OBSERVATIONS ON THE INTERRELATION OF VITAMIN $B_{12}$, FOLIC ACID, AND VITAMIN C IN THE CHICK*

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It is well established that there is a definite relationship between folic acid, vitamin $B_{12}$, and vitamin C. In the past two decades it has become apparent that vitamin C is related, directly or indirectly, to hematopoiesis (1). It has been shown that folic acid and vitamin C in vivo correct the abnormal tyrosine metabolism in the scorbutic guinea pig (2, 3).

Rodney, Swendseid, and Swanson (4) have shown that tyrosine oxidation in livers of folic acid-deficient rats, in which the deficiency was induced by sulfasuxidine, was significantly lower than that of a normal animal. They also showed that either liver extract, vitamin C, or folic acid would correct this abnormality in vivo but that only folic acid would correct it in vitro.

In 1944, Briggs et al. (5) observed a growth stimulation in chicks when vitamin C was added to a purified diet containing crude concentrates of folic acid. In 1946, it was shown that synthetic folic acid (6) and recently that vitamin $B_{12}$ (7) also stimulate growth, in the chick, on a semipurified ration.

It is the purpose of this paper to attempt to clarify, in part, the interrelationship of these three vitamins.

EXPERIMENTAL

Straight run (New Hampshire $\delta^0 \delta^0 \times$ single comb white Leghorns $\Omega \Omega$) cross-bred chicks, which were the progeny of hens fed Diet R-1

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We are indebted to Merck and Company, Inc., Rahway, New Jersey, for crystalline vitamins; to the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for synthetic folic acid; to the Abbott Laboratories, North Chicago, Illinois, for haliver oil; and to E. I. du Pont de Nemours and Company, Inc., New Brunswick, New Jersey, for crystalline vitamin D$_3$.

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described previously (8), were used in all studies. The chicks were housed in electrically heated batteries with raised screen floors. Feed and water were supplied ad libitum. The chicks were wing-banded and weighed at 1 day of age. Weights were recorded at weekly intervals.

All chicks were originally placed on a folic acid-deficient, purified ration containing sucrose 61 gm., alcohol-extracted casein 18 gm., gelatin 10 gm., Salts 5 (9) 6 gm., soy bean oil 5 gm., L-cystine 0.3 gm., thiamine hydrochloride 0.3 mg., riboflavin 0.6 mg., nicotinic acid 5.0 mg., pyridoxine hydrochloride 0.4 mg., calcium pantothenate 2.0 mg., choline chloride 150 mg., biotin 0.03 mg., inositol 100 mg., 2-methyl-1,4-naphthoquinone 0.05 mg., and $\alpha$-tocopherol 0.3 mg. Fortified haliver oil (60,000 U. S. P. units of vitamin A, 6000 U. S. P. units of vitamin D$_3$ per gm.) was given by dropper (2 drops per bird per week).

At the termination of the depletion period, which in all cases was 2 weeks, the chicks were sorted and divided into groups according to weight and per cent gain. A test period of 2 weeks followed, at the termination of which the birds were sacrificed, and the livers removed, frozen immediately, and stored at freezing temperatures until assayed. Individual livers were homogenated in a Waring blendor with enough water to bring the concentration to 0.2 gm. per ml. Aliquots were then taken for both folic acid and vitamin B$_12$ determinations. Folic acid was extracted by autolysis of a 2.5 ml. aliquot of the homogenate in 10 ml. of a citrate-phosphate buffer at pH 4.5 for 18 to 20 hours at 37°C. Autolysis at this pH has been shown to be satisfactory in obtaining maximum release in chick liver tissue (10, 11). Folic acid was measured microbiologically with Streptococcus faecalis R as a test organism with the medium of Luckey et al. (12).

Vitamin B$_12$ was extracted as follows: 2.5 ml. of liver homogenate were added to 10 ml. of 0.8 per cent NaHCO$_3$ solution, boiled for 5 minutes in a boiling water bath, and cooled, and 100 mg. of trypsin (Difco) were added to each sample. Toluene was added as a preservative and the samples were digested 24 to 30 hours at 37°C. After incubation, the samples were neutralized, autoclaved 5 minutes at 120°C, diluted, filtered, and assayed. Blanks were run with each extraction. It has been shown that this method will give maximum release of vitamin B$_12$ in chick liver tissue.$^1$ Vitamin B$_12$ was measured microbiologically with Lactobacillus leichmannii ATCC 4797 with the medium of Skeggs et al. (13), modified by the substitution of 10 ml. of fresh tomato juice per 100 ml. of double strength medium for the enzymatic digest of casein.

In all cases, the terms folic acid and vitamin B$_12$ will refer to Streptococcus faecalis R and Lactobacillus leichmannii activity respectively and

$^1$ Unpublished data from this laboratory.
will include those compounds that are active in promoting growth under the test conditions employed.

In Experiment 1, groups of twelve chicks each were used except in the groups receiving 200 and 500 $\gamma$ of folic acid per 100 gm. of ration in which ten birds were used per group. In Experiment 2 groups of twelve birds were used throughout. Crystalline vitamin $B_{12}$ was injected into the pectoral muscle with a 1 ml. calibrated syringe.

**RESULTS AND DISCUSSION**

The results are presented in Table I. The administration of vitamin C or vitamin $B_{12}$, alone or together, gives a small growth response. Low levels of folic acid produce a greater response, but the addition of vitamin C and vitamin $B_{12}$, or both, gives a further increase. In the series receiving 500 $\gamma$ of folic acid per 100 gm. of ration neither vitamin C nor vitamin $B_{12}$ produces any further response under the conditions of the experiment.

There appears to be a direct correlation between the level of folic acid stored in the liver and the rate of growth obtained. The addition of either vitamin C or vitamin $B_{12}$ with or without folic acid gives consistent if not significant increases in the level of folic acid stored in the liver. Administration of both vitamin C and vitamin $B_{12}$ raises the level of stored folic acid beyond that of either alone. When 500 $\gamma$ of folic acid per 100 gm. of ration are added to the basal ration, the addition of either vitamin C or vitamin $B_{12}$ has no effect on the level of stored folic acid.

As the level of folic acid is increased, the significance of increased folic acid stored in the liver induced by the addition of vitamin C, vitamin $B_{12}$, or both, becomes less. This is in confirmation of the work of Moore et al. (14) who showed that the higher the intake of folic acid the more inefficient is its storage in the liver. This trend is followed by the growth data which show that the stimulation produced by vitamin $B_{12}$, vitamin C, or both is relatively greater with low levels of folic acid and decreases as the folic acid level is increased. From such a comparison, it may be assumed that vitamin $B_{12}$ and vitamin C function, in part, by stimulating the synthesis of folic acid. Thus the administration of either vitamin $B_{12}$, vitamin C, or both, is the equivalent of the addition of a higher level of folic acid. As further evidence, a comparison of Experiments 1 and 2 shows poorer growth in Experiment 2, but a more significant growth stimulation upon the addition of vitamin C, vitamin $B_{12}$, or both. The folic acid stored in the liver follows the same trend. Further comparison of the folic acid stored in the livers of the groups receiving the unsupplemented basal ration shows that the chicks used in Experiment 2 were depleted of folic acid to a greater extent than those in Experiment 1. This accounts, in part, for the differences and again emphasizes the need for ex-
Experimental animals from a controlled source; i.e., chicks which are the progeny of hens on a strictly controlled diet.

**Table I**

Effect of Vitamin B12, Folic Acid, and Vitamin C on Chick Growth and Folic Acid and Vitamin B12 Content of Livers

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Supplement</th>
<th>Weight gain during test (gm)</th>
<th>Vitamin B12 per liver per bird (ug)</th>
<th>Folic acid per liver per bird (ug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>19 (11)</td>
<td>0.05 ± 0.05</td>
<td>2.82 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12 (0.2 γ per day)†</td>
<td>40 (12)</td>
<td>0.12 ± 0.07</td>
<td>5.70 ± 2.76</td>
</tr>
<tr>
<td></td>
<td>Vitamin C (100 mg. %)</td>
<td>33 (12)</td>
<td>0.06 ± 0.05</td>
<td>4.65 ± 1.26</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12 (0.2 γ per day)‡ + vitamin C (100 mg. %)</td>
<td>50 (12)</td>
<td>0.24 ± 0.06</td>
<td>10.04 ± 4.15</td>
</tr>
<tr>
<td></td>
<td>50 γ folic acid per 100 gm. ration</td>
<td>73 (11)</td>
<td>0.16 ± 0.04</td>
<td>4.03 ± 1.78</td>
</tr>
<tr>
<td></td>
<td>50 γ folic acid per 100 gm. ration + vitamin B12 (0.2 γ per day)‡</td>
<td>108 (12)</td>
<td>0.28 ± 0.02</td>
<td>4.27 ± 2.48</td>
</tr>
<tr>
<td></td>
<td>50 γ folic acid per 100 gm. ration + vitamin B12 (0.2 γ per day)‡ + vitamin C (100 mg. %)</td>
<td>90 (11)</td>
<td>0.42 ± 0.18</td>
<td>6.58 ± 2.34</td>
</tr>
<tr>
<td></td>
<td>200 γ folic acid per 100 gm. ration</td>
<td>121 (11)</td>
<td>0.61 ± 0.42</td>
<td>15.13 ± 4.81</td>
</tr>
<tr>
<td></td>
<td>200 γ folic acid per 100 gm. ration + vitamin B12 (0.2 γ per day)‡</td>
<td>128 (10)</td>
<td>0.15 ± 0.06</td>
<td>15.60 ± 3.45</td>
</tr>
<tr>
<td></td>
<td>200 γ folic acid per 100 gm. ration + vitamin C (100 mg. %)</td>
<td>131 (10)</td>
<td>0.15 ± 0.03</td>
<td>17.66 ± 2.44</td>
</tr>
<tr>
<td></td>
<td>200 γ folic acid per 100 gm. ration + vitamin B12 (0.2 γ per day)‡ + vitamin C (100 mg. %)</td>
<td>153 (10)</td>
<td>0.10 ± 0.03</td>
<td>20.70 ± 3.28</td>
</tr>
<tr>
<td></td>
<td>500 γ folic acid per 100 gm. ration</td>
<td>150 (10)</td>
<td>0.18 ± 0.05</td>
<td>22.61 ± 4.49</td>
</tr>
<tr>
<td></td>
<td>500 γ folic acid per 100 gm. ration + vitamin B12 (0.2 γ per day)‡ + vitamin C (100 mg. %)</td>
<td>152 (10)</td>
<td>0.08 ± 0.02</td>
<td>14.05 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>500 γ folic acid per 100 gm. ration + vitamin C (100 mg. %)</td>
<td>147 (10)</td>
<td>0.16</td>
<td>17.50</td>
</tr>
<tr>
<td></td>
<td>500 γ folic acid per 100 gm. ration + vitamin B12 (0.2 γ per day)‡ + vitamin C (100 mg. %)</td>
<td>148 (10)</td>
<td>0.15</td>
<td>13.62</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>8 (7)</td>
<td>0.01 ± 0.00</td>
<td>1.20 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12 (0.1 γ per day)‡</td>
<td>22 (10)</td>
<td>0.17 ± 0.07</td>
<td>2.96 ± 1.43</td>
</tr>
<tr>
<td></td>
<td>Vitamin C (100 mg. %)</td>
<td>14 (8)</td>
<td>0.05 ± 0.04</td>
<td>2.11 ± 0.85</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12 (0.1 γ per day)‡ + vitamin C (100 mg. %)</td>
<td>33 (11)</td>
<td>0.54 ± 0.13</td>
<td>4.44 ± 0.85</td>
</tr>
<tr>
<td></td>
<td>Liver extract (0.5 U. S. P. units per day)‡</td>
<td>87 (11)</td>
<td>0.58 ± 0.16</td>
<td>3.29 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>200 γ folic acid per 100 gm. ration</td>
<td>112 (12)</td>
<td>0.51 ± 0.53</td>
<td>6.87 ± 1.55</td>
</tr>
<tr>
<td></td>
<td>200 γ folic acid per 100 gm. ration + vitamin B12 (0.1 γ per day)‡</td>
<td>134 (12)</td>
<td>0.52 ± 0.18</td>
<td>12.20 ± 2.12</td>
</tr>
<tr>
<td></td>
<td>200 γ folic acid per 100 gm. ration + vitamin C (100 mg. %)</td>
<td>126 (12)</td>
<td>0.30 ± 0.11</td>
<td>11.73 ± 4.30</td>
</tr>
</tbody>
</table>
Comparison of the groups receiving low levels of folic acid shows that
the chick has the ability, although of uncertain efficiency, to store vita-
mamin B$_{12}$.

The ability of the chick to synthesize vitamin B$_{12}$ and to utilize
the product is clearly shown. A comparison of the basal group in either ex-
periment with groups receiving folic acid but no vitamin B$_{12}$ shows a sig-
nificantly higher level of vitamin B$_{12}$ in the livers of the groups receiving
folic acid and no vitamin B$_{12}$ than in groups receiving no folic acid and
no vitamin B$_{12}$. From these observations, it may be concluded that folic
acid stimulates the synthesis of vitamin B$_{12}$. The effect of vitamin C,
either alone or with folic acid, on the synthesis of vitamin B$_{12}$ is doubtful.

That folic acid is synthesized by intestinal flora has been proved beyond
any reasonable doubt. Even in the chick, which requires folic acid on a
highly purified ration, the addition of 1 per cent sulfasuxidine to the diet
increases the folic acid requirement 3-fold (6). It has been shown that
vitamin C or other reducing agents stimulate the growth of certain micro-
organisms in vitro (15, 16). It is apparent that in these cases the action
of vitamin C is that of an oxidation-reduction mechanism rather than
that of a specific vitamin reaction.

The action of vitamin B$_{12}$ in stimulating the synthesis of folic acid is
more obscure. It is improbable that this action is one of a specific vita-
mamin function, but rather it is that of an indirect action. It is possible
that this indirect action is also, in part, that of an oxidation-reduction
mechanism, since it has been shown (17) that the cobalt in vitamin B$_{12}$
appears to be a six group coordination complex and that vitamin B$_{12}$ con-
tains pyrrole-like compounds (18), suggesting a porphyrin nucleus.

### Table I—Concluded

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Supplement</th>
<th>Weight gain* during test</th>
<th>Vitamin B$_{12}$ per liver per bird†</th>
<th>Folic acid per liver per bird†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gm.</td>
<td>γ</td>
<td>γ</td>
</tr>
<tr>
<td>200 γ folic acid per 100 gm. ration + vitamin B$_{12}$ (0.1 γ per day) † + vitamin C (100 mg. %)</td>
<td>140 (12)</td>
<td>0.02 ± 0.00</td>
<td>21.35 ± 2.63</td>
<td></td>
</tr>
<tr>
<td>500 γ folic acid per 100 gm. ration</td>
<td>140 (12)</td>
<td>0.31 ± 0.17</td>
<td>17.97 ± 5.47</td>
<td></td>
</tr>
</tbody>
</table>

* The figures in parentheses represent the number of chicks surviving at the end of the test period.
† The values are the means of five individual livers and one pooled sample of remaining livers in Experiment 1 and seven individual livers and 1 pooled sample of remaining livers in Experiment 2.
‡ Injected.
Both vitamin C and vitamin B_{12} stimulate growth in the chick when fed a semipurified ration with or without folic acid. The addition of both vitamin C and vitamin B_{12} gives a greater growth response than either alone.

Both vitamin C and vitamin B_{12} stimulate the synthesis of folic acid \emph{in vivo}. The addition of both vitamin C and vitamin B_{12} produces an increase of stored liver folic acid, which is in excess of that produced by either alone.

Folic acid stimulates the synthesis of vitamin B_{12} in the chick, as measured by liver storage.

Possible mechanisms and interrelationships between folic acid, vitamin C, and vitamin B_{12} have been discussed.

**BIBLIOGRAPHY**

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