Differentiation between Vitamin B₁₂-deficient and Folic Acid-deficient Megaloblastic Anemias with C¹⁴-Histidine

By Mathews B. Fish, Myron Pollycove and Thomas V. Feichtmeir

MEGALOBLASTIC ANEMIAS result from a relative or absolute deficiency of vitamin B₁₂ or folic acid. Often it is difficult to make this etiologic differentiation, either from clinical observations or by use of routine hematologic procedures. Several methods which have been devised to evaluate folic acid deficiency have been reviewed recently.¹ These include: therapeutic trial of “physiologic doses” of folic acid,² determination of serum folic acid activity,³,⁴ determination of rate of clearance from the plasma of intravenously administered folic acid and determination of amount of folic acid in blood and urine after oral or parenteral doses of folic acid;⁵,⁷ and the urinary formiminoglutaric acid (FIGlu) determination after large oral doses of histidine.⁸,⁹ Some of these methods are helpful in the differentiation of folic acid and vitamin B₁₂ deficiency in megaloblastic anemias, but overlapping results have been noted frequently. As these approaches are primarily diagnostic in nature, the results obtained add little information regarding the basic metabolic abnormalities in folic acid or vitamin B₁₂ deficiency. The determination of urinary FIGlu serves as an index of the folic acid-dependent conversion of formiminoglutaric acid to glutamic acid. However, the large oral dose of histidine administered in this test is unphysiologic and may be responsible for the overlapping results obtained in folic acid deficiency, vitamin B₁₂ deficiency,²,³,¹⁶ and other hematologic and non-hematologic states.¹⁷⁻¹⁹

It is known that folic acid is specifically concerned with the metabolic reactions involving the transfer and utilization of single carbon moieties.²⁰,²¹ Recent studies suggest that vitamin B₁₂ is also required in certain aspects of this metabolic process; it may be essential in methionine methyl neogenesis.²⁰

Histidine is an important contributor to the single carbon moiety pool.²² Some aspects of the metabolism of C¹⁴-labeled histidine [specifically L-histidine 2(ring)-C¹⁴, the 2 carbon atom being contributed to the single carbon-moiety pool] were studied in mice,²³ and monkey, human and rat.²⁴⁻²⁶ These studies demonstrated the very rapid incorporation of radioactivity into visceral protein and the rapid appearance of C¹⁴O₂ in the expired air,²³ the specific

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Supported in part by an American Cancer Society Clinical Fellowship, Public Health Service Grant No. 2G-349, and the United States Atomic Energy Commission.

Portions of this paper were presented to the California Society of Pathologists, San Francisco, December 1961 and the San Francisco branch of the American Federation for Clinical Research, Palo Alto, Calif., May 1962.

Submitted Aug. 24, 1962; accepted for publication Nov. 28, 1962.

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BLOOD, VOL. 21, NO. 4 (APRIL), 1963
Table 1.—Hematologic Laboratory Findings at Time of Initial Examination of Eight Patients with Megaloblastic Anemia and Two Normal Subjects

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Subject</th>
<th>Age</th>
<th>Sex</th>
<th>Weight Kg.</th>
<th>Hb Gm./100 ml. (12-18)</th>
<th>Hct % (37-54)</th>
<th>RBC x 10^9/mm.³ (4.2-6.2)</th>
<th>MCV μg. (82-92)</th>
<th>MCH μg. (27-31)</th>
<th>MCHC % (32-36)</th>
<th>Reticulocytes % (0.5-1.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Control 1</td>
<td>27 M</td>
<td>52</td>
<td>—</td>
<td>48</td>
<td>5.20</td>
<td>90</td>
<td>—</td>
<td>34</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Normal</td>
<td>Control 2</td>
<td>41 M</td>
<td>53</td>
<td>14.0</td>
<td>41</td>
<td>5.10</td>
<td>81</td>
<td>28</td>
<td>34</td>
<td>0.8</td>
<td>—</td>
</tr>
<tr>
<td>Vitamin B₁₂ deficiency</td>
<td>M. F.</td>
<td>73 M</td>
<td>56</td>
<td>9.0</td>
<td>24</td>
<td>2.09</td>
<td>116</td>
<td>42</td>
<td>37</td>
<td>0.9</td>
<td>—</td>
</tr>
<tr>
<td>Vitamin B₁₂ deficiency</td>
<td>W. C.</td>
<td>44 M</td>
<td>53</td>
<td>10.7</td>
<td>33</td>
<td>2.77</td>
<td>119</td>
<td>39</td>
<td>33</td>
<td>0.9</td>
<td>—</td>
</tr>
<tr>
<td>Vitamin B₁₂ deficiency</td>
<td>J. H.</td>
<td>84 M</td>
<td>59</td>
<td>4.2</td>
<td>14</td>
<td>1.08</td>
<td>127</td>
<td>38</td>
<td>30</td>
<td>0.1</td>
<td>—</td>
</tr>
<tr>
<td>Vitamin B₁₂ deficiency</td>
<td>C. K.</td>
<td>86 M</td>
<td>50</td>
<td>11.7</td>
<td>33</td>
<td>3.30</td>
<td>101</td>
<td>36</td>
<td>35</td>
<td>1.6</td>
<td>—</td>
</tr>
<tr>
<td>Folic acid deficiency</td>
<td>R. M.</td>
<td>30 F</td>
<td>55</td>
<td>4.5</td>
<td>14.5</td>
<td>1.50</td>
<td>98</td>
<td>39</td>
<td>31</td>
<td>0.3</td>
<td>—</td>
</tr>
<tr>
<td>Folic acid deficiency</td>
<td>F. M.</td>
<td>25 F</td>
<td>90</td>
<td>3.9</td>
<td>13</td>
<td>1.43</td>
<td>92</td>
<td>28</td>
<td>30</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>Folic acid deficiency</td>
<td>M. G.</td>
<td>59 M</td>
<td>61</td>
<td>6.5</td>
<td>19.8</td>
<td>1.64</td>
<td>121</td>
<td>40</td>
<td>33</td>
<td>1.1</td>
<td>—</td>
</tr>
<tr>
<td>Folic acid block</td>
<td>G. M.</td>
<td>5 M</td>
<td>23</td>
<td>5.8</td>
<td>16.5</td>
<td>1.73</td>
<td>95</td>
<td>34</td>
<td>35</td>
<td>1.0</td>
<td>—</td>
</tr>
</tbody>
</table>

*Hepatic uptake test.*

*Schilling test.*

1Hematologic status at time of initial C¹⁴-histidine study essentially unchanged from that found at time of initial examination.

2Patient responding to vitamin therapy at time of initial C¹⁴-histidine study.

3Patient G. M. developed his anemia after a complete clinical and hematologic remission was obtained while receiving amethopterin, 5 mg./day for 3 months.

activity of various urinary metabolites of C¹⁴-histidine, and the C¹⁴O₂-specific activity in the expired air of rats.²⁴ ²⁶

The present study using C¹⁴-histidine is an attempt to differentiate between folic acid and vitamin B₁₂ deficiency and to investigate the pathologic physiology in patients with megaloblastic anemia.

**METHODS AND MATERIALS**

Blood and bone marrow studies were performed by standard methods.²⁷ The diagnoses of megaloblastic anemia were made by the characteristic blood and marrow findings. Bone marrow hemosiderin samples were prepared²⁸ and graded 0 to 4+ on the basis of the number of hemosiderin granules observed. All patients with a megaloblastic bone marrow also demonstrated the uniformity of hemosiderin distribution characteristic of intramedullary hemolysis.²⁸ Vitamin B₁₂ absorption was measured by either the Schilling test²⁹ or hepatic uptake test.³⁰

The diagnosis of the specific vitamin deficiency was eventually confirmed by the characteristic reticulocyte response to "physiologic doses" of the corresponding vitamin² and/or vitamin B₁₂ absorption studies in patients who had been on folic acid "free" diets (0.2-0.4 mg./d) (table 1). Patient M. G. received a regular diet (folic acid 1-2 mg./d),³¹ ³³ and his reticulocyte count rose from 1.1 per cent at time of initial examination to 2.3 per cent at the time of C¹⁴-histidine study, reaching a peak of 10.9 per cent on the ninth day after admission. Patient W. C. developed a reticulocyte increase from 0.9 per cent (Hb concentration 10.7 Gm./100 ml.) to 7.9 per cent 5 days after parenteral injection of 1000 μg. of vitamin B₁₂ (Schilling test). Patient G. M. had acute lymphatic leukemia and developed megaloblastic anemia after receiving amethopterin 5 mg. daily,
DIFFERENTIATING MEGALOBLASTIC ANEMIAS

Table 1.—(Continued)

<table>
<thead>
<tr>
<th>WBC $\times 10^3$/mm.$^3$</th>
<th>Reticulocyte Response to Physiol. Dose Vitamin B$_2$</th>
<th>Vitamin B$_2$ Absorption</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>(4-12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin</td>
<td>Dose/d</td>
<td>Retic. Day</td>
<td>GI absorption*</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>6.2</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>4.0</td>
<td>B$_2$</td>
<td>1 mg.</td>
<td>14%</td>
</tr>
<tr>
<td>9.5</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>2.2</td>
<td>B$_2$</td>
<td>1 mg.</td>
<td>20%</td>
</tr>
<tr>
<td>7.0</td>
<td>B$_2$</td>
<td>1 mg.</td>
<td>7%</td>
</tr>
<tr>
<td>2.9</td>
<td>folic acid</td>
<td>0.5 mg.</td>
<td>24%</td>
</tr>
<tr>
<td>2.4</td>
<td>reg. diet</td>
<td>---</td>
<td>11%</td>
</tr>
<tr>
<td>6.0</td>
<td>endogenous folic acid</td>
<td>---</td>
<td>32%</td>
</tr>
</tbody>
</table>

for 3 months. When amethopterin therapy was discontinued after the initial C$^{14}$-histidine study, there was a spontaneous reticulocyte increase with a peak of 32 per cent on day 5.

Expired C$^{14}O_2$-specific activity was determined continuously on the breath exhausted from a spherical plastic helmet and passed through an infrared CO$_2$ analyzer and a 22-liter ionization chamber with vibrating reed electrometer similar to that described previously. After baseline measurements were obtained for 20-30 minutes, all subjects tested received an intravenous injection of approximately 25 $\mu$g. of L-histidine-2(ring)-C$^{14}$, specific activity 7.6 mc./millimole, radiochemical purity 97-99 per cent, and breath measurements were continued for 2-4 hours. During this procedure serial blood and urine samples were obtained for the determination of serum folic acid and vitamin B$_{12}$ activity, total radioactivity, and amino acid-specific activities. Results of these blood and urine analyses will be presented elsewhere.

RESULTS

The cumulative excretion of C$^{14}$ in the breath and urine of two normal subjects is presented in figure 1. Breath analyses parameters and hematologic findings in two normal subjects and eight patients with megaloblastic anemia are presented in table 2. Some of the patients were studied initially during relapse, a second time during a period of reticulocytosis in response to vitamin therapy, and a third time during remission after the subsidence of reticulocytosis (figs. 3 and 4); others were studied during one or two of these phases.

The C$^{14}O_2$-specific activity curves of the expired air obtained in a normal subject, a patient with vitamin B$_{12}$ deficiency in relapse, and a patient with folic acid deficiency are shown in figure 2. The specific activity is expressed in $\mu$g. per Gm. of carbon of expired CO$_2$ per 10 $\mu$g. of C$^{14}$ injected. In the normal subject (Control 1) the specific activity rises rapidly and reaches a

*Obtained from Nuclear-Chicago Corporation, Des Plaines, Ill.
maximum at approximately 40 minutes after injection (T_{max} = 40 min.) and then gradually declines so that the cumulative 1-hour excretion of C^{14} in the breath totals 1.18 per cent of the injected C^{14}.

In the vitamin B_{12}-deficient patient (C. K.) the specific activity curve is of the same magnitude and slope characteristics as noted in the normal subject, reaching its maximum at 40 minutes, and in 1 hour 0.73 per cent of the injected activity appears in the breath. By contrast, in the folic acid-deficient patient (R. M.) the specific activity curve rises slowly, reaching a relatively low and delayed maximum at 4 hours, and only 0.06 per cent of the injected activity appears in the breath in 1 hour.

The breath C^{14}O_{2}-specific activity curves in the vitamin B_{12}-deficient patient (M. F.) during relapse, response and remission are shown in figure 3. The graphic insert shows the time relationship of each study to the patient's hematologic course. Prior to the treatment, on day 0, the specific activity curve is of the same magnitude and character as previously shown in the normal subject and in the vitamin B_{12}-deficient patient (fig. 2). On day 6, at a time of increased effective erythropoiesis as evidenced by the marked reticulocytosis, the specific activity curve is quantitatively much lower than on day 0, yet the shape is similar in that a maximum is reached at 28 minutes followed by a gradual decrease. Finally on day 36, when the patient was in
Table 2.—Pulmonary C\textsuperscript{14} Excretion in Normal Subjects and Patients with Megaloblastic Anemia Measured during Relapse, Reticulocyte Response and Remission

<table>
<thead>
<tr>
<th>Subject</th>
<th>Status</th>
<th>Hb Gm./100 ml</th>
<th>Retic. %</th>
<th>Bone Marrow</th>
<th>T\textsubscript{max} min.</th>
<th>Cumulative 1 hr. Breath C\textsuperscript{14} %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>normal</td>
<td>14.5</td>
<td>0.1</td>
<td>—</td>
<td>30</td>
<td>1.18</td>
</tr>
<tr>
<td>Control 2</td>
<td>normal</td>
<td>14.0</td>
<td>0.8</td>
<td>—</td>
<td>50</td>
<td>1.12</td>
</tr>
<tr>
<td>M. F.</td>
<td>remission on</td>
<td>12.1</td>
<td>1.0</td>
<td>normal</td>
<td>29</td>
<td>1.34</td>
</tr>
<tr>
<td>W. C.</td>
<td>remission on</td>
<td>15.0</td>
<td>1.0</td>
<td>—</td>
<td>22</td>
<td>0.79</td>
</tr>
<tr>
<td>G. M.</td>
<td>remission off</td>
<td>10.9</td>
<td>0.3</td>
<td>normal</td>
<td>50</td>
<td>1.02</td>
</tr>
<tr>
<td>M. F.</td>
<td>vitamin B\textsubscript{12} deficient</td>
<td>9.0</td>
<td>0.9</td>
<td>megaloblastic</td>
<td>40</td>
<td>1.16</td>
</tr>
<tr>
<td>J. B.</td>
<td>vitamin B\textsubscript{12} deficient</td>
<td>4.2</td>
<td>0.1</td>
<td>megaloblastic</td>
<td>40</td>
<td>0.71</td>
</tr>
<tr>
<td>C. K.</td>
<td>vitamin B\textsubscript{12} deficient</td>
<td>11.7</td>
<td>1.6</td>
<td>megaloblastic</td>
<td>38</td>
<td>0.73</td>
</tr>
<tr>
<td>M. F.</td>
<td>responding to vitamin B\textsubscript{12}</td>
<td>10.0</td>
<td>13.8</td>
<td>normoblastic eryth. hyperplasia</td>
<td>28</td>
<td>0.12</td>
</tr>
<tr>
<td>W. C.</td>
<td>responding to vitamin B\textsubscript{12}</td>
<td>12.4</td>
<td>7.9</td>
<td>normoblastic eryth. hyperplasia</td>
<td>22</td>
<td>0.05</td>
</tr>
<tr>
<td>G. M.</td>
<td>responding to absence of amethopterin</td>
<td>9.1</td>
<td>11.6</td>
<td>normoblastic eryth. hyperplasia</td>
<td>30</td>
<td>0.32</td>
</tr>
<tr>
<td>J. B.</td>
<td>responding to vitamin B\textsubscript{12}</td>
<td>6.9</td>
<td>10.0</td>
<td>normoblastic eryth. hyperplasia</td>
<td>22</td>
<td>0.06</td>
</tr>
<tr>
<td>J. B.</td>
<td>responding to vitamin B\textsubscript{12}</td>
<td>7.3</td>
<td>2.3</td>
<td>normoblastic eryth. hyperplasia</td>
<td>35</td>
<td>0.27</td>
</tr>
<tr>
<td>M. G.</td>
<td>responding to folic acid</td>
<td>6.5</td>
<td>2.2</td>
<td>normoblastic eryth. hyperplasia</td>
<td>40</td>
<td>0.36</td>
</tr>
<tr>
<td>F. M.</td>
<td>responding to folic acid</td>
<td>6.1</td>
<td>21.2</td>
<td>normoblastic eryth. hyperplasia</td>
<td>27</td>
<td>0.25</td>
</tr>
<tr>
<td>R. M.</td>
<td>folie acid-deficient</td>
<td>4.5</td>
<td>0.3</td>
<td>megaloblastic</td>
<td>240</td>
<td>0.06</td>
</tr>
<tr>
<td>F. M.</td>
<td>folie acid-deficient</td>
<td>6.2</td>
<td>1.0</td>
<td>megaloblastic</td>
<td>140</td>
<td>0.14</td>
</tr>
<tr>
<td>G. M.</td>
<td>folie acid-block</td>
<td>5.8</td>
<td>1.0</td>
<td>megaloblastic</td>
<td>185</td>
<td>0.22</td>
</tr>
</tbody>
</table>

*Sequential order of the studies in each subject.

hematologic remission, the specific activity curve is again of the same magnitude and shape as noted in the normal subject and initially in this vitamin B\textsubscript{12}-deficient patient.

These differences and changes noted in the normal subject, vitamin B\textsubscript{12}-deficient patient, and folic acid-deficient patient during relapse, response and remission were found consistently in all patients studied. Normal subjects, vitamin B\textsubscript{12}-deficient patients in relapse, and patients in remission demonstrated similar T\textsubscript{max} and cumulative 1-hour C\textsuperscript{14} breath excretion: the T\textsubscript{max} occurred within an hour (20-50 minutes) and the cumulative 1-hour C\textsuperscript{14} breath excretion was approximately 1 per cent (0.71 per cent-1.34 per cent) (table 2). Patients studied during a reticulocyte response to vitamin B\textsubscript{12} or folic acid therapy also demonstrated T\textsubscript{max} occurring within an hour.
The maximum permissible total body burden of C\textsubscript{14} for occupational exposure is 400 ftC., as recommended by the National Committee on Radiation Protection, U.S. National Bureau of Standards Handbook 69, 1959.

Fig. 2.—C\textsubscript{14}O\textsubscript{2} specific activity in a normal subject, a patient with vitamin B\textsubscript{12} deficiency megaloblastic anemia, and a patient with folic acid deficiency megaloblastic anemia after intravenous administration of 0.5 mg. L-histidine-2(ring)-C\textsubscript{14} (approximately 25 \textmu c.). (27–40 minutes), but cumulative 1-hour C\textsubscript{14} breath excretion was much less (0.06 per cent–0.35 per cent) (table 2). Folic acid-deficient or folic acid-blocked patients in relapse, on the other hand, demonstrate a markedly prolonged T\textsubscript{max} (140–240 minutes) and markedly decreased cumulative 1-hour C\textsubscript{14} breath excretion (0.06 per cent–0.22 per cent) (table 2).

DISCUSSION

Cumulative pulmonary and renal excretion of C\textsubscript{14} by two normal subjects during the first month after intravenous administration of L-histidine-2(ring)-C\textsubscript{14} approximates 45 per cent (fig. 1). This is somewhat slower than the excretion of C\textsubscript{14} from glycine-2-C\textsubscript{14} which approximates 75 per cent during the first month after administration.\textsuperscript{35} The pattern of excretion for C\textsubscript{14}-histidine is quite similar to that of C\textsubscript{14}-glycine. Should continuing measurements establish that this similarity is maintained, then the average tissue radiation following administration of 100 \textmu c. of C\textsubscript{14} as histidine-2(ring)-C\textsubscript{14} would within a year decrease below the average natural terrestrial and cosmic radiation level.\textsuperscript{*} With the 25 \textmu c. dose used in this study, C\textsubscript{14} tissue radiation corre-

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\textsuperscript{*}The maximum permissible total body burden of C\textsubscript{14} for occupational exposure is 400 \textmu c. as recommended by the National Committee on Radiation Protection, U.S. National Bureau of Standards Handbook 69, 1959.
Fig. 3.—\(^{14}\)O\(_2\) specific activity in a patient with vitamin B\(_{12}\) deficiency megaloblastic anemia occurring after intravenous administration of \(^{14}\)-histidine during relapse (Day 0), reticulocyte response (Day 6), and remission (Day 36).

Fig. 4.—\(^{14}\)O\(_2\) specific activity after intravenous administration of \(^{14}\)-histidine to a patient with megaloblastic anemia occurring as a result of amethopterin therapy; studies were performed during relapse (Day 0), reticulocytosis in response to cessation of amethopterin therapy (Day 13), and remission (Day 30) with respect to the megaloblastic anemia.
sponds to the natural radiation level at approximately 4 months. It thus appears that carbon-14, despite its very long physical half-life (5,600 years), when in the 2(ring) position of histidine, possesses a relatively short biological half-life that permits safe usage for clinical investigation and diagnosis.

The metabolic fate of the 2(ring)-carbon atom of histidine, that which was labeled with C\(^{14}\) in the studies presented, is shown in figure 5. Histidine is metabolized to formiminoglutamic acid (FIGlu)\(^{22}\) and the labeled carbon atom now resides in the formimino group. This formimino group is then transferred to tetrahydrofolic acid (FH\(_4\)), the metabolically active form of folic acid. This transfer, coupled with the release of the formimino nitrogen, results in the formation of C\(^{14}\)-labeled methonyl as N\(^5\), N\(^{10}\)-methenyl tetrahydrofolic acid (N\(^5\), N\(^{10}\)-methenyl-FH\(_4\)), which is one of the metabolically active forms of the monocarbon moiety. This single carbon unit attached to FH\(_4\) may be utilized as (1) a formyl group or its anhydro-derivative, methenyl; (2) a hydroxymethyl group, or its anhydro-derivative, methylene; or (3) a methyl group—depending upon the degree of reduction of this group. It is felt by some that the anhydro-derivatives are probably the active co-enzymes.\(^{20}\) The fate of this active monocarbon moiety is multiple. It may
be coupled with glycine to form serine which in turn, after loss of the amine group, can be oxidized via the tricarboxylic acid cycle to CO\textsubscript{2}, thus labeling the body CO\textsubscript{2} pool with C\textsuperscript{14}O\textsubscript{2}. The active monocarbon unit is used in the formation of methionine from homocystine as it appears in methionine as the methyl group. Three methionine molecules in turn transfer their methyl groups to ethanolamine, probably as phosphatidylethanolamine,\textsuperscript{36} a decarboxylated metabolite of serine, to form choline. Betaine, the oxidation product of choline, can return its methyl groups to the active single carbon moiety pool, with the formation of labeled glycine. This labeled glycine molecule in turn may be oxidized to CO\textsubscript{2}, another site of formation of C\textsuperscript{14}O\textsubscript{2}, or it may combine with still another active single carbon moiety to form serine, which during its decarboxylation to ethanolamine affords another site of C\textsuperscript{14}O\textsubscript{2} formation. As shown in figure 5, the active single carbon moiety is also essential for nucleoprotein synthesis since it is required for incorporation into the 2 and 8 positions of purine and the methyl group of thymidilate. The essential role of folic acid in metabolism is evident; it is the necessary acceptor for the single carbon moiety from histidine and other donors,\textsuperscript{21} and the sole means of transferring this single carbon moiety in the reactions described above. It is also noted that this labeled 2(ring)-carbon atom of histidine is initially involved in two simultaneous and divergent metabolic pathways: (1) the eventual oxidation to C\textsuperscript{14}O\textsubscript{2} and (2) the incorporation into nucleoprotein.

The T\textsubscript{max} occurring within 1 hour and the cumulative 1-hour C\textsuperscript{14} breath excretion of approximately 1 per cent noted in normal subjects, patients in remission, and the vitamin B\textsubscript{12}-deficient patients in relapse (table 2) suggest that the quantitative partition of the 2(ring)-carbon atom of histidine between the above described metabolic pathways is similar in each of these groups of subjects. The markedly delayed T\textsubscript{max} and low cumulative 1-hour C\textsuperscript{14} breath excretion noted in the folic acid-deficient patients in relapse (table 2) are explained by the marked reduction in amount and rate of transfer of the single carbon moiety to that portion of the metabolic pathway allowing for production of C\textsuperscript{14}O\textsubscript{2}. Deficiency of this essential vitamin does not permit release of the formimino carbon atom from FIGlu. The similar results noted in the patient with the megaloblastic anemia (table 2, fig. 4) while receiving amethopterin lends support to this explanation as it appears that the antifolic action of this drug is to prevent the conversion of FH\textsubscript{2} to FH\textsubscript{4}.\textsuperscript{37} A low specific activity curve with T\textsubscript{max} occurring normally within 1 hour and decreased cumulative 1-hour C\textsuperscript{14} breath excretion was noted consistently during response to vitamin therapy (table 2; figs. 3 and 4). The occurrence of this pattern during increased normal nucleoprotein synthesis may be explained by the increased incorporation of the labeled metabolically active single carbon moiety into purine and pyrimidine, leaving a relatively small amount of the active single carbon moiety available for oxidation to C\textsuperscript{14}O\textsubscript{2}.

The specific activity curves obtained in vitamin B\textsubscript{12}-deficient patients with megaloblastic anemia demonstrate a normal T\textsubscript{max} and normal cumulative 1-hour C\textsuperscript{14} breath excretion. The normal values for T\textsubscript{max} are to be expected,
since in the presence of adequate folic acid the active monocarbon units are made available to oxidative pathways at a normal rate of transfer from FIGlu. The unanticipated normal cumulative 1-hour C\(^{14}\) breath excretion is provocative of several intriguing speculations. While there is accumulating convincing evidence that vitamin B\(_{12}\) is required for methionine methyl neogenesis (fig. 5) and deoxyribose formation,\(^{29}\) the latter essential in DNA synthesis, no increase of breath C\(^{14}\) excretion was noted in these vitamin B\(_{12}\)-deficient patients in relapse. Conversely, despite the greatly increased number of giant myeloid cells, megaloblasts, and megaloblastic nucleated red cells with normal or increased DNA content in the marrow,\(^{28}\) many of which were undergoing rapid intramedullary destruction,\(^{39-44}\) and similar cytologic changes in other body cells,\(^{45-48}\) breath C\(^{14}\) excretion was not decreased. Thus, despite greatly increased DNA synthesis, either (1) the total number of monocarbon units required for purine and pyrimidine synthesis in patients with vitamin B\(_{12}\)-deficient megaloblastic anemia is not increased; or, as seems more probable, (2) large numbers of monocarbon units are supplied \textit{in situ} as a result of abnormal cell catabolism and recycling so that the \textit{additional} number of monocarbon units required from the “central” metabolic pool for purine and pyrimidine synthesis is approximately equal to the total number normally required.

Herbert and Zalusky have suggested that in vitamin B\(_{12}\)-deficient subjects “piled up” \textit{Lactobacillus casei}-active folate activity ("probably N\(^{5}\)-methyl-tetrahydrofolic acid") “would tend to reduce the amount of folic acid available for other 1-carbon unit transfers.”\(^{40}\) The tracer studies here presented, however, demonstrate that, in vitamin B\(_{12}\)-deficient patients with megaloblastic anemia, folic acid is not decreased sufficiently to reduce the rate of transfer of the 2(residual)-carbon atom of histidine to the active monocarbon pool or to reduce appreciably monocarbon unit transfer from FH\(_{4}\) to oxidative pathways.

Finding of normal C\(^{14}\)O\(_{2}\) production in vitamin B\(_{12}\)-deficient patients and markedly reduced C\(^{14}\)O\(_{2}\) production in folic acid-deficient patients contrasts with the demonstration of markedly reduced C\(^{14}\)O\(_{2}\) production both in vitamin B\(_{12}\)-deficient and in folic acid-deficient rats by Brown, Silva, Gardiner and Silverman.\(^{29}\) These weanling rats were judged vitamin deficient when urinary FIGlu became increased after 4 weeks of feeding on a synthetic diet in which the appropriate vitamin was omitted. The diet contained 9 per cent casein, thereby limiting the sulfur-amino acids, and was fortified with 0.2 per cent L-histidine. Species difference, special diet, absence of megaloblastic anemia, and lack of evidence of vitamin deficiency, except increased FIGlu excretion, make comparison difficult.

Measurement of urinary FIGlu is the only previously used differential diagnostic test that provides some insight into the nature of the metabolic derangement present in the individual patient. Though the determination of breath C\(^{14}\)O\(_{2}\) and the measurement of urinary FIGlu after histidine loading permit examination of related aspects of the same metabolic process, two important distinctions can be made between these investigative approaches.
The current study uses only a tracer dose of 0.5 mg. histidine as compared with the 2,000-20,000 mg. of histidine used in the urinary FIGlU test. This large unphysiologic dose of histidine may be readily limited in its metabolism by retrograde accumulation of intermediates, including FIGlU, as the result of an enzyme deficiency such as vitamin B12 that would limit the rate of a step subsequent to the transfer to the formimino group from FIGlU to tetrahydrofolic acid. The considerable overlap of urinary FIGlU excretion observed in patients with folic acid deficiency and patients with vitamin B12 deficiency may be a consequence of the large loading dose of histidine. Perhaps little or no overlap of urinary C14-FIGlU values will be observed between these two groups following the administration of tracer amounts of C14-histidine.

Continuous measurement of breath C14O2 discloses two significant parameters, Tmax and cumulative C14 excretion, as compared with the single parameter of the amount of FIGlU excreted in the urine. The time interval between injection and maximal C14O2-specific activity, Tmax, appears to be a sensitive index of the oxidation rate of the 2(ring)-carbon atom of L-histidine to C14O2. This oxidation involves the transference of the formimino moiety from FIGlU to tetrahydrofolic acid. Hence, folic acid deficiency, but not vitamin B12 deficiency, causes marked prolongation of Tmax. If Tmax is normal, then cumulative C14 breath excretion seems to be a significant index of the partition of the active monocarbon moieties between pathways for oxidation and synthetic pathways resulting in the formation of purines and pyrimidines. If these preliminary results were confirmed in a large series of patients, measurements of these parameters would provide specific criteria for rapid differentiation between patients with vitamin B12-deficient or folic acid-deficient megaloblastic anemia. In addition, and more basically, this approach provides a quantitative dynamic representation of metabolic function or malfunction which will be more complete when integrated with measurements derived from current analyses of blood and urine samples.

SUMMARY

Intermediary metabolism of the monocarbon pool and histidine in normal subjects and patients with megaloblastic anemia was studied by continuous measurement of pulmonary excretion of C14O2 and urinary excretion of C14 after injection of L-histidine-2(ring)-C14. Cumulative pulmonary and renal excretion of C14 for 1 month by two normal subjects approximates 45 per cent of the amount injected. Within 4 months after injection of the dose used in this study, the resultant average tissue radiation decreases below the average natural terrestrial and cosmic radiation level.

Simultaneous determination of two parameters, (1) cumulative 1-hour pulmonary C14 excretion and (2) the time of occurrence of maximum C14O2-specific activity (Tmax), may permit rapid and unequivocal differentiation between folic acid deficiency and vitamin B12 deficiency in the pathogenesis of megaloblastic anemia. Folic acid deficiency results in marked diminution of pulmonary C14 excretion (approximately 0.1 per cent of injection C14 in 1 hour) and marked prolongation of C14O2-specific activity Tmax (approxi-
mately 3 hours), while both parameters are normal (approximately 1 per cent and less than 1 hour, respectively) in patients with vitamin B₁₂ deficiency and megaloblastic anemia.

Measurement during periods of reticulocyte response to either folic acid or vitamin B₁₂ demonstrate normal C14O₂-specific activity Tₘₐₓ but decreased pulmonary C14 excretion. These observations suggest that prolongation of C14O₂-specific activity Tₘₐₓ is a sensitive index of folic acid deficiency or block and that if Tₘₐₓ is normal, pulmonary C14 excretion is a sensitive index of the relative partition of the active monocarbon pool between pathways for oxidation and pathways for nucleic acid synthesis.

This type of breath analysis seems to provide a quantitative dynamic representation of metabolic function which may be particularly useful in differentiating between the alterations of intermediary metabolism that occur in patients with folic acid-deficient megaloblastic anemia and in patients with vitamin B₁₂-deficient megaloblastic anemia.

**SUMMARY IN INTERLINGUA**

Le metabolismo intermediari del stock de monocarbon e de histidina in subjectos normal e in pacientes con anemia megaloblastic esseva studiate per le continue mesuration del excretion pulmonari de C14O₂ e del excretion urinari de C14 post le injection de L-histidina-2(anulo)-C₁⁴. Le cumulative excretion pulmonari e renal de C¹⁴ in le curso de 1 mense per duo subjectos normal amontava a approximativemente 45 pro cento del total injicite. Intra 4 menses post le injection del dose usate in le presente studio, le resultante radiation medie de tissu declina a infra le niveiio medie del natural radiation terrestre e cosmic.

Le determination simultanea de duo parametros—(1) le cumulative horal excretion pulmonari de C¹⁴ e (2) le tempore del occurrentia del maxime activitate specific de C14O₂ (Tₘₐₓ) —permitte possibilmente un rapide differentiation inequivoc inter carentia de acido folic e carenti a de vitamina B₁₂ in le pathogenese de anemia megaloblastic. Carentia de acido folic resulta in un marcate diminution del excretion pulmonari de C¹⁴ (approximativemente 0,1 pro cento del injection de C¹⁴ in 1 hora) e in un marcate prolongation del Tₘₐₓ de activitate specific de C14O₂ (approximativemente 3 horas), durante que ambe le paramentros es normal (approximativemente 1 pro cento e minus que 1 hora, respectivemente) in pacientes con carentia de vitamina B₁₂ e anemia megaloblastic.

Mesuraciones effectuate durante periodos de responsa reticulocytic a acido folic o vitamina B₁₂ demonstra normal Tₘₐₓ de activitate specific de C14O₂ sed un reducete excretion pulmonari de C¹⁴. Iste observationes suggere que le prolongation del Tₘₐₓ de activitate specific de C14O₂ es un sensibile indice de carentia de acido folic o de blocage de acido folic e que in le presentia de un normal Tₘₐₓ le excretion pulmonari de C¹⁴ es un sensibile indice del partition relative del active stock de monocarbon inter sequentias de oxydation e sequentias de synthese de acido nucleic.

Il pare que iste typo de analyse del halito provide un quantitative repre-
sentation dynamic del functionamento metabolic que pote esser particula-
mente utile in differentiar inter le alterationes de metabolismo intermediari
le quales occurre in patientes con anemia megaloblastic a carentia de acido
folic e con anemia megaloblastic a carentia de vitamina B12.

ACKNOWLEDGMENT
We are indebted to Dr. Ralph O. Wallerstein for helpful suggestions in the prepara-
tion of this manuscript.

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Differentiation between Vitamin B\textsubscript{12}—deficient and Folic Acid—deficient Megaloblastic Anemias with C\textsuperscript{14}—Histidine

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