STUDIES ON THE RELATIONSHIPS OF VITAMIN B₁₂, FOLIC ACID, THYMINE, URACIL, AND METHYL GROUP DONORS IN PERSONS WITH PERNICIOUS ANEMIA AND RELATED MEGALOBLASTIC ANEMIAS


This article will deal with (1) the effect of refined liver extract, vitamin B₁₂, and thymine in persons with pernicious anemia who relapse hematologically during folic acid therapy, (2) the hematologic effect of uracil in persons with pernicious anemia in relapse, (3) the effect of uracil, methionine, choline and thymine in a patient with pernicious anemia of pregnancy.

The discoveries that folic acid (pteroylglutamic acid) is a potent hematopoietic agent in pernicious anemia² and related macrocytic anemias in doses measured in milligrams³ were made that thymine (S-methyl uracil) can replace folic acid if administered to patients with pernicious anemia in doses of 2.5 grams daily⁴ and that vitamin B₁₂ is active in these conditions in microgram quantities⁵ opened the way for investigations concerning the chemical relationships of these substances in human beings. The therapeutic effects of these substances in human anemias was suggested by the demonstration that they had potent growth-promoting properties for various micro-organisms. It is natural, therefore, that bacterial growth experiments which suggest that vitamin B₁₂ and folic acid may play a part in nucleoprotein metabolism should influence our thinking regarding the possible metabolic relationships of these substances in human beings. The therapeutic effects of these substances in human anemias was suggested by the demonstration that they had potent growth-promoting properties for various micro-organisms. It is natural, therefore, that bacterial growth experiments which suggest that vitamin B₁₂ and folic acid may play a part in nucleoprotein metabolism should influence our thinking regarding the possible metabolic relationships of these substances in human beings. During the last four years we have had the opportunity to test some of the possibilities implied in these relationships. These tests have been carried out in patients with a variety of megaloblastic anemias. In general, they support the proposition that folic acid and at least one other catalyst facilitate the formation of thymine and other essential purines and pyrimidines from intermediary compounds such as uracil; and that vitamin B₁₂ acts at a later stage of a series of reactions which terminate in the synthesis of nucleoprotein.

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Material and Methods

In November, 1945, 42 persons with pernicious anemia were placed on a regimen of folic acid, 30 mg. three times weekly given orally. Thirty-six of them have returned regularly for observation. Of these 36 persons, 11 have been maintained on this dosage in satisfactory hematologic and neurologic state, 4 have expired of disease unrelated to their anemia but were maintained satisfactorily up to their death. Five experienced hematologic relapse. Seven suffered neurologic relapse, 7 showed manifestations of both hematologic and neurologic relapse concomitantly and 2 developed manifestations of hematologic relapse followed shortly by neurologic relapse. Six patients who had definite signs of hematologic relapse with little or no neurologic deterioration were studied with regard to their hematologic responses when thymine was given orally (2 patients), when the dose of folic acid was increased (4 patients), and finally when vitamin B12 or purified liver extract was administered parenterally. When both hematologic and neurologic relapses occurred concomitantly, the effect of vitamin B12 or refined liver extract was observed on both abnormalities. In one instance (Case 3), while the patient was receiving 10 mg. folic acid daily, the urinary output of folic acid was measured before and after a single intramuscular injection of refined liver extract Lederle (10 units) to determine whether, under these conditions, folic acid would be excreted or retained. The manner of performing the hematologic observation will be described below.

Three patients with pernicious anemia in relapse were treated with 15-30 grams of uracil given orally per day for ten days. Hematologic observations were made in the manner described below.

A 36 year old primipara admitted at 6 months gestation with pernicious anemia of pregnancy and moderate hepatic cirrhosis was treated with uracil given orally, 30 grams daily for ten days, choline 3 grams, and methionine 6 grams orally for another ten-day period and finally thymine 15 grams daily by mouth for ten days. Hematologic observations were made during these three periods and for three months after she was delivered of a 7½ month infant by caesarian section necessitated by placenta praevia.

Reference will be made also to a patient previously reported7 whose megaloblastic anemia was refractory to vitamin B12 and refined liver extract. This patient was treated successfully with folic acid 7.5 mg. daily by intramuscular injection for ten days and on a subsequent admission by thymine 13.1 grams by mouth daily for ten days. In this patient, the daily urinary output of folic acid was determined before and for three days following the institution of folic acid therapy to determine if laboratory evidence of folic acid depletion could be found.

All of the patients described above were subjected to the same experimental procedures. Complete medical histories were taken and physical examinations were performed. Particular care was devoted to the nutrition history and to the neurologic examination. The patients with pernicious anemia who were maintained on folic acid were followed in the dispensary at intervals of six weeks to two months. At each visit, hematologic examination of the peripheral blood was performed and the neurologic status of the patient was checked. When signs of hematologic or neurologic relapse appeared, the dose of folic acid was increased to 50 mg. daily and the patient was followed at weekly intervals. Before additional therapy was instituted, the patient was hospitalized, the history and physical examination rechecked, and the peripheral blood and bone marrow were examined. During the periods of therapeutic observations, reticulocyte counts were performed daily. Erythrocyte, platelet, white blood cell and differential white blood cell counts, and hemoglobin and hematocrit determinations were performed every two to four days. The bone marrow was examined at the end of each 10 day experimental period. The patients received a diet free of liver and kidney and low in animal protein.

Gastric analyses after histamine stimulation were performed and direct reacting and total bilirubin were determined in each patient. The gastroenteric tract was examined roentgenologically with the aid of barium. A liver biopsy was performed in one patient when the suspicion of portal cirrhosis arose.

Erythrocyte and white blood cell counts were made with standardized pipets and counting chambers. Hemoglobin was measured as oxyhemoglobin in a Coleman Junior Spectrophotometer. Reticulocyte, and platelet counts were performed by the wet method using a modified Dameshek’s stain. The hematocrit was determined in a Wintrobe tube spun at 3000 r.p.m. for thirty minutes. The anticoagulant was potassium oxalate and ammonium oxalate.
Bone marrow was obtained by aspiration from the sternum or the iliac crest. Coverslip preparations were stained by Wright-Giemsa stain.

Folic acid was determined in the urine microbiologically through the courtesy of Dr. A. L. Franklin, Lederle Laboratories.

| Table 1. — Bone Marrow Counts of Patients after Relapse during Folic Acid Therapy |
|-------------------------------------------------|---------------------------------|-----------|-----------|
| | 1st relapse | After 30 mg. PGA three times weekly |
| | | 2nd relapse | After 50 mg. PGA daily |
| Polymorphonuclear neutrophile | 40.5 | 29.5 | 36.5 | 30.0 | 51.5 | 44.5 |
| Metamyelocyte | 20.5 | 30.0 | 21.5 | 22.0 | 25.5 | 24.5 |
| Myelocyte C | 5.0 | 11.0 | 13.5 | 10.0 | 1.0 | 11.0 |
| Myelocyte B | 3.5 | 4.5 | 5.0 | 3.0 | 1.0 | 3.5 |
| Myelocyte A | 1.0 | 1.0 | 2.5 | 1.0 | 1.0 | 1.0 |
| Myeloblast | 0 | 1.0 | 0 | 0.3 | 0 |
| Lymphocyte | 19.0 | 5.0 | 11.0 | 13.0 | 9.3 | 7.5 |
| Young Lymphocyte | 0 | 0 | 1.5 | 1.0 | 0 | 0 |
| Monocyte | 0 | 0 | 1.0 | 0 | 0.5 | 1.0 |
| Young Monocyte | 0 | 0 | 0 | 0 | 0 |
| Eosinophile | 4.5 | 5.0 | 2.5 | 9.0 | 3.0 | 3.0 |
| Eosinophilic Myelocyte | 2.5 | 0.5 | 3.0 | 7.0 | 4.0 | 1.0 |
| Basophile | 0.5 | 0 | 0.5 | 1.0 | 0.5 | 0 |
| Basophilic Myelocyte | 0 | 0 | 0 | 0 | 0 |
| Plasma Cell | 3.0 | 3.0 | 0 | 1.0 | 2.0 | 0.5 |
| Clastmatocyte | 0 | 0 | 0 | 0 |
| Primitive Cell | 0 | 9.0 | 1.5 | 2.0 | 0 | 2.5 |
| Megakaryocyte | 0 | 0.5 | 0 | 0 | 0 |
| Megaloblasts per 100 WBC | 1.0 | 2.5 | 0 | 0 | 0 |
| Early Erythroblasts per 100 WBC | 8.5 | 14.0 | 4.0 | 4.0 | 0.5 | 2.0 |
| Late Erythroblasts per 100 WBC | 10.5 | 36.0 | 9.5 | 28.0 | 3.5 | 6.0 |
| Normoblasts per 100 WBC | 25.5 | 71.5 | 25.0 | 91.0 | 57.0 | 56.5 |
| Myeloid-Erythroid Ratio | 1:0.45 | 1:1.5 | 1:10.38 | 1:11.2 | 1:10.59 | 1:10.68 |
| Peripheral Blood |
| Erythrocytes (millions) | 2.8 | 1.5 | 2.4 | 2.4 | 3.5 | 3.7 |
| Hemoglobin (grams per 100 cc.) | 10.7 | 8.0 | 10.5 | 9.5 | 12.0 | 12.2 |

RESULTS

Fourteen persons with pernicious anemia had hematologic relapses while taking 30 mg. folic acid three times a week. These relapses occurred on the average of thirty months after the patient’s therapy was changed from liver extract to folic acid in 1945. Erythrocyte counts and hemoglobin levels fell to values as low as 1,500,000 cells per cu. mm. and 7.5 grams per 100 cc. respectively. Macrocystosis appeared and white blood cell counts tended to fall. The bone marrow showed moderate early erythroblastic maturation arrest (table 1). Patients who had severe neurologic relapse as well, were treated with refined liver extract or vitamin B12 and had satisfactory neurologic and hematologic recovery. The other patients
were given varied therapeutic tests. Two received thymine, 15 grams daily in divided doses, for ten days. In one of these subjects (J. S., Case I) reticulocytosis occurred and the erythrocytes and hemoglobin rose from 1,780,000 cells per cu. mm. and 8.9 grams per 100 cc. to 2,950,000 cells per cu. mm. and 11 grams per 100 cc. respectively. Subsequent treatment with refined liver extract did not induce further reticulocytosis and the erythrocytes rose slowly. Signs of neurologic degeneration cleared rapidly. The other patient (A. K., Case II) who received thymine had a rise in reticulocytes from 1.3 per cent to 4.1 per cent, the bone marrow was converted from an early erythroblastic to a normoblastic type, but the erythrocytes remained between 2,800,000 and 3,000,000 cells per cu. mm.

Four patients (R. H., Case III; W. C., Case IV; R. P., Case V; and F. R., Case VI) with mild or no neurologic degeneration were given increased doses of folic acid, 50 mg. daily. Within four weeks after this change in therapy was instituted, the peripheral blood counts rose significantly but macrocytosis usually persisted. After two to eleven months on the increased dose of folic acid the values fell again, this time with the appearance of combined system disease in all patients. When this occurred the patients were admitted to the hospital for further study and therapeutic investigation. There was no hyperbilirubinemia. Examination of the bone marrow showed mild or moderate evidence of erythrocyte maturation factor deficiency. Macrocytic polychromatophilic normoblasts and bizarre metamyelocytes were abundant but in no instance was typical megaloblastic arrest a prominent feature, probably because anemia was mild (table 1).

Two of the 4 patients (R. P., Case V and F. R., Case VI) in whom the neurologic degeneration was most acute were treated with vitamin B12 parenterally. The details of this therapeutic period are recorded in the protocols and graphs which follow. Neurologic improvement was rapid in each instance but the hematologic response was slow.

Patient R. H. (Case III) had relapsed hematologically and had developed neuritis on 30 mg. folic acid three times a week. He improved on 50 mg. folic acid daily only to relapse once more. He was given 10 units of liver extract in a single dose while he was receiving 10 mg. of folic acid daily by intramuscular injection. Within four days after the liver extract, the output of folic acid in the urine fell from 60 per cent to 25 per cent of the injected dose and then rose again. Subsequently he was treated with large doses of refined liver extract because of rapidly progressive cord and cerebral cortex degeneration with gradual hematologic and rapid neurologic improvement.

Patient W. C. (Case IV) had a hematologic relapse and severe peripheral neuritis while taking 30 mg. folic acid three times a week. When the dose of folic acid was increased to 50 mg. daily both a hematologic and neurologic remission occurred. There followed a very severe neurologic relapse but only slight decrease in erythrocytes and hemoglobin. No further improvement in the neurologic state followed another increase in the dose of folic acid to 100 mg. daily but amelioration occurred when vitamin B12 was administered. Brief protocols and graphs of Cases I through VI follow.
Case I, J. S. (fig. 1), a 56 year old Negro with pernicious anemia, developed hematologic, neurologic and glossal relapses while taking folic acid, 30 mg. three times a week, since November, 1945. They were noted first in July, 1946, and became severe by May, 1947. At this time and again at a later date his ability to absorb folic acid given orally was proved by recovering 45 to 50 per cent of a test dose from
the urine in twenty-four hours. Thymine (5 methyl uracil) induced a definite hematologic response, but another test with folic acid with and without B-pyracine lactone was fruitless. Small doses of refined liver extract (1 unit daily for ten days) induced only a slight reticulocytosis and erythrocyte rise. Neurologic symptoms and signs began to abate. Only after several months of intensive therapy with refined liver extract, did the erythrocytes and hemoglobin reach normal values.

This case demonstrates that thymine will induce a hematologic response even though the patient is refractory to folic acid.

Case II, A. K. (fig. 2), a 63 year old Negro woman with pernicious anemia, developed a hematologic relapse after three and one-half years on folic acid, 30 mg. three times a week. Thymine induced a reticulocyte response from 1.3 to 5 per cent on the fifth day and a conversion of the bone marrow morphology from an early erythroblastic to a normoblastic maturation level. Erythrocytes and hemoglobin, how-

ever, did not rise until folic acid, 50 mg. daily, were given. After this rise, a second relapse occurred and had become moderately severe by March, 1950.

This case demonstrates that thymine will induce some hematologic response even though the patient has become refractory to average oral doses of folic acid. It also demonstrates that increasing the dose of folic acid to very large amounts (50 mg. daily) will induce a hematologic response after the patient has become refractory to average doses.

Case III, R. H. (fig. 3), a 66 year old Negro man with pernicious anemia, was given folic acid in place of liver extract in November, 1945. Hematologic and glossal relapse began in fifteen months and became severe in twenty-eight months, when neurologic relapse also began. A temporary remission in the anemia, glossitis and neural manifestations occurred when the folic acid was increased from 30 mg. three times a week to 50 mg. daily. This remission was short lived and when relapse recurred, the bone marrow showed moderate evidence of erythrocyte maturation factor deficiency (table 1). When 10 units of refined liver extract were given parenterally, the urinary output of a 10 mg. daily dose of folic acid decreased from 60 per cent to 35 per cent, followed by a gradual rise to the former level of excretion.
No reticulocyte or erythrocyte response occurred until liver extract was given in 50-unit doses twice, followed by 20 units at three-day intervals for a month. Neurologic and glossal improvement were rapid. After discharge the patient failed to return until January, 1950, when he was in hematologic relapse again. He responded to folic acid 10 mg. daily.

This case demonstrates that greatly increased doses of folic acid will induce a hematologic remission after relapse has occurred on average doses. After a second hematologic relapse, 10 units of liver extract temporarily increased the retention and possibly the utilization of folic acid by the body. Hematologic response did not occur until large doses of liver extract were given, and even then appeared to be delayed.

Case IV, W. C. (fig. 4), a 56 year old Negro with pernicious anemia, developed mild hematologic relapse associated with peripheral neuritis five months after folic acid was begun. A complete hematologic and almost complete neurologic remission occurred when the dose of folic acid was increased to 50 mg. daily. After eight months a very severe neurologic relapse occurred, characterized by peripheral neuritis, paranoia and depression. The hematologic values fell slightly but immediately returned to normal when folic acid 100 mg. daily was given. The neurologic manifestations rapidly grew worse until vitamin B₁₂, 30-50 micrograms daily was given. In six days the depression and paranoia disappeared and the neuritis cleared rapidly.

This case illustrates hematologic relapse, remission and again relapse as the dose of folic acid is increased from average amounts to 100 mg. daily. The first episode of neuritis appeared to improve when the dose of folic acid was increased. The second and most severe neural relapse did not respond until vitamin B₁₂ was given.

Case V, R. P. (fig. 5), a 74 year old white woman with pernicious anemia, developed a severe hematologic relapse two and one-half years after folic acid, 30 mg. three times a week, was begun. A partial remission was induced by increasing the dose to 50 mg. daily. As the hematologic values were beginning to fall again, a severe neurologic relapse occurred. The neurologic symptoms and signs had responded...
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Fig. 5 — Case V

Fig. 6 — Case VI
after a month of therapy with vitamin B₁₂, 5 micrograms every other day; the erythrocytes and hemoglobin rose more slowly and reached normal values for this patient after three months.

This patient demonstrates that a hematologic relapse which occurs while the patient is taking average doses of folic acid will respond to much larger doses. A second hematologic relapse was cut short by vitamin B₁₂ therapy which was required for a severe neurologic relapse. The neurologic symptoms and signs responded much more rapidly than the erythrocyte and hemoglobin values.

Case VI, F. R. (fig. 6), a 78 year old white man with pernicious anemia, developed a severe hematologic relapse after taking folic acid, 30 mg. three times a week, for twenty-six months. When the dose of folic acid was increased to 50 mg. daily, an almost complete remission occurred. A second hematologic relapse was cut short when the sudden onset of severe postero-lateral column disease necessitated therapy with vitamin B₁₂, 5 micrograms every other day in addition to the folic acid. Neurologic symptoms and signs cleared after a month of therapy but erythrocytes, hemoglobin and mean corpuscular volume did not reach normal values for five months. After folic acid was discontinued and for the next six months no further hematologic improvement has been noted.

This patient demonstrates that hematologic relapse on average doses of folic acid is followed by partial remission but continued macrocyosis when the dose of folic acid is increased to 50 mg. daily. A second mild though definite hematologic relapse responded to vitamin B₁₂ more slowly than the neurologic relapse. In this experiment both folic acid and vitamin B₁₂ were continued until hematologic values were entirely normal, since in some of the preceding trials, it appeared as though the hematologic response to vitamin B₁₂ or refined liver extract was inhibited until folic acid was discontinued. Folic acid did not prevent the return to normal in this case.

Hematologic relapse may occur while patients with pernicious anemia are treated with folic acid in doses usually considered adequate (30 mg. three times a week). When the dose of folic acid is increased to 50 mg. daily a partial remission occurs, but a second hematologic relapse invariably follows within a few months. This is a typical mass action effect.

These patients will respond to thymine even though they have become refractory to 30 mg. folic acid three times a week. Ultimately they develop neurologic as well as hematologic relapse. At such a time vitamin B₁₂ or refined liver extract will induce a rapid neurologic response but the hematologic response is slow. These observations suggest relationships between folic acid, vitamin B₁₂ and nucleoprotein metabolism which will be elaborated in the discussion.

The Hematologic Effect of Uracil in Patients with Pernicious Anemia in Relapse

Three patients with pernicious anemia were treated with uracil given orally in doses of 15-30 grams daily for ten days. One of these patients received a second course of 30 grams a day for ten days.

The first subject (J. S., female, Case VII) had a reticulocyte response of 6.8 per cent on the ninth day after the administration of uracil 15 grams daily had been started. Bone marrow taken at this time showed a shift from a predominance of megaloblasts and early erythroblasts to late erythroblasts (table 2). Any erythrocyte rise that might have occurred was obscured by a transfusion given in the operating room following a biopsy procedure. (See protocol and fig. 7.)

The second person treated with 15 grams of uracil daily had no reticulocyte response or erythrocyte rise and examination of the bone marrow on the tenth day showed no change from the original megaloblastic arrest. This patient responded satisfactorily to vitamin B₁₂.

The third patient (N. M., Case VIII) had a reticulocyte rise from 6 per cent to
15 per cent on the ninth day after the administration of 30 grams of uracil per day was begun. During a subsequent ten-day period of uracil administration there was a second reticulocyte response of 1.7 per cent to 7.0 per cent on the eighth day. The bone marrow morphology progressively reverted to a normoblastic type during these two periods (table 2). The erythrocytes and hemoglobin rose from 1,400,000 to 2,840,000 cells per cu. mm. and from 6.3 grams per 100 cc. to 11.6 grams per 100 cc., respectively (fig. 8). The mean corpuscular volume, however, remained elevated.

Case VII, J. S. (fig. 7), a 51-year-old woman, had pernicious anemia and a diffuse and bizarre neurologic disease finally diagnosed by biopsy as diffuse meningioma of the brain stem. She received uracil, 15 grams daily for ten days. A submaximal reticulocyte response occurred on the ninth day and bone marrow showed an increase in late erythroblastic elements in comparison with the marrow specimen prior to treatment (table 1). Unfortunately, shortly after this ten-day period, whole blood was given the patient during a surgical biopsy procedure so that further observations for a rise in hemoglobin and erythrocytes were invalidated.

Case VIII, N. M. (fig. 8), a 72-year-old Negro with pernicious anemia received two ten-day courses of uracil, 30 grams daily. There were two distinct hematologic responses as illustrated in the graph.
conversion of the bone marrow from a megaloblastic to a normoblastic pattern is indicated in table 2. For the past six months, the patient has been maintained in a satisfactory hematologic and neurologic state on folic acid, 15 mg. daily.
These studies indicate that uracil in massive doses will stimulate erythrocytogenesis in some persons with pernicious anemia. They add an additional link to the evidence that the chemical defect in pernicious anemia involves the metabolism of nucleoproteins. It is probable that the variation in absorption and utilization of uracil from person to person explains the ineffectiveness of this pyrimidine in one of our subjects.

Table 3.—Differential Bone Marrow Counts on a Patient with Pernicious Anemia of Pregnancy after Treatment with Various Anti-Anemic Substances

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<th>Before Treatment</th>
<th>After Choline 8 Gm. and Methionine 6 Gm. Daily for 10 Days</th>
<th>After Thymine 15 Gm. Daily for 10 Days</th>
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The Hematopoietic Effect of Uracil, Methionine, Choline and Thymine in a Patient with Pernicious Anemia of Pregnancy

A 33 year old primipara (V. G., Case IX) with pernicious anemia of pregnancy and nutritional cirrhosis was observed during the administration of successive doses of uracil, 30 grams orally per day for ten days; methionine, 6 grams and choline, 3 grams orally per day for ten days; and thymine, 15 grams orally per day for ten days. There was no reticulocytosis, erythrocyte rise or change in the megaloblastic-early erythroblastic bone marrow after uracil administration. Following choline and methionine, reticulocytes increased from 0.1 per cent to 7.4 per cent on the ninth day and erythrocytes increased 800,000 cells per cu. mm. The bone marrow changed from a hypocellular megaloblastic-early erythroblastic type to a hypercellular late erythroblastic and normoblastic type (table 3). During this
course there was a very pronounced leukemoid reaction. The white blood cell count increased from 5,450 cells per cu. mm. to 18,100 cells per cu. mm. and was maintained at this level for the entire ten-day period. The patient experienced considerable improvement in appetite and sense of well-being.

Following the administration of thymine, a secondary reticulocytosis occurred, erythrocytes rose to 2,960,000 cells per cu. mm. and the bone marrow reverted to a normal appearance. This improvement was maintained without further therapy until the patient was delivered of a normal infant by caesarian section. Her post-operative course was satisfactory and she was discharged from the hospital in good health. Unlike most patients with pernicious anemia of pregnancy she returned in hematologic relapse in March, 1950. On this admission glossitis and anemia failed to respond to vitamin B12 given parenterally but responded exceedingly rapidly to oral folic acid.

Case IX, V. G. (fig. 9), a 33 year old white woman, six months pregnant, was found to have severe macrocytic anemia, glossitis, hepatomegaly, free hydrochloric acid in her gastric juice after histamine stimulation, and a total serum bilirubin of 1 mg. per cent with 0.1 mg. per cent giving a prompt reaction. She had a history of alcoholism and portal cirrhosis dating back to 1945. Her liver had been enlarged and her liver function tests moderately abnormal. Needle biopsies of the liver on two occasions showed "moderate fatty degeneration and fatty infiltration of the liver parenchyma with increased proliferation of liver stroma." Her response to therapy, consisting of high carbohydrate, high protein diet, supplemented with vitamins of the B complex and vitamin C in large doses, crude liver extract, and ferrous
sulfate, was excellent. She had been on this regimen for four months before this admission and clinic follow-up had been satisfactory.

Data on the examination of her bone marrow will be found in table 3. A diagnosis of pernicious anemia of pregnancy was made because the megaloblastic bone marrow was not consistent with macrocytic anemia of cirrhosis and there was no foundation for intrinsic or extrinsic factor deficiency. She was treated with uracil, 30 grams daily without response, then with choline, 3 grams and methionine, 6 grams daily for ten days with reticulocytosis and erythrocyte rise as shown in the graph. A leukemoid reaction developed during this period. Finally thymine, 15 grams daily for ten days produced a complete hematologic response. The effects of these therapeutic tests on the bone marrow are shown in table 3.

In August, 1949, she was delivered by cesarian section because of placenta praevia and made an uneventful postoperative recovery. She returned in severe hematologic relapse in March, 1950, failed to respond to vitamin B₁₂, 10 micrograms parenterally, but both glossitis and anemia responded dramatically to 50 mg. of folic acid given orally.

Most patients with pernicious anemia of pregnancy do not respond as well to small doses of refined liver extract or vitamin B₁₂ as do persons with typical pernicious anemia, and the assumption was made that this patient would follow the general rule. In fact, her anemia had developed while she was receiving small amounts of crude liver extract for her cirrhosis. This supposition was proved at a later admission. The observations made in this patient link pernicious anemia of pregnancy to defective nucleoprotein metabolism and suggest that the metabolic defect is somewhat different from that which is responsible for pernicious anemia.

DISCUSSION

During the past four years, various therapeutic tests have been performed on persons with pernicious anemia and related macrocytic anemias in order to investigate the chemical relationships of folic acid, vitamin B₁₂ and thymine and the mechanisms of their action. The plan for these tests was suggested by many investigations in bacterial, animal and human metabolism, and have been altered from time to time over this four-year period as new information became available or because new concepts arose as the testing progressed. By such an evolutionary process, a theory has been developed which links these therapeutic agents which are so effective in the macrocytic anemias to a chemical "chain reaction," which culminates in the synthesis of nucleoprotein. The development of this theory will be described, and the implications of the therapeutic tests just reported will be discussed. Finally, there will be a discussion of the possible causes of the several clinical types of macrocytic anemia based on this theory and the tests of its validity in human beings.

Studies on the growth-promoting properties of folic acid, thymine and vitamin B₁₂ for certain bacteria originally suggested that these substances might be important therapeutic agents in human anemias. These studies have been equally important in suggesting chemical interrelationships.

Large amounts of thymine in the presence of purines will replace folic acid as a growth factor for the *Streptococcus faecalis* R and the *Lactobacillus casei*. Thymine will also overcome the inhibitory effect of methyl folic acid (a folic acid antagonist) on the growth of *L. casei* when purines are available. Furthermore, *L. casei* does not form desoxyribosenucleic acid in normal amounts if the folic
acid content of the media is reduced. This defect can be overcome by the addition of large amounts of thymine but ribonucleic acid production is increased also. These observations have led to the suggestion that folic acid may act as a coenzyme in the biosynthesis of purines and thymine or their equivalents.

Thymidine (thymine nucleoside) can be substituted for vitamin B12 to promote the growth of Lactobacillus lactis dorner when purines are available and for the animal protein factor (a growth factor similar to vitamin B12) in the nutrition of Lactobacillus leischmannii. These observations suggest that vitamin B12 may catalyze a reaction which results in the formation of thymidine.

Vitamin B12 in large doses protects the ribonucleic acid content of the liver cells of rats poisoned with carbon tetrachloride. This observation links vitamin B12 with ribose as well as desoxyribonucleic acid metabolism.

Studies have been reported previously from this laboratory of a patient with megaloblastic anemia refractory to therapy with refined liver extract and vitamin B12. This patient responded to folic acid in one relapse and to thymine in another but folic acid urinary excretion studies did not indicate severe folic acid depletion. The suggestion was made that another factor, possibly the "Wills' factor", was the substance lacking in this person, and that this unknown factor together with folic acid was necessary for the formation of thymine. The available evidence, therefore, links folic acid, vitamin B12 and other unknown factors to nucleic acid metabolism and to the formation of nucleoprotein.

A consideration of possible precursors of thymine and thymidine points to uracil since this substance differs from thymine only by a methyl group in the 5 position. Studies in chicks link the factors under consideration to transmethylation reactions, for a deficiency of animal protein factor which has effects similar to vitamin B12 increases the need for available methyl groups. These considerations and the reports that choline will occasionally promote hematopoiesis in pernicious anemia and related megaloblastic anemias suggested the therapeutic trials with uracil, choline and methionine. Since uracil and other pyrimidines and purines in the diet appear to be poorly utilized for nucleoprotein synthesis, one must postulate that these nitrogenous bases are built up from simple amino nitrogen and carbon sources. It is also probable that endogenous nucleic acid metabolism contributes nucleosides and nucleotides for resynthesis into nucleic acid.

From these suggestive bits of evidence one may postulate the existence of a chain type reaction resembling the pattern shown in figure 10. According to such a scheme, folic acid together with unknown substances activates the formation of purines and pyrimidines from amino acids and probably facilitates the interconversion of these nitrogenous bases. An interconversion reaction of this type is illustrated by the methylation of uracil to form thymine (fig. 11). Vitamin B12 on the other hand probably activates the formation of nucleosides from the purines and pyrimidines. Other unknown factors carry the reaction through further stages to the formation of nucleic acid.

* The "Wills' factor" is a term applied to an unknown hematopoietic substance found in some crude liver extracts and proteolyzed liver which is effective in certain tropical macrocytic anemias and in pernicious anemia of pregnancy. As far as is known, it is not folic acid.
Our studies are compatible with the general hypothesis proposed in figure 10. Vitamin $B_{12}$ and folic acid are probably involved in closely related chemical reactions since refined liver extract containing vitamin $B_{12}$ temporarily decreased the excretion of folic acid in the urine of a patient with pernicious anemia who had relapsed while taking folic acid.

It is likely that folic acid induces hematologic remissions in persons with pernicious anemia by a "mass action" effect, for the same dose that induced the remission will not maintain it indefinitely.* When relapse occurs, an increase in the dose will induce another temporary remission. The concept of "mass action" in turn suggests that folic acid is a substrate in the formation of another coenzyme† which accelerates the synthesis of thymine as well as other essential pyrimidines

\[
\text{NH}_2 \text{ and C sources (from amino acids?)} \\
\text{Step I} \\
\text{Folic Acid + unknown substances} \\
\text{(Wills' factor?)} \\
\text{(xanthine oxidase?)} \\
\text{Purines and Pyrimidines (Thymine)} \\
\text{Ribose and Desoxyribose} \\
\text{Step II} \\
\text{Vitamin } B_{12} \\
\text{Nucleosides (Thymidine)} \\
\text{Nucleotides} \\
\text{Nucleic Acid}
\]

* Hematologic relapses in patients with pernicious anemia treated with folic acid have been reported previously by Hansen-Pruss,† Vilter, Vilter and Hawkins∥ and Schwartz, Kaplan and Armstrong.¶

† Sauberlich has recently isolated a substance active for growth of Leuconostoc _citrovorum_ 8081 which occurs in the urine of animals and humans receiving folic acid. This substance, called "the _citrovorum factor," is chemically related to folic acid, and may be an intermediate metabolite in the conversion of folic acid to the hypothetical hematopoietically active coenzyme. Preliminary tests on persons with pernicious anemia do not suggest that it is the active substance, itself.
probable that the depletion of the scant supply of vitamin B₁₂ available to patients with pernicious anemia may be the reason why every patient developed neurologic relapse as the dose of folic acid was increased in attempts to combat the hematologic relapse. Other factors necessary for cell maturation are depleted also. We have evidence for this in the desultory hematologic responses which occurred in these multi-depleted patients when they were treated with refined liver extract or vitamin B₁₂. One of the substances depleted by folic acid may be the "Wills' factor" and there may be others as yet unnamed.

An analogous situation has been described in pigs nineteen and twenty made deficient in folic acid and extrinsic factor (vitamin B₁₂) by a vitamin-free casein and synthetic nutrient diet. The macrocytic anemia which developed in these pigs responded poorly or not at all to refined liver extract. When a hematologic response did occur, relapse followed in short order and optimum improvement occurred only when folic acid was given. Our patients were probably deficient in vitamin B₁₂ and unknown substances such as the "Wills' factor," but not in folic acid. The deficiency was by no means so complete, however, as that which would be induced by a synthetic ration. Therefore, the responses to refined liver extract and vitamin B₁₂ were retarded but not abolished.

Thymine will induce a response in patients with pernicious anemia even after folic acid has lost its effect. This observation may be interpreted in two ways: Either thymine acts through a parallel reaction and circumvents the need for folic acid and the enzyme systems related to it; or thymine is the product of a reaction activated by folic acid and unknown substances such as the "Wills' factor" which are depleted by the continuous administration of folic acid. The latter theory is consistent with previously discussed observations and with studies in bacterial metabolism (fig. 10). We favor it over the former theory, which has no experimental support as yet.

The effectiveness of uracil in very large doses in persons with pernicious anemia in relapse suggests that it may be a precursor of thymine (5 methyl uracil) and that the process involved may be one of transmethylation (fig. 11). Another interpretation suggests that uracil may stimulate the formation of ribonucleic acid as thymine seems to stimulate formation of deoxyribonucleic acid. Studies on the patient with pernicious anemia of pregnancy support the former
Patients with pernicious anemia of pregnancy are often refractory to treatment with refined liver extract and vitamin B12 but respond to some crude preparations of liver extract, proteolyzed liver or to folic acid. The suggestion has been made that the primary deficiency in such patients may be in the "Wills' factor," since folic acid deficiency per se could not be established, and folic acid was not always found in sufficient quantity in the preparations to which these patients responded. A similar metabolic defect has been suggested to explain the macrocytic anemia refractory to refined liver extract and vitamin B12 which has been observed in one male patient, to whom reference has been made previously.

These considerations and the experiments reported in this paper suggested that the unknown substance called the "Wills' factor" might function together with folic acid in the formation of purines and pyrimidines or in the interconversion of these bases through reactions such as transmethylation. An example of the type of reaction proposed is the methylation of uracil to thymine with folic acid and the "Wills' factor" forming the system which activates the reaction (fig. 11). If this theory is correct, the patient with pernicious anemia of pregnancy and a deficiency of the "Wills' factor" should not respond to uracil unless a much larger dose than 30 grams a day could be given. The anemia might respond to methyl donors and should respond to folic acid or to thymine. It should not respond to vitamin B12. The results of the tests completely fulfilled the theoretic possibilities. Uracil did not induce a response, the methyl donors, choline and methionine induced a partial response too rapidly to be explained by direct effect on liver function through lipotrophic action, and thymine induced a complete response. In another relapse, the anemia failed to respond to vitamin B12 but did respond to folic acid. These studies support the hypothesis proposed above.

Conversely, these studies and those published by other investigators help to define the probable chemical defects in persons with the various types of megaloblastic anemia.

Pernicious anemia seems to be a conditioned deficiency of vitamin B12 due to failure of absorption of this essential nutrient from the gastroenteric tract. A deficiency of an enzyme, the intrinsic factor, which seems to be essential for the absorption of vitamin B12, appears to be the conditioning factor (fig. 11). According to this theory, vitamin B12 is both extrinsic factor and the factor in refined liver extract. In bacterial metabolism, vitamin B12 is essential to the conversion of thymine to its nucleoside, thymidine, and this may be the ultimate chemical defect in persons with pernicious anemia (Step II, fig. 10). So far, only small ineffective doses of thymidine have been given to persons with pernicious anemia. The hematologic effect of folic acid and thymine in this disease would appear to be by "mass action."

In some patients with pernicious anemia it appears that liver extract facilitates the degradation of conjugates of folic acid, found in food perhaps by inactivation of a conjugase inhibitor. On the basis of this data, the suggestion has been made that in pernicious anemia, the deficiency of the liver factor or vitamin B12 in turn may induce a deficiency of folic acid. If this proves to be true, it provides an additional explanation for the effectiveness of folic acid in patients with pernicious anemia in hematologic relapse.
Pernicious anemia of pregnancy, refractory megaloblastic anemia and/or achrestic anemia appear to occur because of a defect in the formation or utilization of thymine and probably other pyrimidines and purines. Folic acid and unknown substances, possibly the 'Wills' factor,' activate these reactions (fig. 12). A defect at any stage of the reaction for the formation of the nucleic acids proposed in this paper is theoretically capable of inducing megaloblastic anemia. If this defect cannot be rectified by refined liver (vitamin B_{12}) the anemia can be classified according to our present nomenclature as refractory.

Nutritional macrocytic anemia and sprue may be mixed dietary deficiencies of these various catalysts due to dietary inadequacies and failure of absorption of these substances from the gastro-intestinal tract (fig. 12). In nutritional macrocytic anemia a dietary deficiency of vitamin B\textsubscript{12} predominates, while in sprue the principle deficiency seems to be one of folic acid.\textsuperscript{24} The macrocytic anemia of idiopathic steatorrhea seems to be due to a deficiency of these essential substances secondary to various diseases of the small bowel. There is insufficient evidence upon which one might attempt to classify megaloblastic anemia of infancy,
though the diets of these infants are usually inadequate and the anemia responds more rapidly to folic acid than to vitamin B_{12}.*

Vitamin B_{12} may be intimately involved also in similar types of chain reactions upon which the integrity of the nervous system and the metabolism of bilirubin depend. Combined system disease and indirect reacting bilirubinemia are common to pernicious anemia but very rare in the other types of macrocytic anemia. Folic acid may be involved in nervous system metabolism also since temporary remissions in neuritis were observed in several patients when folic acid dosage was increased. All the factors discussed in this paper are involved in maintenance of a normal tongue, oral, and probably gastroenteric mucous membrane; for glossitis occurs in all types of macrocytic anemia. It is improved when any one of these factors is prescribed, but may recur during the period of administration of any factor except vitamin B_{12}. The fundamental chemical reaction may involve formation and conversion of nucleic acid nucleotides to codehydrogenase nucleotides (nicotinamide coenzymes). Such a concept provides chemical links between the glossitides of pernicious anemia, sprue and pellagra.

These speculations are still highly theoretic, only partially proved and replete with assumptions. It is possible that additional work may show that the observations made on these patients may lend themselves to other interpretations. There are reports that thymine and purines will not substitute for folic acid in the folic acid-deficient rat and mouse on synthetic rations. Studies with thymine antagonists do not wholly support the idea that folic acid is an activator for the biosynthesis of thymine. Because of these experiments the suggestion has been made that folic acid and thymine act as alternatives rather than as two components of an anabolic system. Our data do not eliminate this possibility.

It is probable that folic acid and the other essential nutrients discussed in this paper have many functions in addition to these which have been mentioned. The metabolism of tyrosine and phenyl alanine is abnormal in folic acid-deficient animals and this metabolic abnormality which is also observed in scorbutic guinea pigs may be reversed by folic acid. Folic acid also seems to play a part in the activation of "dopa" decarboxylase which relates it to amino acid metab-

* Since this paper was submitted, May, Nelson, Salmon, Lowe, Lienke and Sundberg have demonstrated that monkeys fed milk diets deficient in vitamin C and folic acid develop anemia characterized by megaloblastic maturation arrest in the bone marrow. Folic acid but not vitamin B_{12} led to rapid reversion of the bone marrow to a more normal type of maturation. Administration of vitamin C led to similar but less dramatic changes but did not prevent death of some of the animals. Macrocytic anemia of infancy, which occurs so commonly in association with scurvy and in infants consuming unsupplemented milk diets, has many features in common with this monkey anemia. By analogy, these authors believe that macrocytic anemia of infancy is caused by defective utilization potentiated by vitamin C deficiency of small amounts of folic acid available in milk diets.

Luhby and Wheeler in a recent publication, offer clinical evidence from a study of 16 cases in support of this theory. Conversely, deficiencies of folic acid and vitamin B_{12} are factors which probably condition the appearance of the normocytic or slightly macrocytic anemia of adult persons with scurvy.† A recent publication by Morris, Harper and Goldblum indicates that abnormal metabolites of tyrosine, "tyrosyl derivatives" which are found in the urine of scorbutic infants, are eliminated by folic acid administration. However, folic acid in doses of 10 mg. daily did not have this effect in 1 adult scorbutic patient.†
olism and in the regulation of the activity of xanthine oxidase and other flavin-containing enzymes. It is probable that interference with these basic functions may explain some of the bizarre effects observed in animals and human beings treated with aminopterin.

At the present time a way is not readily found to fit these observations into a single functional scheme. It is probable that these substances are essential for many diverse cellular metabolic reactions which may or may not be related to the reaction discussed in this paper. Nonetheless, the relationship to nucleoprotein metabolism seems clear though the exact steps are uncertain.

**Summary and Conclusions**

1. Patients with pernicious anemia who are maintained on folic acid, 30 mg. three times a week, for two to three years may have a hematologic relapse which will remit satisfactorily if refined liver extract is given, or partially if the dose of folic acid is increased to 50 mg. daily, or if thymine is given.

2. The hematologic remission succeeding the increased dosage of folic acid is followed within several months by a second relapse. At this time the response of these patients to liver extract or vitamin B12 is retarded. Recovery occurs after two to four months.

3. These experiments suggest that folic acid exerts its effect by "mass action" in pernicious anemia and that it is essential to the formation of thymine and other pyrimidines and purines or facilitates the utilization of these substances.

4. Posterolateral column disease or peripheral neuritis occurred in every person with pernicious anemia who received increasing doses of folic acid to maintain an hematologic remission. This observation suggests that folic acid, by pushing a chemical reaction through to completion in the face of a serious deficiency of vitamin B12, depleted the supply of this factor even more and led to the development of combined system disease.

5. Uracil produced a hematologic response in 2 of 3 persons with pernicious anemia in relapse when given in doses of 15–30 grams daily. The data suggest that uracil may be a precursor of thymine.

6. A patient with pernicious anemia of pregnancy failed to respond to uracil, 30 grams daily, but did respond partially when choline, 3 grams, and methionine, 6 grams were given. Thymine induced a complete response. The partial response to methionine and choline and the better response to thymine suggest that choline and methionine supplied methyl groups for the formation of thymine, but that activating substances for the methylating process were missing.

7. Reference is made to a patient previously reported from this laboratory who had liver extract and vitamin B12-refractory megaloblastic anemia but who responded to folic acid and on a second relapse to thymine. Studies on the output of folic acid in the urine of this patient did not support the possibility of folic acid deficiency, and the suggestion was made that another substance, possibly the "Wills' factor," was deficient, and that this factor together with folic acid activated the formation of thymine. These two factors correspond to the activators of the transmethylation reaction mentioned in the preceding paragraph.
8. These studies on human beings and similar ones in bacterial metabolism suggest that folic acid, liver extract and vitamin $B_{12}$ are essential to the formation of nucleic acid and nucleoprotein through a chemical chain reaction. The suggestion is made that the megaloblast common to pernicious anemia and related macrocytic anemias is a primitive erythroblast with an abnormality in the metabolism of nucleic acid and nucleoprotein. The so-called maturation arrest in all marrow elements occurs because of this abnormality which may be induced by a deficiency of vitamin $B_{12}$, folic acid, the "Wills' factor," and probably other chemical activators of this reaction.

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STUDIES ON THE RELATIONSHIPS OF VITAMIN B₁₂, FOLIC ACID, THYMINE, URACIL, AND METHYL GROUP DONORS IN PERSONS WITH PERNICIOUS ANEMIA AND RELATED MEGALOBLASTIC ANEMIAS

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