DU spectrophotometer, according to the method of Rimington and Sveinssson (39).

Day-old Pekin ducklings were fed the vitamin B₆-deficient diet of Hegsted and Rao (26) or the pantothenic acid-deficient diet of Hegsted and Perry (40). Control diets contained 6 mg. of pyridoxine hydrochloride and 30 mg. of calcium pantothenate per kilo of diet, respectively.

Blood was drawn from the heart into a heparinized syringe under ether anesthesia. 2 ml. of either pooled or individual blood samples were mixed
with 0.1 ml. of glycine-2-C\(^{14}\) (20 \(\mu\)moles, 147,500 c.p.m. per \(\mu\)mole), sodium succinate-2-C\(^{14}\) (20 \(\mu\)moles; 125,000 c.p.m. per \(\mu\)mole), or \(\delta\)-aminolevulinic acid-2,3-C\(^{14}\) (2 \(\mu\)moles, 14,500 c.p.m. per \(\mu\)mole). Usually the ALA incorporation was tested with hemolyzed cells prepared by centrifuging the blood at 6000 r.p.m. for 5 minutes in a Servall refrigerated centrifuge and replacing the volume of plasma by an equal volume of water and allowing 1 to 2 hours for hemolysis. KCl and MgCl\(_2\) were added as suggested by Shemin and Kumin (8). The mixture was incubated at 38° in a Dubnoff metabolic shaker in air for 2 hours and then chilled to 0° to stop the reactions. 780 mg. of hemoglobin in 5 ml. of saline were added as heme carrier to each beaker, and hemin was isolated by a modification of Fisher's method (41), as described below.

The contents of the incubation beaker were added dropwise to 30 ml. of boiling glacial acetic acid, containing 0.5 ml. of saturated NaCl solution, over a 10 minute period. The temperature was not allowed to drop below 90°. The flask was placed on a steam bath for at least 2 hours, after which time the contents were transferred to a 40 ml. centrifuge tube. After the hemin crystallized, the solution was spun at 2000 r.p.m. for 30 minutes to separate the crystals from the mother liquor. The washings were performed as those described by Fisher, care being taken to suspend the crystals in each wash liquid before spinning. If the crystals are not completely dispersed in each wash liquid, the final product may be contaminated by some radioactive impurity. Usually the preparations were counted without recrystallization, since, in those instances in which the hemin was re-crystallized, the samples gave essentially the same specific activities as the original crystalline preparations. All samples were counted in duplicate in a windowless flow counter and corrected to infinite thinness. The results are reported as counts per minute per mg. of hemin \(\pm\) the standard error of the mean.

In early experiments with whole blood, the cells were washed with saline after incubation to remove unchanged labeled substrates. It was found, however, that the specific activity of the isolated hemin was unaffected by the above washing.

### Results

In a preliminary communication (34) it was reported that heme was synthesized in the red cells of vitamin B\(_6\)-deficient ducklings from glycine-2-C\(^{14}\) at a rate which was half, or less than half, that found with control ducklings and that the addition of pyridoxal-5-phosphate \textit{in vitro} stimulated the ability of the deficient cells to synthesize heme. From additional experiments with vitamin B\(_6\)-deficient ducklings it appeared that the cells became less able to incorporate radioactive glycine into heme with increas-
ing duration of the deficiency period (Table I). Day-old ducklings were fed Purina chick starter for 1 day and then the experimental diets for 5 or 10 days. The specific activity of the hemin from 5 day-deficient bloods was 253 c.p.m. per mg. of hemin, but only 133 c.p.m. after 10 days of deficiency. The percentage stimulation over the deficiency levels by added pyridoxal phosphate was comparable in the two groups. In the 5 day-deficient blood samples, added coenzyme increased the value to 416 (61 per cent). In the 10 day group, four of the five blood samples were stimu-

Table I

<table>
<thead>
<tr>
<th></th>
<th>5 days on diet</th>
<th>10 days on diet</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Plus B&lt;sub&gt;6&lt;/sub&gt;-PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Plus B&lt;sub&gt;6&lt;/sub&gt;-PO&lt;sub&gt;4&lt;/sub&gt;*</td>
</tr>
<tr>
<td>Control whole blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with glycine-2-C&lt;sup&gt;14&lt;/sup&gt;</td>
<td>594 ± 63 (4)</td>
<td>579 ± 111 (3)</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt;-deficient whole blood with glycine-2-C&lt;sup&gt;14&lt;/sup&gt;</td>
<td>253 ± 20 (5)</td>
<td>410 ± 52 (5)</td>
</tr>
<tr>
<td>Control hemolysate with ALA-2,3-C&lt;sup&gt;14&lt;/sup&gt;</td>
<td>130 ± 4 (4)</td>
<td>‡</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt;-deficient hemolysate with ALA-2,3-C&lt;sup&gt;14&lt;/sup&gt;</td>
<td>121 ± 29 (3)</td>
<td>‡</td>
</tr>
</tbody>
</table>

* 0.3 mg. of pyridoxal phosphate monohydrate was added to each vessel containing 2 ml. of duck blood. The total incubation volume was 2.3 ml.
† The numbers in parentheses refer to the number of ducklings.
‡ In other experiments the addition of pyridoxal-5-phosphate did not increase the ALA incorporation.

Table I also shows the effects of vitamin B<sub>6</sub> deficiency on the incorporation into heme of radioactive ALA by red cell hemolysates. After 5 days there was little difference, and after 10 days the specific activity of the heme from the deficient bloods was approximately 60 per cent of that of the control values. Pyridoxal-5-phosphate did not stimulate ALA incorporation into heme.

Studies with sodium succinate-2-C<sup>14</sup> gave results very similar to those found with glycine (Table II). Therefore, it appears that pyridoxal-5-phosphate acts specifically in the utilization of glycine and succinate for ALA synthesis.
0.1 to 0.5 mg. of pyridoxal phosphate added to 2 ml. of deficient blood gave optimal stimulation; 1 mg. did not stimulate the deficient blood to the same extent as did the lower levels. When added to control bloods, 1 mg. of pyridoxal-5-phosphate caused a slight depression. Pyridoxamine phosphate was at least as effective as pyridoxal phosphate when added to deficient bloods; 1 mg. levels were not inhibitory to control bloods. Pyridoxal hydrochloride, pyridoxamine dihydrochloride, and pyridoxine hydrochloride did not stimulate labeled glycine incorporation.

The results with glycine, succinate, and ALA with and without pyridoxal-5-phosphate were qualitatively similar whether vitamin B₆-deficient whole blood or hemolysates were used (Table II). Glycine and succinate were incorporated into heme more efficiently by intact cells, whereas ALA was utilized better by hemolysates, which is in agreement with other reports (42). Glycine and succinate incorporation was stimulated by pyridoxal-5-phosphate in both the intact deficient cells and the hemolysates, but no such effect was observed with ALA.

To test whether the anemia seen in vitamin B₆-deficient ducks is due solely to a retarded synthesis of ALA, day-old ducklings fed the deficient diet were injected subcutaneously with half neutralized sterile solutions of ALA hydrochloride (10 mg. in 0.2 ml. of saline daily) during the last 7 of the 11 days on which they were maintained on the deficient diet. Four ducklings were used in each of the following: (1) control, (2) vitamin B₆-deficient, and (3) vitamin B₆-deficient treated with ALA. At the end of 11 days, the average weights were 208, 80, and 73 gm., respectively. Hemoglobin levels were 9.4, 6.7, and 6.1 per cent, respectively, showing that the ALA did not correct the anemia. The amount of ALA administered probably was not limiting. The daily dose of ALA could provide heme for 120 mg. of hemoglobin daily, provided that none was diverted. The hemo-

### Table II

<table>
<thead>
<tr>
<th>Glycine, Succinate, and ALA Incorporation in Vitamin B₆-Deficient Whole Blood As Compared to Hemolysates*</th>
</tr>
</thead>
<tbody>
<tr>
<td>The values are given in counts per minute per mg. of hemin.</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Succinate</td>
</tr>
<tr>
<td>ALA</td>
</tr>
</tbody>
</table>

* The figures represent the average of two experiments carried out with pooled blood from several ducklings.
globin deficit in the anemic duck at the end of the experiment, when compared to control hemoglobin levels, can be calculated to be approximately 0.21 gm. (9.4 - 6.7 = 2.7 gm. per 100 ml. or 200 mg. per 8 ml., assuming the volume of blood to be approximately 10 per cent of the body weight of 80 gm.). Since the ALA was administered for 7 days, the utilization would have needed to be 0.21/0.84 or about 25 per cent efficient. Administering ALA to control ducklings did not influence the hemoglobin levels.

In a second similar experiment in which three ducklings were injected with ALA, the livers and intestines were examined for free porphyrin content. 10 mg. of ALA were injected daily for the first 6 days and 20 mg. daily for 2 additional days. 1 day later the animals were killed. Although the deficient ducks remained anemic, the livers and intestines from each contained an average of 72 and 24 γ of porphyrin, respectively. This porphyrin was of a single kind, as demonstrated by paper chromatography in lutidine-water-NH₃ (43), and was identified as protoporphyrin 9 by paper chromatography of the methyl ester (44), absorption spectrum (45), and melting point (45). Similar results were obtained with control ducklings.

Administered ALA has been found to be a precursor of heme (15, 16). From the appearance of protoporphyrin 9 in the tissues due to ALA administration, it would seem that the anemia in vitamin B₆-deficient ducklings is not due solely to a deficiency of precursors of protoporphyrin 9. Therefore, although pyridoxal phosphate appears to be necessary for the utilization of glycine and succinate for ALA and heme synthesis, this vitamin must also be essential elsewhere in the process of red cell formation.

The decreased incorporation of glycine and succinate into heme when incubated with bloods from pantothenic acid-deficient ducklings is shown in Table III. When the measurements were made with blood from ducklings after only 2.5 days on the deficient diet (after 1 day on starter mash), the specific activity of the hemin from incubation with glycine was 420 c.p.m. per mg. of hemin as compared to 803 c.p.m. for normal bloods. There was no difference in specific activities when hemolysates from deficient and control bloods were incubated with ALA. The weights of the deficient and control ducklings were 78 and 101 gm., respectively. On a prolonged deficiency of 6 or 7 days after 5 days of starter mash, the weights of the deficient and control animals were 114 and 280 gm., respectively. The glycine incorporation with either whole blood or a hemolysate fell to very low levels (approximately one-fifth of normal), and, under these conditions, ALA incorporation was also decreased, but by no more than 50 per cent in one experiment or 25 per cent in another. It is apparent that the system utilizing glycine and succinate for heme synthesis is more
susceptible to pantothenic acid deficiency than is the system which utilizes ALA. This would be expected if the succinate is converted to succinyl

**TABLE III**

*Incorporation of Glycine, Succinate, and ALA into Heme by Blood from Pantothenic Acid-Deficient Ducklings*

The values are given in counts per minute per mg. of hemin.

<table>
<thead>
<tr>
<th>Days on diet</th>
<th>Glycine</th>
<th>ALA</th>
<th>Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole blood</td>
<td>Hemolysate</td>
<td>Whole blood</td>
</tr>
<tr>
<td></td>
<td>Deficient</td>
<td>Control</td>
<td>Deficient</td>
</tr>
<tr>
<td>Whole blood</td>
<td>Deficient pooled</td>
<td>Control pooled</td>
<td>Deficient pooled</td>
</tr>
<tr>
<td>1</td>
<td>2.5</td>
<td>420 ±45 (5)*</td>
<td>803 ±82 (4)</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>107 ±24 (3)</td>
<td>662 ±162 (3)</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
<td>222 ±35 (4)</td>
<td>904 ±81 (4)</td>
</tr>
</tbody>
</table>

The ducklings were not anemic in 7 days, since the hemoglobin levels were 7.83 and 8.0 per cent, respectively, for the control and deficient animals. The average weights were 280 and 114 gm.

* The numbers in parentheses indicate the number of experiments.

**TABLE IV**

*Effect of Calcium Pantothenate on Restoration of Heme Synthesis in Deficient Bloods*

The values are given in counts per minute per mg. of hemin.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>No. of ducks</th>
<th>Glycine</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + 1 mg. (in vitro) Ca pantothenate</td>
<td>6</td>
<td>579 ± 36</td>
<td>60</td>
</tr>
<tr>
<td>Deficient + 1 mg. (in vitro) Ca pantothenate</td>
<td>5</td>
<td>551 ± 46</td>
<td>56</td>
</tr>
<tr>
<td>Deficient + 5 &quot; (in vivo) &quot;</td>
<td>7</td>
<td>422 ± 12</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>426 ± 14</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>579 ± 51</td>
<td>60</td>
</tr>
</tbody>
</table>

* The samples in this experiment were counted in a Nuclear flow counter with a Micromil window which had 72 per cent of the counting efficiency of the windowless flow counter used for the other radioactive assays.

coenzyme A before reacting with glycine, as suggested by Shemin and Russell (15). Attempts were made to restore the rates of glycine and succinate uptake to normal by the addition of coenzyme A in vitro to the deficient bloods, but without success.

Olson and Kaplan (46) found that pyruvate oxidation by pantothenic
acid-deficient duck liver was not stimulated by the addition of coenzyme A or calcium pantothenate in vitro, but the injection of calcium pantothenate 1 to 2 hours before the animals were killed was effective. To test whether these techniques could restore heme synthesis, blood from 7 day-old pantothenic acid-deficient ducklings (4 days on mash, 3 days on the deficient diet) was incubated with radioactive glycine or ALA with and without 1 mg. of calcium pantothenate; other ducklings were injected subcutaneously with 5 mg. of the vitamin 1 hour before the bloods were drawn (Table IV). When calcium pantothenate was injected, the rate of glycine incorporation into heme was restored to that found with the control bloods. However, the addition of calcium pantothenate in vitro was ineffective.

**SUMMARY**

1. The rate of heme synthesis from glycine-2-C\(^{14}\) or succinate-2-C\(^{14}\) in the red cells of vitamin B\(_{12}\)- and pantothenic acid-deficient ducklings was much lower than that in normal cells, whereas \(\delta\)-aminolevulinic acid incorporation was essentially normal. The addition of pyridoxal-5-phosphate in vitro stimulated the ability of the vitamin B\(_{12}\)-deficient cells to synthesize heme from glycine and succinate. This property was shared by pyridoxamine phosphate, but not by pyridoxal, pyridoxamine, or pyridoxine. The incorporation of \(\delta\)-aminolevulinic acid was unaffected by the addition of pyridoxal-5-phosphate. The injection of pantothenate into pantothenic acid-deficient ducklings 1 hour before the bloods were drawn restored the glycine incorporation to control values; the addition of calcium pantothenate in vitro was ineffective.

2. The administration of \(\delta\)-aminolevulinic acid to vitamin B\(_{12}\)-deficient ducklings did not prevent anemia.

**BIBLIOGRAPHY**

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