Relative Folate Deficiency of Erythrocytes in Pernicious Anemia and its Correction with Cyanocobalamin

By Bernard A. Cooper and Louis Lowenstein

The determination of folate activity in serum with Lactobacillus casei allows recognition of patients with folate deficiency.1-3 It has been observed that much of the L. casei-active folate in human serum consists of N5-methyl tetrahydropteroyl monoglutamate,4 and that the concentration of folate activity in serum is increased in some patients with pernicious anemia in relapse.5 However, not all such patients have high serum folate activity.

Determinations of folate activity in whole blood have revealed that folate activity of erythrocytes is higher than that of serum, but most of the folate activity of erythrocytes is not available for assay with L. casei without pre-incubation with serum or plasma.6,7 It has been reported that whole blood folate activity is decreased in patients with folate deficiency8,9 and in some patients with pernicious anemia.10

We have reported that in patients with vitamin B12 deficiency, the erythrocyte folate activity is lower relative to the serum folate activity than in normal subjects.11 Because this relative deficiency of erythrocyte folate for the corresponding serum folate activity was reversed by specific therapy with cyanocobalamin, we felt that detailed study of this phenomenon might contribute to understanding of the interrelationships of folate and vitamin B12 in megaloblastic anemia.

The conversion of N5-methyl tetrahydropteroyl monoglutamate to tetrahydrofolate utilizes a vitamin B12 dependent co-enzyme.5,12 It has been suggested that megaloblastic anemia occurs in pernicious anemia because of interference with this reaction.5,11-12 If conversion of methyl-folate to folate is required to allow transport of folate compounds into maturing erythroid cells, then folate deficiency of erythroid cells should appear in all patients with megaloblastic anemia due to deficiency of vitamin B12. If vitamin B12 is necessary for folate metabolism within the cells, then the relative concentrations of the different folate co-enzymes within erythroid cells might differ in vitamin B12 deficiency from the distribution in normal subjects.

We have undertaken to investigate this problem by studying the serum and erythrocyte folate activity in patients with vitamin B12 deficiency before and after specific therapy with cyanocobalamin. In order to evaluate the
results, it was deemed desirable to determine the reproducibility of the assay technic, and to compare the serum and erythrocyte folate activity in normal subjects and in patients with certain deficiencies. Finally an attempt was made to separate certain of the folate co-enzymes within the erythrocyte in normal subjects and in patients with vitamin B₁₂ deficiency before and after therapy.

METHODS

Folate activity was determined utilizing L. casei by the technic of Usdin et al.¹³ and Jukes¹⁴ as modified by Baker et al.¹ and by Herbert.¹⁵ The detailed procedure utilized in our laboratory has been described previously.³ Serum folate was assayed without preincubation of the diluted serum, but determinations of whole blood folate activity included 90 minutes of preincubation prior to precipitation of proteins by autoclaving. The frozen blood was thawed, diluted 1:5 with ion-free water, then diluted 1:10 in ascorbate-phosphate buffer (containing 150 mg. per cent or 8.5 mM. ascorbate), incubated and assayed. The whole blood values were calculated per ml. of erythrocytes by the formula:

\[
\text{erythrocyte folate} = \frac{\text{whole blood folate} - \text{serum folate}}{\text{ml. erythrocytes/ml. of blood}} + \text{serum folate}
\]

In this way the erythrocyte folate activity was determined after incubation of the hemolyzed erythrocytes with plasma.

Assay with Streptococcus faecalis utilized a modification of the technic described by Teply and Elvehjem.¹⁷ The medium was a commercially available mix.* Blood was taken by venipuncture into a pair of acid washed sterile tubes. One tube contained 20 mg. of sodium citrate and provided an unclotted simple. Hematocrit was determined on the unclotted sample prior to freezing. Samples to be assayed within 1 week were frozen without the addition of ascorbate. Other samples, including sequential samples taken from a single subject were mixed with 5 mg. of ascorbic acid per ml. of sample before storage at -20 C. Samples containing ascorbate were adjusted to pH 6.1 with 2N sodium hydroxide prior to assay. No attempt was made to have the subject fasting when blood was taken; indeed, most of the samples were obtained from subjects who had completed breakfast.

Serum vitamin B₁₂ level was determined with Euglena gracilis var. bacillaris.¹⁸ DEAE cellulose chromatography utilized the technic of Johns et al.¹⁰ Blood was autoclaved after incubation in ascorbate-phosphate buffer for 90 minutes. The clear supernatant obtained after autoclaving was applied to the column. After stepwise elution with phosphate, compounds remaining adsorbed to the column were eluted with 0.1 N sodium hydroxide. Effluent fractions were refrigerated immediately, and added to previously prepared tubes of medium which then were autoclaved, inoculated with either Str. faecalis or L. casei and assayed.

Vitamin B₁₂ absorption in vivo was measured by the Schilling test utilizing two consecutive 24-hour urine collections as described previously.²⁰ When possible, the patients studied were fed a diet with a low folate content. This diet was adjusted for the caloric needs and for the food preferences of each patient. A sample diet is shown in table 1. The folate activity of the diet after preparation for eating was assayed with L. casei without deconjugation.

Formiminoglutamic acid (FIGlu) was determined in urine specimens after the oral administration of a single dose of 10 Gm. of 1-histidine. Urine was collected for 12 hours in bottles containing 2 to 3 ml. of concentrated hydrochloric acid and was assayed electro-

*Purchased from Difco Corporation.
Table 1.—Typical Folate Deficient Diet

<table>
<thead>
<tr>
<th>Quantity (Gm. or ml.)</th>
<th>Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakfast</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>Orange juice</td>
</tr>
<tr>
<td>10</td>
<td>Oatmeal</td>
</tr>
<tr>
<td>30</td>
<td>Egg white</td>
</tr>
<tr>
<td>30</td>
<td>Bread</td>
</tr>
<tr>
<td>1</td>
<td>Butter</td>
</tr>
<tr>
<td>10</td>
<td>Jam</td>
</tr>
<tr>
<td>100</td>
<td>Milk</td>
</tr>
<tr>
<td>15</td>
<td>Sugar</td>
</tr>
<tr>
<td>Lunch</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>Minced sirloin (broiled)</td>
</tr>
<tr>
<td>70</td>
<td>Potato</td>
</tr>
<tr>
<td>75</td>
<td>Turnips</td>
</tr>
<tr>
<td>15</td>
<td>Bread</td>
</tr>
<tr>
<td>12</td>
<td>Gelatin dessert</td>
</tr>
<tr>
<td>150</td>
<td>Milk</td>
</tr>
<tr>
<td>5</td>
<td>Sugar</td>
</tr>
<tr>
<td>Dinner</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>Breast of chicken (steamed)</td>
</tr>
<tr>
<td>70</td>
<td>Potato</td>
</tr>
<tr>
<td>60</td>
<td>Carrots</td>
</tr>
<tr>
<td>15</td>
<td>Bread</td>
</tr>
<tr>
<td>100</td>
<td>Canned peaches</td>
</tr>
<tr>
<td>150</td>
<td>Milk</td>
</tr>
<tr>
<td>10</td>
<td>Sugar</td>
</tr>
<tr>
<td>Evening snack</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>Egg</td>
</tr>
<tr>
<td>120</td>
<td>Milk</td>
</tr>
<tr>
<td>15</td>
<td>Sugar</td>
</tr>
<tr>
<td>3</td>
<td>Vanilla</td>
</tr>
</tbody>
</table>

Calories, 1550; protein, 65 Gm.; fat, 50 Gm.; carbohydrate, 201 Gm. and folate, 35 µg. by assay with L. casei without deconjugation.

The diet was adjusted to the caloric requirements and food preferences of each patient. The intake was maintained constant throughout each study. The diet was adjusted for increased appetite after therapy by increasing caloric intake with bread and jam. All vegetables used were cooked.

Approximate quantitation was effected by inspecting the FIGlu spot visually and comparing the intensity of color with two standard FIGlu spots (50 µg./ml. and 100 µg./ml.) run concurrently. With this technic, we have successfully detected FIGlu in concentrations as low as 25 µg./ml. of urine.

Patients Studied

Two groups of patients were utilized for the studies described in this report: one group included most of the patients with typical megaloblastic bone marrow. These patients received a diet which was carefully controlled and were repeatedly studied during a control period and during planned, carefully supervised therapy, usually on a metabolic ward. The second group was selected from approximately 300 patients in which routine determinations of serum-vitamin B₁₂ and folate and erythrocyte-folate activity were carried out between January, 1961 and June, 1963. The normal subjects,
the patients with iron deficiency, and some of the patients with deficiencies of folate and vitamin B12 were selected from this group.

Normal Subjects

Twenty-four patients were selected as “normal.” Of these, six were normal in all respects and bone marrow examination was not carried out. Seven patients were studied because of neurologic abnormalities and the subsequently-unconfirmed suspicion of subacute degeneration of the spinal cord, but were otherwise normal. The remaining 11 included two with neoplasia whose dietary intake was adequate, one with polycythemia vera, one with reticulocytosis of 4.4 per cent and hemoglobin of 9.9 Gm. per cent following acute hemorrhage, two with pernicious anemia in remission who were receiving monthly injections of 100 μg. of cyanocobalamin, one with myxedema, two with mild hypoplastic anemia, of which one subsequently recovered spontaneously, and two with bronchopneumonia and mild secondary anemia. These 11 patients had normoblastic bone marrow with normal hemosiderin in the marrow. The values of these 24 patients were added to those of 9 normal laboratory personnel to make up the “normal” group.

Iron Deficiency

Twenty patients were found to have severe iron deficiency with adequate clinical information available to assess dietary intake. In all of these patients, severe hypochromic anemia was present, serum iron was low, unsaturated iron binding capacity was increased and iron stain of the bone marrow revealed absent iron stores. Five of these patients also had macrogranulocytosis of the bone marrow, manifested by giant metamyelocytes and band neutrophils without obvious megaloblastic features in the erythroid cells.

Folate Deficiency

Patients with folate deficiency were recognized by the presence of megaloblastic and macrogranulocytic bone marrow and satisfactory evidence of inadequate diet or of malabsorption syndrome. Fourteen such patients were found. The patients with inadequate diet admitted to ingesting a diet usually devoid of folate-rich foods such as liver, kidney, and vegetables other than potatoes. Several probably were “tea and toasters.” The patients with malabsorption syndrome were characterized by steatorrhoea, abnormal xylose absorption, and abnormal jejunal biopsy. In one patient with typical sprue, attempts to biopsy the jejunal mucosa were unsuccessful. In all of these patients, the serum vitamin B12 level was greater than 200 pg./ml. except for one whose diet had consisted almost entirely of carbohydrate. This patient was admitted with megaloblastic bone marrow and with hemoglobin of 5.1 Gm. per cent. Serum vitamin B12 level on admission was 96 pg./ml. and serum and erythrocyte folate were less than 2.5, and 46.5 ng./ml. respectively. Serum iron was 317 μg./100 ml. This patient received a standard ward diet for 5 days prior to being placed on a folate deficient diet, and underwent a spontaneous remission with reticulocytosis to 30 per cent. As-
sociated with conversion to normoblastic bone marrow, serum iron decreased to 49 μg./100 ml. of serum, and serum vitamin B₁₂ level increased spontaneously to 200 pg./ml. Serum folate remained less than 2.5 ng./ml. throughout the period of observation, but erythrocyte folate rose to 169 ng./ml. on the twentieth hospital day, 5 days after the peak of the reticulocytosis. Schilling test was normal. This patient obviously had severe folate deficiency. Possibly he also had associated deficiency of vitamin B₁₂. In all of the folate deficient patients, reticulocytosis or conversion of megaloblastic to normoblastic bone marrow occurred on diet alone or on small doses of folic acid. Many of these patients were not studied on the metabolic ward and so control of the total quantity of folate inducing remission in these patients was not possible. Of the patients with folate deficiency, two showed evidence of classical malabsorption syndrome diagnosed by abnormal xylose absorption and steatorrhoea, and abnormal jejunal biopsy in one. In the others, no evidence of malabsorption was detected and dietary history was consistent with the appearance of folate deficiency.

**Vitamin B₁₂ Deficiency**

All of the patients with vitamin B₁₂ deficiency had low serum vitamin B₁₂ levels with megaloblastic bone marrow. The highest serum vitamin B₁₂ level in this group was 86 pg./ml. and the lowest was 12. Of the 21 vitamin B₁₂ deficient patients, all but two were found to have classical pernicious anemia on the basis of typical Schilling test and response to vitamin B₁₂ therapy. One patient was found to have a normal Schilling test and had taken a diet devoid of animal produce for several years. He probably had developed vitamin B₁₂ deficiency on a nutritional basis. Another patient was found to have developed vitamin B₁₂ deficiency with subacute combined degeneration of the cord after resection of approximately 5 feet of ileum because of trauma. This patient had iron deficiency, normal gastric acid after histamine and low excretion of radioactivity in the urine during Schilling test with cyanocobalamin alone, with cyanocobalamin and hog intrinsic factor concentrate, after tetracycline, and with sodium bicarbonate. He had not previously received hog intrinsic factor concentrate. The fat content of his stools was normal.

**Observations**

To evaluate the reproducibility of the assay, 4 blood samples were obtained per day from each of three normal fasting subjects for 4 consecutive days. In this way, 16 samples were obtained from each subject. The folate activity of plasma and of erythrocytes was assayed on 3 different days. The mean plasma folate concentrations of these three subjects were 10.8, 8.6, and 6.45 ng./ml. with a single standard deviation representing 13.3, 16.2 and 20.9 per cent of the mean respectively. Whole blood folate activity expressed per ml. of erythrocytes revealed mean values of 286, 223 and 152 ng./ml. respectively with a single standard deviation representing 26.4, 14.1 and 13.8 per cent of the mean. Thus the average standard deviation of plasma
Mean serum folate (ng./ml.) of two subjects before and after eating a normal meal which included neither liver nor kidney. The values represent the mean of four experiments.

To determine if samples might be taken from non-fasting subjects, serum folate determinations were carried out on two subjects before and after lunch on 4 consecutive days. Lunch consisted of ground beef, cheese, eggs, potato, cooked tomato, string beans and canned fruit, but did not include liver or kidney. The mean serum folate values before and after eating are shown in table 2. It will be seen that although the postprandial value appeared to be slightly higher than the fasting value, the difference was not statistically significant. Other studies of folate feeding were carried out on a single normal fasting subject. The serum folate rose less than 18 per cent following the feeding of 526 Gm. of cottage cheese which represented 49 µg. of folate activity and rose from 4.6 to 8.0 ng./ml. 1½ hours after feeding 200 Gm. of cooked frozen lima beans representing 152 µg. of folate activity. When the same subject was fed 60 Gm. of broiled calf’s liver, representing 700 µg. of folate activity, the serum folate rose from the fasting value of 5.0 to 51 ng./ml. 1½ hours after the meal. The folate values of these foods were determined by assay with L. casei without deconjugation.

In order to determine if serum and erythrocyte folate activity correlated with states of clinical deficiency, the serum and erythrocyte folate values were compared in “normal” subjects and in patients deficient in iron and in folate (fig. 1). Most of the patients with folate deficiency were found to have serum folate values below 3.0 ng./ml. as we have reported previously⁴ and to have erythrocyte folate values below 175 ng./ml. There was some overlap between the erythrocyte folate activity in the “normal” subjects and in the patients with folate deficiency. In the 33 “normal” subjects, a correlation was found between the serum folate and the erythrocyte folate value (r = 0.389, p <0.05). The mean serum folate value for the normal group was 8.1 with a standard deviation of 3.1. However, the points were skewed so that the median value was 7.1 ng./ml. The mean erythrocyte folate for the normal group was 422 ng./ml. of erythrocytes with a standard deviation of 160 and median of 379. Many of the patients with iron deficiency and normoblastic

| Table 2.—The Effect of Food on Serum Folate |
|-------------------------------|---|---|
|                                | A  | B  |
| Fasting                       | 5.02 | 4.5 |
| 1½ hours after meal           | 5.4 | 5.3 |
| t                             | 1.15 | 2.06 |
| p                             | 0.33 | 0.13 |

Fasting serum folate values was 16.8 per cent of the mean and of erythrocyte folate values 18.3 per cent of the mean. Similar results were obtained when aliquots of serum were assayed repeatedly over several months. This large standard deviation indicates that one would expect only two-thirds of folate determinations on a single sample of blood or serum to fall within 18 per cent of the mean value for that sample. This variability of the assay in our hands must be considered when correlating folate values with the clinical state.
Fig. 1.—Relationship of serum folate and erythrocyte folate in normal subjects, in patients with iron deficiency, and in patients with folate deficiency. Patients with folate deficiency are differentiated from "normal" subjects by the presence of low serum and erythrocyte folate. Most of the patients with iron deficiency appear to cluster to the left of the graph, indicating that some of these subjects appeared to have relative deficiency of serum folate for the corresponding erythrocyte folate. Patients with combined iron deficiency and macrogranulocytosis of the bone marrow were observed to show somewhat higher serum and erythrocyte folate activities than did patients with uncomplicated folate deficiency.

marrow appeared to differ somewhat from the normal group by having relatively lower serum folate for the corresponding erythrocyte folate. Careful history revealed that a number of patients with severe iron deficiency had lost their appetite during the weeks prior to admission to the hospital. It may be, therefore, that the decrease of serum folate in this group might represent developing folate deficiency in subjects in whom adequate time had not elapsed for depletion of erythrocyte folate. It will be noted that five of the iron deficient patients were found to have macrogranulocytosis of the bone marrow which disappeared on adequate diet. One of these patients received iron therapy while receiving a folate deficient diet and the macrogranulocytosis of the bone marrow did not disappear. The serum folate values of the five patients with iron deficiency anemia and macrogranulocytosis of the bone marrow ranged from < 2.5 to 3.9 ng./ml. In two of these patients, the serum folate was above 3.0 but below 4.0 ng./ml. The erythrocyte folate values of all five patients were normal. It would appear that in patients with early, mild deficiency of folate associated with iron deficiency, macrogranulocytosis of the bone marrow may be observed when the serum folate falls below 4.0 ng./ml. at a time when the erythrocyte folate is in the normal range.

As shown in figure 2 when values of serum and erythrocyte folate of
RELATIVE FOLATE DEFICIENCY IN PERNICIOUS ANEMIA

Fig. 2.—Serum and erythrocyte folate activity in pernicious anemia. Patients with vitamin B₁₂ deficiency show relative accumulation of folate activity in the serum compared to the erythrocyte folate activity. The range of 33 “normal” subjects is indicated by the area with vertical hatching and that of most of the patients with folate deficiency by the area with horizontal hatching. The line was drawn by the method of least squares.

patients with vitamin B₁₂ deficiency were plotted, the points fell below the area of the results of the normal group. A clear correlation was found between the serum and whole blood values in the vitamin B₁₂ deficient patients \((r = 0.81, p < 0.001)\). The erythrocyte folate activity of the patients with vitamin B₁₂ deficiency was lower for the corresponding serum folate value than was observed in the normal group.

In figure 3 are plotted the serum and erythrocyte folate activity of 10 patients with pernicious anemia before therapy and 14 days after therapy at which time the reticulocytosis had abated and the bone marrow was normoblastic. It will be noted that the relationship between the erythrocyte and serum folate activity altered during therapy so that post-treatment values fell within the same area as did the “normal” subjects. In one patient (patient no. 9), determinations of serum and erythrocyte folate were carried out after admission, after 12 days of folate deficient diet, after 8 days of therapy with 3 μg. of cyanocobalamin per day, and on day 21 of therapy, 9 days after receiving 1000 μg. of cyanocobalamin. This patient (J. H. in figure 4) showed an initial fall of serum folate without change of erythrocyte folate while on folate deficient diet and before vitamin B₁₂ administration. This was followed by a gradual rise of erythrocyte folate and further decrease of serum folate after vitamin B₁₂ was administered.

To exclude the possibility that relatively low erythrocyte folate in pernicious anemia might be due to interference with activation of erythrocyte
folate by $B_{12}$ deficient plasma, blood was obtained from three patients with pernicious anemia, and from subjects without vitamin $B_{12}$ deficiency. The plasma and erythrocytes were separated by centrifugation, and the erythrocytes were washed three times with cold isotonic saline solution. Erythrocyte folate was determined on mixtures of washed erythrocytes and plasma from the $B_{12}$ deficient and non-deficient subjects. As shown in table 3, the use of homologous plasma was without effect on the erythrocyte folate activity, indicating that the apparent erythrocyte folate deficiency in pernicious anemia is not due to an abnormality of pernicious anemia plasma which prevents activation of erythrocyte folate.

In figure 4 are plotted reticulocyte count and serum and erythrocyte folate activity of two patients with pernicious anemia maintained on a folate deficient diet and treated with 3 $\mu$g. of cyanocobalamin daily. Both patients showed complete conversion of the megaloblastic erythropoiesis to normoblastic within six days on this regimen, although some macrogranulocytosis persisted for several additional days. It is apparent that the erythrocyte folate activity increased following the reticulocytosis in J. H. and serum folate fell. Repeated bone marrow aspirations were carried out during the experiment and revealed no change before the institution of therapy. Patient J. H. was only slightly anemic (hematocrit 37 per cent), but showed typical manifestations of combined system disease. The other patient, E. C., was mildly anemic (hematocrit 32 per cent) and developed iron deficiency during
Fig. 4.—Patients with pernicious anemia treated with small doses of vitamin B₁₂.
Following the institution of specific therapy, mild reticulocytosis was observed in both patients followed by a rise of whole blood folate. Patient J. H. showed a gradual decrease of serum folate during the control period which continued following therapy with vitamin B₁₂.

the 4 weeks following therapy as the hematocrit rose to 36 per cent. It is apparent that the serum folate of J. H. decreased progressively during the control period, during which he received a folate deficient diet, and daily injections of 0.5 ml. of normal saline. Associated with this placebo therapy,
Table 3.—Effect on Erythrocyte Folate Activity of Exchanging Plasma and
Erythrocytes of Patients with Pernicious Anemia and
Normal Subjects Prior to Assay

<table>
<thead>
<tr>
<th>Cells</th>
<th>Plasma</th>
<th>Folate/ng./ml. RBC's</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>558</td>
</tr>
<tr>
<td>Normal</td>
<td>P.A.</td>
<td>543</td>
</tr>
<tr>
<td>P.A.</td>
<td>P.A.</td>
<td>259</td>
</tr>
<tr>
<td>P.A.</td>
<td>Normal</td>
<td>234</td>
</tr>
</tbody>
</table>

He noted decrease of the paraesthesias in his fingers, and the area of hypoesthesia diminished. The rate of regression of the area of hypoesthesia in the fingers and the rate of decrease of the serum folate did not alter following the substitution of injections of 3 μg. of cyanocobalamin for the saline injections, although the bone marrow become normoblastic, and mild reticulocytosis was observed (fig. 4). This patient’s diet had been excellent prior to admission to the hospital, with large quantities of leafy green vegetables and meat taken daily.

The increase of erythrocyte folate in E. C. (fig. 4) following cyanocobalamin therapy was striking, and suggests that if this increase was due only to the first group of new erythrocytes entering the circulation, then these erythrocytes may have had a higher folate content than normal.

Figure 5 demonstrates the changes in reticulocyte count and serum and erythrocyte folate activity in a woman with severe megaloblastic anemia and subacute combined degeneration of the cord. Hematocrit on admission was 11 per cent, and serum vitamin B₁₂ level was 50 pg./ml. She was treated with vitamin B₁₂, initially with 10 μg. followed by 1000 μg. per day for 2 days associated with a Schilling test and then 100 μg. per day. She showed a satisfactory rise of erythrocyte values, a striking reticulocytosis, and some improvement of her neurologic disease. Associated with therapy she showed a marked decrease of serum folate activity together with a rise of erythrocyte folate activity.

Figure 6 illustrates the changes of folate activity of serum and erythrocytes in a patient with pernicious anemia treated with a single oral dose of 15 mg. of folic acid. The rise of erythrocyte folate following the reticulocytosis induced by folic acid therapy is apparent as is the further rise following the administration of vitamin B₁₂. This patient was admitted with mild anemia (hematocrit 32 per cent) and was found to have megaloblastic bone marrow. Serum vitamin B₁₂ level was 15 pg./ml. The decrease of erythrocyte folate during the initial period of therapy before the reticulocytosis was not associated with a decrease of hematocrit. It is possible that this decrease may have been associated with the removal from the circulation of the remaining erythrocytes formed before the vitamin B₁₂ deficiency became severe, or may be coincidental and due to the variability of the method. This experiment indicates that the folate activity of vitamin B₁₂ deficient erythrocytes can be increased somewhat by the administration of a large quantity of folic acid.

Figure 7 illustrates the course of a patient with nutritional folate deficiency
RELATIVE FOLATE DEFICIENCY IN PERNICIOUS ANEMIA

Fig. 5.—A patient with marked anemia and combined system disease treated with large doses of vitamin B₁₂. Therapy resulted in reticulocytosis and marked decrease of serum folate with rise of erythrocyte folate.

with malnutrition due to anorexia. Serum vitamin B₁₂ was 220 pg./ml. and bone marrow showed intermediate megaloblastic changes. She showed a satisfactory rise of erythrocyte folate activity while receiving 0.2 mg. of folic acid per day by injection. Her bone marrow was converted from intermediate megaloblastic change to normoblastic on this therapy.

Figure 8 illustrates the course of a patient with osteogenic sarcoma of the skull who received three courses of amethopterin therapy. Megaloblastic erythropoiesis was observed during all courses of therapy. She received 50 mg. per day of amethopterin by intra-arterial infusion together with 24 mg. of folinic acid per day by intramuscular injection during the amethopterin therapy. FIGlu excretion rose to 25 and 50 mg. per day respectively during the first two courses of therapy, but no FIGlu was detected in the urine during the third course. It is of interest that although the serum folate activity was measurable shortly after the first and third courses of therapy, and FIGlu excretion returned to normal after each course, her erythrocyte folate activity was unmeasurable (less than 0) because of inhibition of growth of L. casei for 7 weeks following the first course of therapy. Two other patients receiving 5 mg. per day of amethopterin for psoriasis also showed unmeasurable folate activity (less than 0) because of inhibition of growth of L. casei when whole blood was assayed for several weeks following therapy. This indicates that amethopterin probably was incorporated into the erythrocytes which matured during the amethopterin therapy, and acted as
Fig. 6.—Effect of therapy with folic acid on serum and erythrocyte folate in pernicious anemia. The patient received 15 mg. of folic acid by mouth. Following the reticulocytosis induced by this therapy, a slight rise of whole blood folate activity was observed. The whole blood folate activity increased further following administration of vitamin B₁₂. A different scale for serum folate from that used in other figures has been employed in this graph.

Fig. 7.—Effects of folic acid therapy in folate deficiency. The patient was treated with 0.2 mg. per day of folate acid by injection. This resulted in a gradual rise of whole blood folate activity following the reticulocytosis with conversion of the bone marrow to normal.
a folate antagonist during the assay of whole blood with *L. casei*. The determination of erythrocyte folate involved the determination of the folate content of whole blood and so the erythrocyte folate values are not reliable until after the disappearance of the amethopterin from the plasma. Hence, the rapid decrease of erythrocyte folate to less than zero after the institution of amethopterin therapy (fig. 8) does not necessarily indicate that amethopterin appeared immediately in the erythroid cells.

Because of the possibility that the low erythrocyte folate in pernicious anemia might be a reflection of a predominantly old erythrocyte population, patients with hypoplastic anemia, and five with hemolytic anemia were examined for evidence of abnormal relationship between serum and erythrocyte folate.

These studies were inadequate to determine if the folate content of an old erythrocyte population differs from that of a young population, but no clear relationship between bone marrow activity and the relationship of serum and erythrocyte folate was found.

Hemolyzed whole blood was fractionated on DEAE cellulose as described in "Methods." The chromatograms obtained on patient J. H. before and after therapy with vitamin B₁₂ are shown in figure 9. These chromatograms do not differ significantly from those of blood obtained from normal subjects. The only difference between the pre-treatment and post-treatment chromatograms was a relative decrease after therapy in the concentration of the fraction eluted by 0.1 M. sodium hydroxide after the completion of the
Fig. 9.—DEAE chromatography of folate co-enzymes of erythrocytes in pernicious anemia. Chromatogram of folate activity (L. casei) of whole blood obtained from a patient with pernicious anemia (J. H.) before and 15 days after therapy. Slight Str. faecalis activity was observed associated with the first peak at 0.2 M phosphate buffer, but even this highest Str. faecalis activity was too low to be read quantitatively.

elution with phosphate buffer. The significance of this difference remains to be determined. The remainder of the chromatogram is similar to that described by a number of workers for the fractionation of erythrocyte folate co-enzymes.

It is of interest that chromatography resulted in recovery of 250–300 per cent of the folate activity originally applied to the column. This suggests that some folate activity of erythrocytes may become available for L. casei during passage through the column. It also is possible that conjugates of folate such as polyglutamates are present in the erythrocytes, and that these conjugates do not support the growth of L. casei identically with folic acid.

DISCUSSION

The relationship which has been demonstrated between the folate content of erythrocytes and that of serum indicates either that one is the precursor of the other or that both are derived from a common source. The altered relationship between serum and erythrocyte folate activity in patients with vitamin B₁₂ deficiency, however, suggests that the erythrocyte folate probably is derived from the folate of the plasma and that vitamin B₁₂ may be necessary to allow penetration of folate from the plasma into the maturing erythroid cell. The previously described failure of folic acid to penetrate mature erythrocytes together with the rise which we have demonstrated of erythrocyte folate in patients with vitamin B₁₂ deficiency following the reticulocytosis.
induced by therapy is in agreement with the suggestion that only very early erythroid cells are capable of incorporating folate. The two subjects treated with small quantities of cyanocobalamin showed a modest rise of erythrocyte folate associated with treatment (fig. 4). The subsequent rise of erythrocyte folate after treatment with larger doses of cyanocobalamin was observed at a time when the bone marrow was normoblastic. This suggests that some block of folate utilization may have persisted even after therapy which was adequate to reverse the megaloblastic changes of the bone marrow, and that this was corrected by therapy with larger doses of vitamin B₁₂. However, it also is possible that this late rise of erythrocyte folate was associated with removal of folate deficient erythrocytes from the circulation as they reached the end of their life span.

It has not been possible to exclude the possibility that the low erythrocyte folate in pernicious anemia is due to the presence of a population of aged or defective erythrocytes, or that the folate content of the erythrocytes decreases with age. Preliminary studies of erythrocyte and serum folate in patients with hemolytic anemia and aplastic anemia has not been conclusive in determining the relationship of serum and erythrocyte folate in the presence of erythrocyte populations of different ages. The pattern of decrease of erythrocyte folate in experimental folate deficiency, however, suggests that the folate content of the erythrocyte does not decrease with age. The observations of Cox et al. and Nieweg et al. indicate that the folate of reticulocytes differs qualitatively from that of mature erythrocytes, but no evidence of quantitative differences has been reported.

An increase of *L. casei*-active whole blood folate in some patients with vitamin B₁₂ deficiency treated with cyanocobalamin has been reported previously. The data presented above confirms this observation and demonstrates that the increase of erythrocyte folate following therapy in these patients persists after the reticulocytosis. The one patient studied who showed no increase of erythrocyte folate following therapy with cyanocobalamin showed a decrease of serum folate so that a "normal" relationship between serum and erythrocyte folate was established.

It is possible that the relative folate deficiency of erythrocytes in pernicious anemia is secondary to disordered folate metabolism of the erythroblast, rather than a direct result of vitamin B₁₂ deficiency. If this is the case, then other states of abnormal erythrocyte folate metabolism should be discovered with deficiency of erythrocyte folate relative to the serum folate concentration. The data presented above, however, are consistent with a direct effect of vitamin B₁₂ deficiency on folate transport into erythroid cells.

Waters and Mollin have indicated that the serum folate concentration in pernicious anemia is directly proportional to the hematocrit. We have not found a statistically significant correlation of hematocrit and serum folate in the pernicious anemia patients studied (0.20 > p > 0.10). If the relationship reported by Waters & Mollin is valid then the correlation between serum and erythrocyte folate described here would indicate that patients with higher hematocrits would have higher erythrocyte folate activity. The
severity of the anemia in vitamin $B_{12}$ deficiency might be regulated by the quantity of folate available for incorporation into maturing erythroblasts.

The patient with pernicious anemia who underwent a partial remission after administration of a large dose of folic acid (fig. 6) showed a small rise of erythrocyte folate activity during and after the reticulocytosis. This supports the hypothesis that large doses of folic acid may provide enough of the transport form of folate to allow entry of some folate into maturing erythroblasts despite deficiency of vitamin $B_{12}$.

The marked variability of the assay indicates that efforts must be made to improve the reproducibility of this technic. This variability may explain occasional patients whose serum folate activity does not correlate with their clinical state. The variability of the assay may mask small effects of normal food intake upon serum folate levels. It is of interest that the rise of serum folate activity following the ingestion of high folate foods was approximately proportional to the folate activity of the foods measured directly by *L. casei* without deconjugation. It may be, then, that much of the folate in food which is available to *L. casei* also is available to the human organism.

Assay of the folate of either serum or whole blood readily differentiated between patients with anemia due to folate deficiency and normal subjects. The slightly higher serum folate values found in two iron deficient patients with macrogranulocytosis of the bone marrow, and the normal erythrocyte folate in all five of these patients are similar to the results observed in pregnant women with macrogranulocytosis of the bone marrow. Thus although severe folate deficiency is readily recognized by serum folate below 3.0 ng./ml. and/or erythrocyte folate below 175 ng./ml., patients with early or mild folate deficiency may have serum folate as high as 3.9 ng./ml. and normal erythrocyte folate.

Chromatography of erythrocyte folate activity following activation of erythrocyte folate by plasma, revealed no striking alteration of the distribution of the various folate co-enzymes in pernicious anemia. It is possible, however, that some abnormality of distribution may not have been recognized by this technic which separates only approximately five components and which includes incubation and autoclaving prior to chromatography. The components fractionated by the DEAE cellulose appear not to correspond precisely with the folate co-enzymes chromatographed as standards (viz., tetrahydrofolic acid, $N_5$-formyltetrahydrofolic acid, folic acid, and terop-terin$^*$) and may represent conjugates of $N_5$-methyltetrahydrofolic acid as suggested by Noronha and Aboobaker. The chromatogram is not identical to that reported by them, but the technic differs from theirs primarily in that they did not pre-incubate the blood samples prior to protein precipitation.

The significance of the sodium hydroxide peak in the DEAE cellulose chromatography is unknown. This peak has been reported previously by Johns et al. in solutions of commercial folic acid. It is postulated that the

*We are grateful to Dr. D. Johns of the Montreal General Hospital for supplying this material.
sodium hydroxide peak in that material might represent a folate dimer. Precise identification of this peak in biological material must await further studies.

**Summary**

1. A relationship has been observed between the serum and erythrocyte folate in normal subjects.

2. Patients with folate deficiency were more readily differentiated from normal subjects by determinations of serum folate activity than by determinations of erythrocyte folate activity.

3. In pernicious anemia in relapse, accumulation of serum folate and/or depletion of erythrocyte folate was observed in all patients, resulting in a disturbance of the normal relationship between the serum and erythrocyte folate activity. This altered relationship was corrected following the reticulocytosis induced by vitamin B12 therapy.

4. Patients receiving amethopterin therapy appear to incorporate the folate antagonist into maturing erythroid cells, since the erythroid cells so formed showed antibacterial activity for several weeks following the administration of the antagonist.

5. Preliminary fractionation of folate co-enzymes in erythroid cells of a patient with pernicious anemia before and after therapy revealed no significant difference in the distribution of the co-enzymes induced by therapy. A significant proportion of the folate activity of the erythroid cells was found in a fraction eluted from the DEAE column by sodium hydroxide.

6. The data presented are consistent with a direct effect of vitamin B12 on folate transport into the erythroblast.

**Summario in Interlingua**

1. Ha essite observate un relation inter le folate de sero e illo de erythrocytos in subjectos normal.

2. Patientes con carentia de folato esseva plus prestemente differentiate ab subjectos normal per determinationes de activitate de folato in le sero que per determinaciones de activitate de folate in le erythrocytos.

3. In anemia perniciose in recidiva, le acumulation de folato in le sero e/o le depletion de folato in le erythrocytos esseva observate in omne le patientes, con le resultato de un disturbance del relation normal inter le activitate de folato in le sero e le activitate de folato in le erythrocytos. Iste alterate relation esseva corrige post le induction de reticulocytosis per therapia a vitamina B12.

4. Patientes recipiente therapia a amethopterina pare incorporar le antagonist de folato ad in le maturante cellulas erythroide, proque le cellulas erythroide assi formate monstrava activitate antibacterial durante plure septimanas post le administration del antagonist.

5. Un fractionation preliminari de co-enzimas de folato in cellulas erythroide de un paciente con anemia perniciose ante e post le therapia revelava nulle significative differentia in le distribution del co-enzimas in consequentia del therapia. Un proportion significative del activitate de folato in le cellulas
erythroide esevva trovate in un fraction eluite per hydroxuro de natrium ex le columnna de cellulosa a diethylaminoethanol.

6. Le datos presentate es congrue con un effecto directe de vitamina B₁₂ super le transporto de folato ad in le erythroblasto.

ACKNOWLEDGMENTS

We are grateful to Mrs. H. Girey, Mrs. E. Jonas, Mrs. T. Gabor, and Mrs. J. Shefner for technical assistance, and to dietitians Miss A. Christen, and Mrs. E. Lanjelier.

REFERENCES


20. Cooper, B. A., and Lowenstein, L.: An immunologic basis for acquired re-
sistance to oral administration of hog
intrinsic factor and vitamin B\textsubscript{12} in
pernicious anemia. J. Clin. Invest. 40:
Westall, R. G.: Simplified method for
detecting formiminoglutamic acid in
urine as a test of folic-acid deficiency.
22. Zalusky, R., and Herbert, V.: Urinary
formiminoglutamic acid as a test of
folic-acid deficiency. Lancet 1:108,
1962.
23. Noronha, J. M., and Aboobaker, V. S.: Studies on the folate compounds of
human blood. Arch. Biochem. 101:
24. Herbert, V.: Experimental nutritional
folate deficiency in man. Tr. A. Am.
25. Cox, E. V., Meynell, M. J., Cooke, W.
T., and Gaddie, R.: Folic acid ac-
tivity during blood regeneration.
26. Nieweg, H. O., Faber, J. G., de Vries,
J. A., and Kroese, W. F. S.: The rel-
ationship of vitamin B\textsubscript{12} and folic
acid in megaloblastic anemias. J.
27. Waters, A. H., and Mollin, D. L.: Ob-
servations on the metabolism of folic
acid in pernicious anaemia. Brit. J.
Haemat. 9:319, 1963.
28. Lowenstein, L., Brunton, L., Cooper,
B. A., Milad, A., and Hsieh, Y. S.: The relation of erythrocyte and
serum L. casei folate activity to folate
deficiency in certain megaloblastic
anemias. Proc. IX Cong. European
29. Johns, D. G., Pledgerleith, I. H., and
Cooper, B. A.: A folic acid-active
compound strongly bound by DEAE-
cellulose. Biochem. Pharmacol. 12:
388, 1963.

Bernard A. Cooper, M.D., Assistant Professor of Medicine and
Clinical Medicine, McGill University, Medical Research As-
sociate of the Medical Research Council of Canada, and
Assistant Physician, Royal Victoria Hospital.

Louis Lowenstein, M.D., Associate Professor of Medicine and
Clinical Medicine, McGill University, Physician and Haema-
tologist-in-Charge, Royal Victoria Hospital.
Relative Folate Deficiency of Erythrocytes in Pernicious Anemia and its Correction with Cyanocobalamin

BERNARD A. COOPER and LOUIS LOWENSTEIN