Subnormal Serum Folate and Macrocytosis Associated with Anticonvulsant Drug Therapy

By FREDERICK A. KLIPSTEIN

Since the original description by Mannheimer in 1952 of two cases of megaloblastic anemia occurring as a probable complication of anticonvulsant drug therapy with hydantoin derivatives,1 more than 60 cases have now been described in the literature.2-35 The clinical and hematologic aspects have been reviewed in detail elsewhere.25,31,34 It is now well established that megaloblastic anemia may occur as a complication of the administration of diphenylhydantoin (Dilantin, Phenytoin, Epanatim) either alone6,10,17 or in combination with other anticonvulsant agents,2,3,5,7,9,15,16,17,18,21,23,24,30,31,34,35 of Mysoline (primidone) alone4,12,13,20,24,26 and of barbiturates alone.14,22,27,32,33

The development of megaloblastic anemia associated with anticonvulsant drug therapy is considered to be the result of folic acid deficiency. In the past, the presence of folic acid deficiency has been implied by the finding of a megaloblastic anemia in association with normal serum vitamin B12 concentrations.2,4,5,14,17,25,29 More recently, direct evidence of folic acid deficiency has been obtained: subnormal values of serum folate, measured by assay with Lactobacillus casei, have been recorded in two patients36,37 increased urinary excretion of formiminoglutamic acid (FIGLu) has been reported in two subjects31,37 and lastly, Chanarin and his associates have observed an abnormally rapid plasma clearance of intravenous test doses of folic acid in some, but not all, patients.28,31,37 Studies of the absorption of folic acid12,14,25,27,28,33 and vitamin B122,4,10,18,25,27,29,31,35 have been uniformly normal. Since such causes of folic acid deficiency as malnutrition, malabsorption, and increased utilization of the vitamin have not been incriminated in these patients, it has been postulated that the anticonvulsant drugs produce folate deficiency by acting as weak folic acid antagonists.12,36,38,39 The studies of Hawkins and Meynell have suggested that deranged folic acid metabolism may not be limited exclusively to those patients who develop a megaloblastic anemia. These workers have reported mild macrocytosis in 45 per cent of a group of epileptics receiving anticonvulsant drug therapy. The macrocytosis disappeared following treatment with folic acid.10,41

The original observations of Baker, Herbert and their colleagues48,51 that the modified L. casei microbiological assay for folic acid factors in the serum relates well with the presence or absence of folic acid deficiency states has
now been confirmed by a number of laboratories.\textsuperscript{44-46,72} Herbert, Larrabee, and Buchanan have shown that the greater part of the activity measured in the serum by the \textit{L. casei} assay is due to 5-methyl-tetrahydrofolic acid.\textsuperscript{42} Subnormal concentrations of serum \textit{L. casei} folate are regularly observed in those megaloblastic anemias associated with folic acid deficiency.\textsuperscript{42-46,64,72} Two recent reports have suggested that subnormal serum folate levels may reflect partial depletion of total body stores of folic acid and may be observed prior to the onset of overt hematologic changes.\textsuperscript{47-48} Subnormal serum \textit{L. casei} folate values may also reflect deranged folic acid metabolism as is observed following the administration of such folic acid antagonists as Methotrexate\textsuperscript{45} and pyrimethamine.\textsuperscript{49}

The present report concerns the results of the \textit{L. casei} assay for serum folate, the \textit{L. leichmannii} assay for serum \textit{B12}, and observations of hematologic changes in a group of 65 epileptics, 60 of whom were receiving anticonvulsant drug therapy. In addition, results of in vivo folic acid clearance studies and in vitro studies of \textit{L. casei} growth following the addition of anticonvulsant agents are presented.

\textbf{METHODS AND MATERIALS}

The majority of the subjects studied were attending the outpatient Seizure Clinic of Vanderbilt Clinic; they were seen in the afternoon and hence blood samples were obtained 1 to 3 hours postprandially. Sixteen subjects were receiving Dilantin alone and an additional 37 subjects were receiving Dilantin plus one or more other anticonvulsant agents. Five subjects were receiving Mysoline and single additional patients were taking Mesantoin alone and phenobarbital alone. Five subjects (Cases 60-65) were epileptics who were not receiving anticonvulsant therapy at the time of the study.

The degree of macrocytosis was estimated on Wright-stained smears of peripheral blood by two independent observers who were unaware of the results of the serum folate assay at the time. Since the estimation of macrocytosis is a subjective approximation at best, no attempt at precise quantitation of the degree of macrocytosis was made and slides were reported as “none” if less than 2 per cent of the red cells were macrocytic, “slight” for 2 to 6 per cent, and “moderate” for greater than 6 per cent macrocytes. Serum \textit{B12} assays were performed using \textit{L. leichmannii};\textsuperscript{54} normal values in this laboratory range from 140 to 900 \mu g./ml. Serum \textit{L. casei} folate activity was determined by a modification\textsuperscript{45} of the technic described by Baker, Herbert, and their colleagues,\textsuperscript{51} with the further modification that the final volumes assayed were 6 ml. Samples were assayed on at least three different occasions and the averages are reported. In this laboratory, values of \textless{} 5 \mu g. per ml. are considered indicative of folic acid deficiency; values of 5 to 7 \mu g./ml. are diagnostically indeterminate; values of 7 to 18 \mu g./ml. are normal; and values greater than 25 \mu g./ml. are found in subjects on folic acid therapy or with primary vitamin \textit{B12} deficiency. Folic acid clearance studies were performed by the technic described by Chanarin, Mollin, and Anderson\textsuperscript{52} using a dosage of 15 \mu g. folic acid per Kg. body weight; the serum folic acid activity of the 3- and 15-minute samples were assayed with both \textit{Streptococcus faecalis} and \textit{L. casei}. In vitro studies of anticonvulsant drugs were conducted by the addition of 1 ml. of the drug in increasing concentrations to 1 ml. of a 150 mg. per cent ascorbic acid-phosphate buffer, 3 ml. of water, and 5 ml. of basal medium. The turbidity of the \textit{L. casei} growth was assayed after 18 hours incubation.

\textbf{RESULTS}

\textit{Serum Folate Levels}

The results of the hematologic studies and serum \textit{L. casei} folate and vitamin
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</tr>
<tr>
<td>48</td>
<td>31</td>
<td>F</td>
<td>11</td>
<td>Mysoline</td>
<td>1125</td>
<td>13.0</td>
<td>7,050</td>
<td>none</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>42</td>
<td>F</td>
<td>12</td>
<td>phenobarbital</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>23</td>
<td>M</td>
<td>12</td>
<td>phenobarbital</td>
<td>120</td>
<td>14.0</td>
<td>7,500</td>
<td>none</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>20</td>
<td>F</td>
<td>12</td>
<td>phenobarbital</td>
<td>1000</td>
<td>13.2</td>
<td>7,350</td>
<td>slight</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>52</td>
<td>17</td>
<td>F</td>
<td>13</td>
<td>phenobarbital</td>
<td>90</td>
<td>11.4</td>
<td>5,300</td>
<td>slight</td>
<td>5.2</td>
<td>550</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>55</td>
<td>F</td>
<td>15</td>
<td>phenobarbital</td>
<td>100</td>
<td>10.6</td>
<td>5,850</td>
<td>none</td>
<td>7.2</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>14</td>
<td>M</td>
<td>—</td>
<td>none</td>
<td>1000</td>
<td>15.7</td>
<td>7,500</td>
<td>none</td>
<td>7.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>66</td>
<td>F</td>
<td>—</td>
<td>none</td>
<td>1000</td>
<td>12.9</td>
<td>5,350</td>
<td>none</td>
<td>5.7</td>
<td>410</td>
<td></td>
</tr>
<tr>
<td>56</td>
<td>16</td>
<td>F</td>
<td>—</td>
<td>none</td>
<td>750</td>
<td>13.4</td>
<td>7,200</td>
<td>none</td>
<td>5.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>68</td>
<td>M</td>
<td>—</td>
<td>none</td>
<td>750</td>
<td>14.7</td>
<td>6,750</td>
<td>slight</td>
<td>3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>32</td>
<td>F</td>
<td>—</td>
<td>none</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>59</td>
<td>14</td>
<td>M</td>
<td>—</td>
<td>none</td>
<td>1200</td>
<td>14.1</td>
<td>3,650</td>
<td>slight</td>
<td>13.7</td>
<td>380</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>47</td>
<td>F</td>
<td>—</td>
<td>phenobarbital</td>
<td>100</td>
<td>12.7</td>
<td>5,600</td>
<td>slight</td>
<td>12.2</td>
<td>900</td>
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</table>
Table 2.—Incidence of Subnormal Folate Levels and Macrocytosis in Relation to Dosage of Dilantin

<table>
<thead>
<tr>
<th>Dosage of Dilantin (mg./day)</th>
<th>Total No. Subjects</th>
<th>Percentage of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>200</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>300</td>
<td>18</td>
<td>61</td>
</tr>
<tr>
<td>400</td>
<td>22</td>
<td>68</td>
</tr>
<tr>
<td>500</td>
<td>5</td>
<td>20</td>
</tr>
</tbody>
</table>

B₁₂ assays performed on the 60 subjects studied who were receiving anticonvulsant therapy are presented in table 1. Subnormal serum folate levels of less than 5.0 µg./ml. were observed in 31 of the 53 patients who were receiving Dilantin therapy, an incidence of 58 per cent. An identical percentage of subnormal folate levels was observed in patients taking Dilantin alone and patients receiving additional anticonvulsant drug therapy. Thus, serum folate levels were subnormal in 10 of 17 (58 per cent) subjects who were receiving only Dilantin and in 21 of 36 (58 per cent) subjects who were taking additional anticonvulsant drugs. Mysoline was the additional anticonvulsant agent in 10 subjects; serum folate levels were subnormal in six. The occurrence of subnormal folate levels in relation to dosage and duration of therapy with Dilantin are tabulated in tables 2 and 3. While the incidence of subnormal folate levels appeared to bear no relationship to the dosage of Dilantin, the incidence was greater in those patients who had taken the drug for periods of greater than 5 years (table 3).

Seven subjects were studied who were receiving anticonvulsant drugs other than Dilantin. Serum folate levels were subnormal in one subject receiving Mysoline and phenobarbital and were normal in four other subjects taking Mysoline as well as in single patients taking Mesantoin alone and phenobarbital alone. Five epileptics who were not receiving anticonvulsant therapy are not included in table 1. All had normal blood counts, no evidence of macrocytosis and normal serum folate and vitamin B₁₂ levels.

Folic Acid Clearance Studies

Folic acid clearance studies were performed in nine subjects taking Dilantin and two subjects on Mysoline. The results are presented in table 4. Serum folic acid concentrations measured with Str. faecalis were within the normal range of 75 to 186 µg./ml. at 3 minutes and 21 to 80 µg./ml. at 15 minutes in six patients on Dilantin and the two subjects studied taking Mysoline. Abnormally rapid clearances were observed in three patients: Case 28, a non-anemic patient who had a subnormal fasting serum L. casei folate concentration; Case 33, a 22-year old mentally retarded, quadriplegic male with a history of poor nutritional intake whose fasting L. casei folate level was normal; and Case P. V. The latter patient has not been included in table 1 due to the
Table 3.—Incidence of Subnormal Folate Levels and Macrocytosis in Relation to Duration of Dilantin Therapy

<table>
<thead>
<tr>
<th>Duration of Therapy (yr.)</th>
<th>Total No. Subjects</th>
<th>Percentage of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>&lt;1</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>1–5</td>
<td>18</td>
<td>33</td>
</tr>
<tr>
<td>5–10</td>
<td>13</td>
<td>77</td>
</tr>
<tr>
<td>&gt;10</td>
<td>16</td>
<td>75</td>
</tr>
</tbody>
</table>

Table 4.—Folic Acid Clearance Studies Following Intravenous Administration of 15 μg. FA/Kg.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Drug</th>
<th>Serum L. casei Folate activity (mug./ml.)</th>
<th>Serum Str. faecalis activity (mug./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dilantin</td>
<td>12.0</td>
<td>120</td>
</tr>
<tr>
<td>23</td>
<td>Dilantin</td>
<td>5.6</td>
<td>166</td>
</tr>
<tr>
<td>31</td>
<td>Dilantin</td>
<td>15.3</td>
<td>120</td>
</tr>
<tr>
<td>32</td>
<td>Dilantin</td>
<td>11.0</td>
<td>156</td>
</tr>
<tr>
<td>33</td>
<td>Dilantin</td>
<td>11.0</td>
<td>122</td>
</tr>
<tr>
<td>13</td>
<td>Dilantin</td>
<td>5.0</td>
<td>148</td>
</tr>
<tr>
<td>28</td>
<td>Dilantin</td>
<td>2.0</td>
<td>12.5</td>
</tr>
<tr>
<td>47</td>
<td>Dilantin</td>
<td>1.6</td>
<td>55</td>
</tr>
<tr>
<td>P. V. *</td>
<td>Dilantin</td>
<td>&gt;32</td>
<td>51</td>
</tr>
<tr>
<td>55</td>
<td>Mysoline</td>
<td>5.7</td>
<td>108</td>
</tr>
<tr>
<td>56</td>
<td>Mysoline</td>
<td>5.3</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*See text for details on this patient.

fact that she received folic acid prior to being studied. She was a well nourished 18 year old girl who had been on a variety of anticonvulsants, including Dilantin, for at least 10 years. Found to have a megaloblastic anemia by her local physician, she had taken approximately two or three 5 mg. folic acid tablets 1 week prior to being seen at the Neurological Institute. At that time, abnormal bone marrow changes were limited to a few intermediate megaloblasts and giant metamyelocytes. Serum vitamin B₁₂ concentration was 240 μg./ml. Serum folate was 32 mug./ml. Formiminoglutaric acid (FIGLu) was present in the urine following oral histidine load of 15 Gm.

Concomitant assay of the sera obtained at 3 and 15 minutes with L. casei in eight of these patients showed the L. casei folate concentrations to parallel the values obtained with the Str. faecalis assay to a degree that was within the limits previously described for normal subjects by Herbert and Zalusky.

Serum B₁₂ Levels

Serum B₁₂ concentrations were assayed in 32 patients and found to be normal in all except two subjects, in both of whom the etiology of the sub-
normal B₁₂ levels was unrelated to the anticonvulsant drug therapy. These two patients have not been included in this series.

Hematologic Changes

Slight or moderate macrocytosis was observed in 27 of the patients (51 per cent) receiving Dilantin. A significantly higher incidence of macrocytosis was noted in those subjects with low folate levels. Thus, 23 of the 31 patients (74 per cent) taking Dilantin who had subnormal folate levels had increased macrocytes, whereas macrocytosis was noted in only 4 of 22 subjects (18 per cent) on Dilantin who had normal folate concentrations. Macrocytosis was also observed in four of the five subjects receiving anticonvulsants other than Dilantin.

Leukopenia with white blood cell counts of less than 4000 was observed in one subject receiving Dilantin and Tridione (Case 57) and in one patient (Case 36) on Mesantoin. No neutrophils with greater than five lobes were observed on the differential count, which was carried out in all subjects in this study. Ten subjects, all females, had hemoglobin concentrations of less than 12 Gm. per cent; in the 11 to 12 Gm. per cent range, in eight subjects; and 10.6 to 11.0 Gm. per cent, in two subjects. Peripheral smears in all of these subjects showed moderate degrees of hypochromia. Treatment with folic acid, 5 mg. daily, did not result in a significant increase in hemoglobin concentration in five of these patients so treated.

In Vitro Studies

Representative results of in vitro studies are presented in table 5. Increasing concentrations of pyrimethamine, Dilantin, Mysoline, and phenobarbital were added to known concentrations of folic acid and to aliquots of serum from subjects receiving Dilantin with known normal or subnormal folate concentrations, and growth of L. casei assayed. Pyrimethamine (Daraprim) is a known folic acid antagonist which has been associated with megaloblastic anemia and subnormal serum folate levels and has been demonstrated to have an in vitro antimicrobial action against the growth of both L. casei and Str. faecalis. Pyrimethamine serum levels after therapeutic doses in man have been found to be in the order of magnitude of 1–10 μg./ml. In the present study, concentrations of 1 μg./ml. and greater were found to significantly inhibit the growth of L. casei when added to folic acid as well as to serum. This inhibition was modified by increased amounts of folic acid. Dilantin plasma levels, on the average daily dosage schedule of 300 mg. per day, range from 5 to 20 μg./ml. Concentrations of this drug as high as 100 μg./ml.

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*One patient had been on 400 mg. Dilantin for 2½ years. She had a megaloblastic anemia and a subnormal serum vitamin B₁₂ concentration of 50 μg./ml. on the basis of regional enteritis and malabsorption of vitamin B₁₂. Serum folate was 9.2 μg./ml. The second patient had been on 400 mg. of Dilantin for 8 years. Although not anemic, the white blood cell count was 3800 with hypersegmented neutrophils. A subnormal serum B₁₂ concentration of 125 μg./ml. was shown by Co⁶⁰B₁₂ absorption study to be secondary to latent pernicious anemia. Serum folate was 2.8 μg./ml.
had no inhibitory effect on the growth of *L. casei*; indeed, lesser concentrations appeared to mildly potentiate growth. Phenobarbital in concentrations as high as 1.0 mg./ml. did not interfere with the growth of *L. casei*. Mysoline in concentrations of 100 μg./ml. had a distinct inhibitory effect on growth when added to both folic acid and serum samples; however, this growth-inhibitory effect was shown to be due to the solution used to solubilize the drug. Mysoline is relatively insoluble except in organic solvents and the solution of 50 per cent ethanol with a few drops of KOH required to solubilize Mysoline in concentrations of 100 μg./ml. was shown to inhibit bacterial growth even without addition of Mysoline. No inhibition was noted when lesser concentrations were used.

**Discussion**

It is now accepted that folic acid deficiency is the basis of the development of megaloblastic anemia in patients receiving anticonvulsant drug therapy. This was assumed in earlier studies on the basis of the finding of normal serum B₁₂ concentrations in patients with a megaloblastic anemia associated with anticonvulsant drugs.² ⁴ ⁵ ⁶ ⁷ ¹⁴ ¹⁷ ¹⁹ ²⁵ ²⁷ ²⁹ ³² ³⁴ ³⁶ Recent evidence now confirms this impression and a number of direct observations indicating folic acid deficiency have been made in subjects receiving Dilantin. Serum *L. casei* folate concentrations were subnormal in the two reported cases in which this assay was performed.³⁵ ³⁷ In addition, Dr. David Mollin and the author have studied four epileptic patients at the Hammersmith Hospital who had a megaloblastic anemia associated with anticonvulsant drug therapy.³⁸ Serum assays of the two patients who had been receiving Dilantin and the third, who was on barbiturates, showed serum vitamin B₁₂ levels of 135, 730, and 215 μg./ml. and serum *L. casei* folate concentrations of 0.7, 1.3, and 0.9 mg./ml., respectively. The fourth patient, whose megaloblastic anemia was associated with Mysoline, had normal serum concentrations of vitamin B₁₂ (150 μg./ml.) and *L. casei* folate (6.0 mg./ml.). Increased urinary excretion of formiminoglutamic acid (FIGLu) has been noted in two patients.³¹ ³⁷ Chanarin and his associates have reported an abnormally rapid plasma clearance of folic acid in two patients who developed severe megaloblastic anemia while receiving Dilantin, as well as three patients who received various combinations of anticonvulsant drugs.²⁶ On the other hand, two patients with megaloblastic anemia following the use of primidone and one following barbiturate therapy had normal folic acid clearances.²⁹ ³³ Studies of folic acid absorption have been uniformly normal. Employing the technic described by Girdwood of assaying the urinary excretion of folic acid following an oral test dose, normal absorption was observed in five patients with megaloblastic anemia and in five non-anemic patients on anticonvulsants who had macrocytosis.³⁶ Chanarin and his associates reported subnormal serum vitamin B₁₂ concentrations have been reported in five patients.¹⁸ ²⁰ ³⁰ ³¹ It is likely that the B₁₂ deficiency was on the basis of nutritional deficiency in three of these patients.¹⁸ ²⁰ ³⁰
Table 5.—Growth of L. casei Following Addition of Anticonvulsant Drugs to Varying Concentrations of Folic Acid or Serum

<table>
<thead>
<tr>
<th>Folic acid (mg./ml.)</th>
<th>Pyrimethamine (μg./ml.)</th>
<th>Dilantin (μg./ml.)</th>
<th>Mysoline (μg./ml.)</th>
<th>Phenobarbital (μg./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>128</td>
<td>155</td>
<td>155</td>
<td>30*</td>
</tr>
<tr>
<td>0.3</td>
<td>183</td>
<td>205</td>
<td>245</td>
<td>30*</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate normal</td>
<td>125</td>
<td>135</td>
<td>35*</td>
<td>135</td>
</tr>
<tr>
<td>Folate low</td>
<td>105</td>
<td>100</td>
<td>40*</td>
<td>105</td>
</tr>
</tbody>
</table>

*Similar growth inhibition obtained when a solution of 50 per cent ethanol without Mysoline was added to these systems.
found normal peak serum concentrations of folic acid measured by Str. faecalis following an oral test dose in two patients with megaloblastic anemia. Dr. David Mollin and the author have observed peak serum folic acid concentrations of 57 and 70 μg./ml. (normal is greater than 40 μg./ml. at 1-3 hours) in two of the patients who had megaloblastic anemia.

Although treatment with folic acid has resulted in a hematologic remission in every case, little information pertaining to folic acid deficiency has been gained from therapeutic trials, since the dosages employed have been in the large “pharmacologic” range of from 15 to 60 mg./day. At these dosages, a hematologic response would be anticipated with either vitamin B12 or folate deficiency. A “physiological” dose has been employed in one patient described by Druskin, Wallen, and Bonagura, a 49 year old female with poor dietary intake, normal jejunal biopsy and absorption, whose megaloblastic anemia responded to the administration of 25 μg. folic acid daily despite continuation of Dilantin therapy. Dosage levels of 25 to 50 μg. have been found to be adequate to produce a hematologic remission in conditions of uncomplicated folic acid deficiency due to kwashiorkor, cirrhosis, and scurvy, as well as some cases of tropical sprue. Response to doses of this magnitude are considered to be specific for folic acid deficiency and will not produce a remission if the megaloblastic anemia is due to vitamin B12 deficiency. Larger doses of folic acid are required to produce a response in the megaloblastic anemia due to folic acid deficiency associated with congenital hemolytic anemia in which there is an increased rate of folic acid utilization, as well as in some instances of combined folic acid and vitamin B12 deficiency.

In some instances additional factors other than the ingestion of anticonvulsant drugs appear to be operative in the development of sufficient folate deficiency to produce a megaloblastic anemia. A number of patients have been noted to be on an inadequate diet. In addition, increased utilization of folic acid may play a role: Gatenby has reported that megaloblastic anemia is more likely to develop when anticonvulsants are used in pregnancy. However, in view of the significant number of patients in whom no contributing factors have been elicited other than the ingestion of anticonvulsant drugs, the most likely suggestion has been that the anticonvulsant drugs produce folate deficiency on the basis of a block in folic acid metabolism. A number of clinical observations support this concept: (1) normal folic acid clearance studies have been observed in some patients who developed a megaloblastic anemia associated with Mysoline and phenobarbitone therapy; (2) in those few cases where the anticonvulsant drug has been withdrawn, a remission has occurred without therapy with vitamin B12 or folic acid; (3) a number of patients have had recurrence of the megaloblastic anemia following resumption of anticonvulsant therapy; (4) in at least 12 instances where patients have been treated with vitamin B12 alone, in dosages as high as 100 μg. daily, without withdrawing the anticonvulsant therapy, there has been no hematologic response. In view of the fact that vitamin B12 in doses of the order of magnitude of 100 μg. daily will produce a hematologic response in uncomplicated folic acid deficiency, the following observations are of interest:...
deficiency, the failure of such therapy in subjects maintained on anticonvulsant drugs suggests that vitamin $B_{12}$ alone is unable to compensate for a block in folic acid metabolism. This is further supported by the observations of Hawkins and Meynell that the macrocytosis associated with anticonvulsant drug therapy was noted to disappear following either withdrawal of drug therapy or institution of folic acid therapy, but not after the administration of vitamin $B_{12}$ in dosages of 100 μg. weekly over a 3-month period.

The present study indicates that an abnormality of folic acid metabolism does exist in a significant number of subjects taking anticonvulsant drugs without overt hematologic manifestations other than macrocytosis. Thirty-one of 53 patients taking Dilantin and one of five subjects receiving Myoline had serum $L. casei$ folate levels of less than 5.0 μg./ml. Folate levels were normal in single patients studied who were taking Mesantoin alone and phenobarbital alone, and in five epileptics who were not receiving anticonvulsant therapy at the time of the study. Herbert and Zalusky have also reported observing subnormal folate levels in 11 subjects who had received Dilantin therapy for periods in excess of 6 months. In the present study, the frequency of occurrence of subnormal folate levels appeared to bear no relationship to the dosage of Dilantin (table 2) nor to whether other anticonvulsant drugs were being taken concomitantly with Dilantin. However, a significantly higher incidence of subnormal folate levels was observed in those subjects who had taken Dilantin for a period of 5 years or longer (table 3).

Hematologic changes were limited to the presence of macrocytosis. Slight or moderate macrocytosis was observed in 51 per cent of the subjects taking Dilantin. The incidence of macrocytosis was significantly higher in those subjects with subnormal folate levels: 74 per cent in subjects with subnormal folate levels versus 18 per cent in subjects with normal levels. Hawkins and Meynell, in studying a large group of epileptics, noted a “slight but definite” macrocytosis in 45 per cent of subjects receiving Dilantin and phenobarbital, and 27 per cent of patients on Dilantin alone. In view of the association of macrocytosis with subnormal folate levels in three-quarters of the cases in this study and the observation by Hawkins and Meynell that treatment with folic acid will cause the disappearance of the macrocytosis, it seems likely that the presence of macrocytosis in patients receiving anticonvulsant drug therapy is indeed a reflection of deranged folic acid metabolism.

The cause of the subnormal $L. casei$ folate levels in these subjects is unknown and the site of a possible block in folic acid metabolism remains to be elucidated. Some indirect evidence is available, however. The normal metabolism of folic acid has been recently reviewed elsewhere. Its principal role in metabolism appears to be as a donor and acceptor of one-carbon fragments. A simplified version of the major metabolic pathway of folic acid is presented in figure 1. The formula for folic acid and the site at which the various one-carbon fragments are attached to form the coenzymes are shown in figure 2. Following reduction of folic acid to tetrahydrofolic acid (FAH₄) in a system requiring the enzyme folic acid reductase, FAH₄ can accept a single-carbon unit to form one of these coenzymes and may be further converted to 5, 10-methylene-FAH₄ via any of three pathways: (1) formiminoo-
SUBNORMAL SERUM FOLATE AND MACROCYTOSIS

FOLIC ACID

FA reductase

FAH₂

FAH₄

FA CO-ENZYMES

5,10-METHYLENE

FAH₄

5-METHYL-FAH₄

Formimino-glutamic Acid

Glutamic Acid

5-FORMIMINO-FAH₄

Fig. 1.—Simplified version of the major metabolic pathway of folic acid. Formulae for folate coenzymes are given in fig. 2.

Mysoline  Phenobarbital  Dilantin

Fig. 2.—Formulae of the anticonvulsant drugs studied and folic acid. Broken lines indicate the basic 1-carbon acceptor site of folic acid, and the various 1-carbon-donating coenzymes which may be formed there, are listed (after Herbert).
glutamic acid or formiminoglycine can transfer -CHNH to the 5 position of FAH₄ to form-5-formino-FAH₄, which then loses ammonia and cyclizes to 5, 10-methenyl-FAH₄; this compound can then be hydrogenated to form 5, 10-methylene-FAH₄; (2) formate can transfer a -CHO to the 10-position of FAH₄ to form 10-formyl-FAH₄ which is reduced and dehydrated to form 5, 10-methylene-FAH₄; (3) formaldehyde or serine can transfer CH₂OH to the 10 position of FAH₄, followed by cyclization and loss of water to form 5, 10-methylene-FAH₄. The 5, 10-methylene-FAH₄ so formed in any of these three ways is then reduced to 5-methyl-FAH₄, which then transfers its methyl group to homocysteine to form methionine. Herbert, Larrabee and Buchanan have demonstrated that 5-methyl-FAH₄ is the major folate coenzyme in the serum responsible for the growth of L. casei.⁴²

Possible Sites of Metabolic Block

In vitro studies with Str. faecalis and L. casei.—Comparison of the effect of the addition of anticonvulsant drugs on the growth of bacterial systems of Str. faecalis and L. casei with that produced by the folic acid antagonists, Aminopterin and pyrimethamine, suggest that the anticonvulsant drugs do not block folic acid metabolism at the stage of reduction of FAH₂ to FAH₄. Both Aminopterin and pyrimethamine act primarily by inhibiting dihydrofolic reductase, the enzyme necessary for the reduction of FAH₂ to FAH₄.⁷⁰,⁷¹ Both drugs inhibit the growth of Str. faecalis and L. casei when added in vitro to these bacterial systems.⁷³,⁷⁴ Serum samples obtained from patients receiving therapeutic doses of Methotrexate show complete inhibition of bacterial growth when assayed with L. casei.⁴⁵,⁴⁹ We have recently studied a patient receiving therapeutic doses of pyrimethamine. Serum folate assay with L. casei was normal after 1 week of therapy and subnormal (3.0 µg./ml.) 3 weeks later, at which time the bone marrow was overtly megaloblastic. In vitro studies by Chanarin⁷⁵ and Broquist⁷⁶ have failed to show growth inhibition of Str. faecalis following the addition of Mysoline or Dilantin. In the present studies, as well as those reported by Baker et al.,⁵⁷ no inhibition of L. casei growth was observed following the in vitro addition of Dilantin, Mysoline, or phenobarbital to culture media containing folic acid or to serum from patients on Dilantin with normal or low folate concentrations. It would thus seem unlikely that the anticonvulsant drugs inhibit the reduction of FAH₂ to FAH₄, although the implications of in vitro studies with these bacteria may not necessarily be analogous to in vivo actions in man.

Plasma clearance studies.—Studies of the plasma clearance of intravenously administered test doses of folic acid suggest that the metabolic block induced by Dilantin may differ from that of Mysoline and the barbiturates. In the present study, a normal rate of plasma clearance as measured by Str. faecalis was observed in six patients receiving Dilantin and in two patients on Mysoline. Abnormally rapid clearances were found in two non-anemic patients taking Dilantin, one of whom had a subnormal fasting serum L. casei folate, and in a patient who had a partially treated megaloblastic anemia associated with Dilantin. It is noteworthy that the five patients studied by Chanarin et al. whose megaloblastic anemia was associated with a fast clearance of Str. faecalis folate were all receiving Dilantin, whereas clearance studies...
were normal in two patients with Mysoline-induced megaloblastic anemia.36

In addition to the abnormally rapid Str. faecalis folate clearance observed in Dilantin-induced megaloblastic anemia, abnormal clearance patterns may be associated with two conditions in which a metabolic block of folic acid is thought to exist: (1) Chanarin and Bennett have reported a relatively slow Str. faecalis clearance, with a high 15-minute serum level, in a patient who had just completed a course of Methotrexate, suggesting accumulation of unreduced folic acid in the presence of blocked folic acid reductase.82 (2) Herbert and Zalusky have observed a parallel decline of serum Str. faecalis and L. casei folate factors in normal subjects following the intravenous administration of a physiologic dose of folic acid. The disappearance of serum L. casei values was delayed in some subjects with vitamin B12 deficiency. This has been interpreted to indicate the presence of a metabolic block in the conversion of 5-methyl-FAH4 to FAH4.1 Neither of these types of abnormal clearance patterns were observed in the present study.

Possible Sites of Action of Dilantin

The observations summarized above have suggested that the loci of metabolic inhibition related to Dilantin are not at the stages of reduction of FAH2 to FAH4, nor the conversion of 5-methyl-FAH4 to FAH4. Herbert has recently emphasized the close resemblance of the 5-membered ring of 5, 10-methenyl-FAH4 to the hydantoin ring of diphenylhydantoin (Dilantin) (fig. 2) and speculated that megaloblastic anemia may be induced in patients by Dilantin on the basis of a weak competitive inhibition of the conversion of 5, 10-methenyl-FAH4 to 5-methyl-FAH4.39 To date no evidence is available to support this hypothesis, although the finding of low serum L. casei folate levels (i.e., low serum levels of 5-methyl-FAH4) in our patients is not inconsistent with it. An alternative mechanism might be the displacement of folic acid from its carrier plasma protein by the analogues of hydantoin, as has been observed in the case of thyroxine.80 Studies with tritium-labeled folic acid by Johns and his associates have indicated that an average of 64 per cent of circulating folic acid is bound to plasma protein.81 Protein-binding of plasma folic acid is also suggested by our observation that only negligible amounts of folic acid appear in ascitic transudates following intravenous injection of 15 μg. folic acid per Kg. body weight.83 Further studies of possible displacement of folate from its carrier protein by Dilantin are presently in progress in this laboratory.

Studies with Barbiturates

Girdwood12 was among the first to comment on the structural similarities between phenobarbital and folic acid, both of which contain a 6-membered pyrimidine ring (fig. 2). Recent evidence has been presented by Biswas and Broquist to suggest that a metabolic block may be induced in pyrimidine synthesis by barbituric acid.17,84 It was found that the antibacterial effect of barbituric acid on the growth of Escherichia coli K-12 could be counteracted by uracil, cytosine, and thymine as well as their respective nucleosides and nucleotides, whereas the pyrimidine precursors, orotic acid and orotidyl acid, were without effect. These observations were interpreted as indicating
a block at the level of conversion of orotidylic acid to uridylic acid. The clinical implications of these observations are suggested by possible analogy to the patient described by Huguley and his associates, in which case a congenital defect in pyrimidine metabolism produced a megaloblastic anemia associated with orotic aciduria. This defect has been shown by Smith et al. to be on the basis of a congenital deficiency of the enzymes, orotidylic pyrophosphorylase and orotidylic decarboxylase, with resultant inability to convert orotic to uridylic acid. However, unlike patients with barbiturate-induced megaloblastic anemia, the administration of folic acid did not induce a remission in this patient.

Studies with Mysoline

Other than the one non-anemic patient reported in the present study who was taking Mysoline and phenobarbital and had a subnormal serum L. casei folate level, no direct observations have been reported in patients indicating folic acid deficiency associated with Mysoline usage. Unlike studies of patients taking Dilantin, plasma Str. faecalis folic acid clearance studies have been normal in patients with megaloblastic anemia associated with this anti-convulsant agent. Nor have in vitro studies implicated a Mysoline-induced block in folic acid metabolism. However, Baker and his associates have recently presented evidence which suggests that Mysoline may interfere with pteridine metabolism. These workers observed that the addition of Mysoline to in vitro systems produced a growth inhibition of both Crithidia fasciculata, a flagellate whose folic acid requirement may be satisfied by unconjugated pteridines, and E. coli I, a thymine- or thymidine-requiring bacteria. Growth inhibition of Crithidia was annulled by folic acid as well as unconjugated pteridine. Thymidylic acid was significantly more efficient in overcoming the inhibiting effect of Mysoline on E. coli growth than were thymine and thymidine. These results were interpreted as indicating interference by Mysoline in unconjugated pteridine metabolism and in the phosphorylation of thymidine to thymidylic acid. At the present time it is difficult to assess the significance of these observations since there is no direct evidence that unconjugated pteridines serve as precursors of the pteridine ring of folic acid in higher animals.

SUMMARY

Hematologic studies and microbiological assay of serum folate levels with L. casei and serum vitamin B12 concentrations with L. leichmannii were performed on 60 subjects receiving anticonvulsant drug therapy and five epileptics receiving no treatment. Subnormal serum folate levels of less than 5.0 μg./ml. were observed in 58 per cent of 53 subjects receiving Dilantin and one of seven patients on other anticonvulsant agents. The incidence of subnormal serum folate values was greater in those subjects who had been taking Dilantin for periods of time greater than 5 years, but did not appear to bear any correlation to the dosage of the drug. Slight or moderate macrocytosis was observed in 71 per cent of subjects receiving Dilantin who had subnormal serum folate levels and in 18 per cent of subjects with normal levels. Serum B12 concentrations were within the normal range.

The growth of L. casei was not inhibited by anticonvulsant drugs when
added to culture media containing folic acid or serum from patients on Dilantin with normal or low folate concentrations. Folic acid clearance studies were abnormally rapid in three of nine patients studied who were receiving Dilantin.

Possible sites of metabolic inhibition resulting in disturbances in folic acid metabolism during therapy with anticonvulsant medications are discussed.

SUMMARY IN INTERLINGUA

Studios hematologic e assayos microbiologic del nivellos seral de folato con le uso de Lactobacillus casei e del concentrationes seral de vitamina B12 con le uso de Lactobacillus leichmannii esseva executate pro 60 subjectos sub tractamento con pharmacos anticonvulsive e pro cinque epilepticos non recipiente ulle tractamento. Subnormal nivellos seral de folato, amontante a minus que 5.0 mg per ml. esseva observate in 58 pro cento del 53 subjectos recipiente Dilantina e in un de septe subjectos recipiente altere agents anticonvulsive. Le incidentia de subnormal nivellos seral de folato esseva plus alte in le subjectos qui habeva essite sub tractamento con Dilantina durante periodos de plus que 5 annos, sed ille incidentia non pareva esser in correla-
tion con le dosage del pharmaco. Leve o moderate grados de macrocytosis esseva observate in 71 pro cento del subjectos recipiente Dilantina qui habeva subnormal nivellos seral de folato e in 18 pro cento del subjectos con nivellos normal. Le concentrationes seral de vitamina B12 esseva intra le limites del norma.

Le crescentia de L. casei non esseva inhibite per drogas anticonvulsive quando istos esseva addite al medios cultural continente acido folic o sero ab patientes sub tractamento con Dilantina con normal o basse concentrationes de folato. Studios del clearance de acido folic revelava anormal rapidititates in tres de novem patientes studiate le quales esseva omnes sub tractamento con Dilantina.

Es discutite le possibile sitos del inhibition metabolic que resulta in disturbarion del metabolismo de acido folic durante curso therapeutic con medicationes anticonvulsive.

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Subnormal Serum Folate and Macrocytosis Associated with Anticonvulsant Drug Therapy

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