RELATION OF DIETARY FOLIC ACID AND VITAMIN B₁₂ TO ENZYME ACTIVITY IN THE CHICK

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The relationship of folic acid (PGA) to certain enzyme systems has recently been the subject of investigation by workers in several laboratories. In 1948 Kalckar and Klenow (1-3) demonstrated the inhibitory effect of folic acid upon milk xanthopterin oxidase in vitro. Subsequently Keith et al. (4), studying the effect of dietary PGA upon enzymes, observed that activity of xanthine oxidase in chick liver was strongly depressed as the concentration of folic acid was increased in the ration. Quite recently Kelley (5) observed that rat liver d-amino acid oxidase activity was decreased to some extent by feeding PGA.

Although the relationship of folic acid and vitamin B₁₂ to hemoglobin production is quite well known (6), some of the basic metabolic functions of these two vitamins, particularly of vitamin B₁₂, are at present obscure.

In view of this information the effects of variations in dietary intake of vitamin B₁₂ and folic acid on the following enzyme systems were investigated: xanthine oxidase, d-amino acid oxidase, endogenous respiration, and catalase. Since we have experimental evidence¹ that folic acid strongly inhibits endogenous respiration of rat liver in vitro, the effects of dietary folic acid and vitamin B₁₂ upon endogenous oxidative activity have been studied. The influence of the two vitamins in question upon liver catalase activity has been investigated for several reasons. First, the porphyrin group is common to both catalase and hemoglobin; second, catalase destroys metabolic hydrogen peroxide, which is produced by oxidation of d-amino acids and xanthine; and third, metabolic hydrogen peroxide is believed to be involved in the destruction of hemoglobin (7).

As a preliminary investigation into the metabolic disorder producing paralytic symptoms in chicks on a folic acid-deficient diet containing

¹ Williams, J. N., Jr., and Elvehjem, C. A., in press.
vitamin $B_{12}$ (6), blood glucose determinations were made on the various groups of experimental animals.

**EXPERIMENTAL**

Straight run (New Hampshire males $\times$ single comb white Leghorn females) cross-bred chicks which were the offspring of hens fed Diet B-1 (8) were used as experimental animals. The chicks were housed in electrically heated batteries with raised screen floors and were fed and watered *ad libitum*. Weights and hemoglobin values of the chicks were recorded just prior to use in the enzyme studies. Blood samples for hemoglobin and glucose determinations were taken by direct puncture of a wing vein.

Throughout the feeding period all chicks received a folic acid-deficient ration containing sucrose 61, alcohol-extracted casein 18, gelatin 10, Salts V (9) 6, soy bean oil 5, L-cystine 0.3, and fortified haliver oil (60,000 U. S. P. units of vitamin A, 6000 A. O. A. C. units of vitamin $D_{3}$ per gm.) 0.4 per cent; thiamine hydrochloride 0.3, riboflavin 0.6, nicotinic acid 5.0, pyridoxine hydrochloride 0.4, calcium pantothenate 2.0, biotin 0.02, choline chloride 150, inositol 100, 2-methyl-1,4-naphthoquinone 0.05, and $\alpha$-tocopherol 0.3 mg. per cent.

Four groups of chicks were maintained on the synthetic ration for 4 weeks before being used in the enzyme studies. When supplemented with folic acid or vitamin $B_{12}$, the chicks received 200 $\gamma$ of folic acid$^2$ per 100 gm. of ration and 0.7 per cent charcoal-vitamin $B_{12}$ concentrate$^2$ (equivalent to approximately 3 $\gamma$ of vitamin $B_{12}$ per 100 gm. of ration). Some of the chicks were placed on the basal ration for 2 weeks before supplementation with PGA or vitamin $B_{12}$. The remainder received the supplements throughout the 4 week period. Each of the four groups of chicks received the following supplements to the basal ration, respectively: Group I, folic acid plus vitamin $B_{12}$; Group II, folic acid alone; Group III, vitamin $B_{12}$ alone; and Group IV no supplement. In addition to these four groups of chicks an additional group was maintained on a good stock ration for 4 weeks.

When used in the enzyme experiments, the animals were decapitated and bled. The livers were removed immediately, chilled in cracked ice, blotted free of moisture, and weighed. A portion of each liver was homogenized for 3 minutes in a Potter-Elvehjem homogenizer with 5 volumes of ice-cold 0.039 M sodium potassium phosphate buffer (10) and strained through gauze.

$^2$ We are indebted to Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for the folic acid and to Merck and Company, Inc., Rahway, New Jersey, for the vitamin $B_{12}$ concentrate.
Xanthine oxidase activity of the homogenates was determined by a method outlined by Keith et al. (4). D-Amino acid oxidase was measured by the method of Axelrod, Sober, and Elvehjem (11), and catalase by the method of Jolles (12). The first 10 minute oxygen uptake before the substrate was added in the d-amino acid oxidase determinations was taken as a measure of endogenous respiration of the livers.

Liver nitrogen and dry weight determinations were made in most cases. Whenever possible, enzyme activity, blood glucose, nitrogen, and dry weight determinations were made on the same animals.

### Table I

<table>
<thead>
<tr>
<th>Ration supplement</th>
<th>Liver enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA, vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>0.07 Units = μl. O&lt;sub&gt;2&lt;/sub&gt; uptake per hr. per gm. liver at 30°</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>1.00 Units = μl. O&lt;sub&gt;2&lt;/sub&gt; uptake per hr. per gm. liver at 30°</td>
</tr>
<tr>
<td>None</td>
<td>3.95 Units = μl. O&lt;sub&gt;2&lt;/sub&gt; uptake per hr. per gm. liver at 30°</td>
</tr>
<tr>
<td>PGA, vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>2.0 Units = mg. uric acid formed per 1½ hrs. per gm. liver at 25°</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>1.6 Units = mg. uric acid formed per 1½ hrs. per gm. liver at 25°</td>
</tr>
<tr>
<td>None</td>
<td>5.3 Units = mg. uric acid formed per 1½ hrs. per gm. liver at 25°</td>
</tr>
<tr>
<td>PGA, vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>2.4 Units = mg. uric acid formed per 1½ hrs. per gm. liver at 25°</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>2.4 Units = mg. uric acid formed per 1½ hrs. per gm. liver at 25°</td>
</tr>
<tr>
<td>None</td>
<td>5.3 Units = mg. uric acid formed per 1½ hrs. per gm. liver at 25°</td>
</tr>
<tr>
<td>PGA, vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>12.10 Units = μl. O&lt;sub&gt;2&lt;/sub&gt; uptake per hr. per gm. liver at 30°</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>18.80 Units = μl. O&lt;sub&gt;2&lt;/sub&gt; uptake per hr. per gm. liver at 30°</td>
</tr>
<tr>
<td>None</td>
<td>24.10 Units = μl. O&lt;sub&gt;2&lt;/sub&gt; uptake per hr. per gm. liver at 30°</td>
</tr>
<tr>
<td>PGA, vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>94 Units = monomolecular reaction constant at 0° × dilution of liver</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>97 Units = monomolecular reaction constant at 0° × dilution of liver</td>
</tr>
<tr>
<td>None</td>
<td>108 Units = monomolecular reaction constant at 0° × dilution of liver</td>
</tr>
</tbody>
</table>

### RESULTS AND DISCUSSION

The results of the enzyme determinations are presented in Table I. Enzyme activities for the two lots of chicks given supplements of the vitamins in question throughout the 4 week feeding period or for only the last 2 weeks have been averaged together, since both lots gave essentially the same results.

Calculation of the enzyme activities in terms of wet weight, nitrogen, or dry weight of the liver gives essentially the same picture. For this reason we have reported the results upon a wet weight basis only.

When both PGA and vitamin B<sub>12</sub> are absent from the diet, activity of
n-amino acid oxidase in the liver is about 4 times greater than that observed when PGA is present. However, if vitamin B₁₂ is present without PGA in the diet, a 7-fold increase in n-amino acid oxidase activity is observed. It appears, therefore, that vitamin B₁₂ and folic acid have dissimilar effects upon this enzyme. When folic acid is included in the diet, however, the stimulatory effect of vitamin B₁₂ disappears.

In contrast to the results with n-amino acid oxidase, xanthine oxidase activity is not increased by the level of vitamin B₁₂ fed, even if PGA is omitted from the ration. In fact, vitamin B₁₂ appears to depress activity of this enzyme slightly when PGA is absent from the diet. Since both n-amino acid oxidase and xanthine oxidase are flavin enzymes, the apparent inhibition of activity by folic acid can possibly be explained by a direct action of the vitamin upon the enzyme protein or prosthetic group. This action is perhaps competitive in nature in view of the structural similarities of the pterin nucleus both to the purines and to the flavin group. However, the fact that n-amino acid oxidase activity is higher when vitamin B₁₂ is included in the diet (in the absence of PGA) is perhaps explainable by an increased synthesis of enzyme protein rather than a direct stimulation of the enzyme already present. This point remains to be checked by direct in vitro studies with vitamin B₁₂, however.

From the results in Table I, it appears that dietary PGA at the levels employed has only a negligible influence upon endogenous respiration. These results are contrary to those obtained when PGA is added directly to a flask containing rat liver homogenate. It is possible that the effects of the relatively low dietary levels upon endogenous oxygen consumption are obscured by dilution of the vitamin in the tissues of the intact animal. It is also conceivable that the depressant effects of folic acid occur mainly in relation to specific flavin enzymes. Consequently, if most of the endogenous respiration in chick liver is due to oxidation of metabolites not involving the flavin enzymes in question, the depressant effect of PGA upon endogenous oxygen consumption may not be easily apparent.

From the results in Table I, it appears that catalase activity is independent of either dietary PGA or vitamin B₁₂. It was thought that perhaps the activity of catalase would increase when a greater need for it appeared, as in a folic acid deficiency. On the other hand, there is certainly no decrease in catalase activity when PGA is absent from the diet. It would appear, therefore, that the decrease in blood hemoglobin concomitant with a folic acid deficiency is due to an effect other than inadequate porphyrin synthesis.

The results of the enzyme determinations for the group of stock chicks are somewhat different in endogenous respiration and catalase activity from the results of the chicks on synthetic rations, although all of the animals employed were comparable in age and heredity. The reasons
for these differences are not clear, although they possibly reflect a healthier status of the animals on stock ration because of the presence of unknown dietary factors.

Values for average chick weights, hemoglobin, ratios of liver to body weight, and blood glucose for the different groups of chicks on synthetic ration are reported in Table II. The results from those animals receiving supplements of PGA or vitamin B\textsubscript{12} throughout the 4 week period are presented, since blood glucose was determined only upon these chicks. It appears that, although vitamin B\textsubscript{12} alone exerts little influence upon hemoglobin formation, it has a strong effect in increasing the body weight of the chicks. Folic acid alone has a similar but greater effect upon chick weight. Under our experimental conditions weight response is optimal only when both vitamins are given.

From the work of Keith et al. (4) it appears that dietary folic acid is inversely related to the ratio of liver to body weight. Our results indicate

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
Ration supplement & Average weight of chicks & Hemoglobin & Ratio of liver to body weight & Blood glucose \\
& & & & \\
\hline
Folic acid, vitamin B\textsubscript{12} & 309 & 9.1 & 0.028 & 181 \\
Vitamin B\textsubscript{12} & 263 & 8.6 & 0.029 & 180 \\
None & 146 & 5.2 & 0.036 & 246 \\
None & 90 & 6.8 & 0.036 & 238 \\
\hline
\end{tabular}
\caption{Effect of Dietary Folic Acid and Vitamin B\textsubscript{12} on Weight and Blood Constituents in Chicks}
\end{table}

\* Six chicks in each group.

this to be the case, and that vitamin B\textsubscript{12}, even in the absence of PGA, has little influence upon this ratio.

The results of blood glucose determinations indicate that in the absence of dietary folic acid blood sugar concentration is increased. Dietary vitamin B\textsubscript{12}, however, at the levels employed appears to have little effect.

The apparently synergistic action of folic acid and vitamin B\textsubscript{12} upon weight and hemoglobin of the chick is not clearly indicated among the metabolic factors we have studied in this paper. In only one case, D-amino acid oxidase, do we find a positive effect of vitamin B\textsubscript{12} upon enzyme activity, and this effect is unlike that of folic acid.

\textbf{SUMMARY}

Studies on the influence of dietary folic acid and vitamin B\textsubscript{12} on D-amino acid oxidase, xanthine oxidase, catalase, and endogenous respiration of chick liver have been presented.
Reports that dietary folic acid is related inversely to the two flavin enzymes have been confirmed. It appears that catalase and endogenous respiration are not directly controlled by either vitamin B_{12} or PGA.

Chicks fed a diet supplemented with vitamin B_{12} without folic acid show higher D-amino acid oxidase activity than those not receiving vitamin B_{12}.

Vitamin B_{12} augments the effect of PGA in weight responses.

Dietary folic acid is inversely related to blood glucose, while under the conditions of our experiments vitamin B_{12} appears to have little direct relationship to this blood component.

**BIBLIOGRAPHY**

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