STUDIES ON THE BIOCHEMICAL DEFECT OF PERNICIOUS ANEMIA.

I. IN VITRO OBSERVATIONS ON OXYGEN CONSUMPTION, HEME SYNTHESIS AND DEOXYRIBONUCLEIC ACID SYNTHESIS BY PERNICIOUS ANEMIA BONE MARROW 1, 2

BY E. DONNALL THOMAS AND HARRY L. LOCHTE, Jr. 3

[From the Mary Imogene Bassett Hospital (Affiliated with Columbia University), Cooperstown, N. Y.]

(Submitted for publication August 8, 1957; accepted October 14, 1957)

In microbiological systems, folic acid and vitamin B12 appear to be important in the synthesis of purines and pyrimidines, in the synthesis of methyl groups, and possibly in the synthesis of deoxyribose (1–3). Studies at the clinical level, summarized by Mueller and Will (4), indicate that similar biochemical processes may be involved in patients with pernicious anemia (P. A.). Because such studies on patients are difficult to carry out and interpret, precise methods of in vitro assessment of the biochemical activity of P. A. marrow cells are needed.

Direct studies on P. A. marrow have been confined, for the most part, to observations of morphologic changes and cell counts in tissue culture (5–12). In attempting to avoid the difficulties inherent in evaluating these changes in tissue culture and in an effort to secure more direct biochemical information, P. A. marrow has been studied in our laboratory by the following techniques: 1) oxygen consumption, 2) heme synthesis as measured by the rate of incorporation of C14-glycine into heme, and 3) deoxyribonucleic acid (DNA) synthesis by measurement of the rate of incorporation of C14-formate into thymine.

With these techniques, which permit measurements of biochemical activity over short periods of time and with small amounts of bone marrow, we have attempted to answer the following questions: 1) Does vitamin B12 have a direct effect on P. A. marrow cells? 2) Why has the liquid culture-vaccine vial technique failed to demonstrate an effect of vitamin B12? 3) Is there an inhibitory factor in P. A. serum? 4) What is the effect of folic acid on P. A. cells?

METHODS

Bone marrow was obtained by aspiration biopsy from patients with classical pernicious anemia in relapse. The marrow was dispersed by passage through wire screens, and aliquots were pipetted into incubation vessels. The suspending media were varied according to the conditions being studied. In making the various dilutions, care was taken to keep the concentration of serum and of radioactive substrate constant in all aliquots of any given experiment. The methods for measuring oxygen consumption, heme synthesis and DNA synthesis have been described (13, 14). In measuring heme synthesis, the concentration of glycine-2-C14 was 1.4 μM per ml of medium, and the specific activity was 0.2 mc. per mM. In measuring DNA synthesis, the concentration of C14-formate was 0.5 μM per ml of medium, and the specific activity was 2.0 mc. per mM. At the end of the period of incubation, aliquots of carrier rabbit bone marrow were added to the incubation vessels to facilitate the subsequent isolation of thymine. In any one experiment, therefore, the absolute value of the specific activity of the isolated thymine is a function of the amount of carrier added. Crystalline vitamin B12, folic acid and folinic acid were obtained through the courtesy of Lederle laboratories.

RESULTS

Table I illustrates a comparison, with regard to heme synthesis and oxygen consumption, of P. A. bone marrow incubated in P. A. serum, normal serum, and P. A. serum plus 0.1 γ of vitamin B12 per ml. As was indicated previously (13), the comparison of different serum samples is difficult because of a wide range of variability among different normal serum samples from hematologically normal donors. This difficulty is apparent in attempting to compare P. A. serum to
**TABLE I**

<table>
<thead>
<tr>
<th>EXPERIMENT</th>
<th>COMPARISON OF PERNICIOUS ANEMIA SERUM TO NORMAL SERUM AS 100%</th>
<th>COMPARISON OF PERNICIOUS ANEMIA SERUM PLUS 0.1 mg of vitamin B₁₂ per ml. TO PERNICIOUS ANEMIA SERUM ALONE AS 100%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEME SYNTHESIS</td>
<td>OXYGEN CONSUMPTION</td>
</tr>
<tr>
<td>1</td>
<td>127</td>
<td>120</td>
</tr>
<tr>
<td>2</td>
<td>112</td>
<td>99</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
<td>93</td>
</tr>
<tr>
<td>4</td>
<td>126</td>
<td>113</td>
</tr>
<tr>
<td>5</td>
<td>195</td>
<td>102</td>
</tr>
<tr>
<td>6</td>
<td>125</td>
<td>108</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>112</td>
</tr>
<tr>
<td>8</td>
<td>95</td>
<td>91</td>
</tr>
<tr>
<td>9</td>
<td>51</td>
<td>94</td>
</tr>
<tr>
<td>10</td>
<td>108</td>
<td>109</td>
</tr>
<tr>
<td>11</td>
<td>112</td>
<td>102</td>
</tr>
<tr>
<td>12</td>
<td>141</td>
<td>100</td>
</tr>
<tr>
<td>MEAN</td>
<td>113</td>
<td>104</td>
</tr>
<tr>
<td>S.D.</td>
<td>36</td>
<td>9</td>
</tr>
</tbody>
</table>

* Each experiment represents a different P. A. marrow. The time of incubation for heme synthesis was 10 hours with oxygen consumption being measured during the first 3 hours.

normal serum, as indicated by the large standard deviation. In comparing the effect of P. A. serum with and without added vitamin B₁₂, we used aliquots of the same serum in each of the experiments, with the result that the standard deviation is much smaller. It is apparent that, within the limits of the technique, there is no effect of either

**TABLE II**

<table>
<thead>
<tr>
<th>CONCENTRATION</th>
<th>CPM/μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>2830</td>
</tr>
<tr>
<td>0.01</td>
<td>3970</td>
</tr>
<tr>
<td>0.001</td>
<td>4030</td>
</tr>
</tbody>
</table>

The effect of various concentrations of vitamin B₁₂ on deoxyribonucleic acid synthesis by pernicious anemia marrow in pernicious anemia serum incubated for five hours

**FIG. 1. THE EFFECT OF VITAMIN B₁₂ ON DEOXYRIBONUCLEIC ACID SYNTHESIS BY PERNICIOUS ANEMIA MARROW IN PERNICIOUS ANEMIA SERUM**

In Experiments 1 and 2, the period of incubation was 10 hours; in Experiments 3 through 7, 5 hours. In Experiments 1 through 5 the concentration of vitamin B₁₂ was 0.1 mg per ml; in Experiments 6 and 7, 0.01 mg per ml. In each experiment the control was an aliquot of the P. A. marrow incubated in P. A. serum without added vitamin B₁₂.
normal serum or vitamin B$_{12}$ on these two processes.

Figure 1 illustrates the effect of vitamin B$_{12}$ on DNA synthesis by P. A. marrow cells in P. A. serum. The results illustrated in this figure show that vitamin B$_{12}$ does have a direct and consistent effect at the cellular level. The amount of vitamin B$_{12}$ used in these experiments is considerably above the normal range of 100 to 900 $\mu$g. per ml. (15). The data in Table II show that 0.001 $\mu$g. per ml. also produces an optimal effect. In three experiments the addition of vitamin B$_{12}$ to normal serum produced no significant increase in the rate of DNA synthesis by P. A. marrow.

Figure 2 illustrates the effect of folic acid on DNA synthesis by P. A. marrow cells in P. A. serum. In three experiments there was no effect, in one there was a moderate stimulation, and in one there was marked stimulation. In one other experiment, the addition of folic acid to P. A. mar-

row in normal serum also produced a marked increase in DNA synthesis. It appears, therefore, that P. A. marrow cells differ considerably in their response to folic acid. In three experiments, no difference between the effect of folic acid and of folinic acid was observed.

Table III illustrates two experiments that have been done to test the effect of vitamin B$_{12}$ and folic acid singly and in combination. In Experiment A the marrow was stimulated markedly by folic acid, whereas in Experiment B the folic acid had a barely detectable effect. The effect of the combination of vitamin B$_{12}$ with folic acid was roughly the sum of the individual effects alone. One interpretation of these limited data is that the two substances are not related in their mode of action.

The experiments illustrated in Figures 3A, 3B and 3C were designed to determine whether or not there is an inhibitor of DNA synthesis in P. A. serum. Figure 3A compares the effect of dilution of P. A. serum and normal serum on DNA synthesis by normal marrow, and Figure 3B illustrates a similar experiment using P. A. marrow. DNA synthesis by P. A. marrow and normal marrow in various mixtures of P. A. serum and normal serum is illustrated in Figure 3C. It is apparent that none of these experiments shows any evidence of an inhibitor of DNA synthesis in P. A. serum.

Studies of DNA synthesis over a 48 hour period are illustrated in Figure 4. Using the vac-

![Figure 2](image-url)

**FIG. 2. THE EFFECT OF FOLIC ACID ON DEOXYRIBONUCLEIC ACID SYNTHESIS BY PERNICIOUS ANEMIA MARROW IN PERNICIOUS ANEMIA SERUM**

In Experiments 1 and 2 the period of incubation was 10 hours; in Experiments 3 through 5, 5 hours. The concentration of folic acid was 0.2 $\gamma$ per ml. In each experiment the control was an aliquot of the P. A. marrow incubated in P. A. serum without added folic acid.

<table>
<thead>
<tr>
<th>EXPT.</th>
<th>SUPPLEMENT</th>
<th>CPM/$\mu$m THYMINE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NONE</td>
<td>265</td>
</tr>
<tr>
<td></td>
<td>B$_{12}$</td>
<td>713</td>
</tr>
<tr>
<td></td>
<td>FOLIC ACID</td>
<td>1,705</td>
</tr>
<tr>
<td></td>
<td>B$_{12}$ &amp; FOLIC ACID</td>
<td>2,410</td>
</tr>
<tr>
<td></td>
<td>NONE</td>
<td>8,900</td>
</tr>
<tr>
<td></td>
<td>B$_{12}$</td>
<td>11,300</td>
</tr>
<tr>
<td></td>
<td>FOLIC ACID</td>
<td>9,500</td>
</tr>
<tr>
<td></td>
<td>B$_{12}$ &amp; FOLIC ACID</td>
<td>12,690</td>
</tr>
</tbody>
</table>
cine vial method of Osgood and Brownlee (5), as modified by Lajtha (8), we incubated aliquots of normal marrow in 25 per cent normal serum in Gey's solution. After the various times of incubation, $^{14}$C-sodium formate was added, and incubation was carried on for an additional four hours. It is apparent from Figure 4 that the ability of marrow cells to synthesize DNA is markedly reduced after 24 hours and has virtually ceased at 48 hours.

**DISCUSSION**

These short term experiments with P. A. marrow show a definite effect of vitamin B$_{12}$ on DNA synthesis. No effect was demonstrated on either oxygen consumption or heme synthesis. It is possible that longer experiments would show some effect on heme synthesis, as suggested by the experiments of Walsh, Thomas, Chow, Fluharty, and Finch (16). In the first few hours, vitamin B$_{12}$ may permit proliferation of cells and then later, as these cells mature, heme synthesis may begin to increase.

Results given here are of particular interest in that they show a definite action of vitamin B$_{12}$ on P. A. marrow cells. Using the liquid suspension culture technique, Lajtha (7) and Thompson (10) observed that folic acid, but not vitamin B$_{12}$, had a maturing effect on megaloblasts. These observations led to the speculation that vitamin B$_{12}$ might combine with an intrinsic factor or be altered in some way to form a hematopoietic factor in serum (10). However, the experimental data reported here demonstrate that vitamin B$_{12}$ in P. A. serum has a direct effect on P. A. marrow cells, indicating that an intrinsic factor is not essential to

**FIG. 3A. THE EFFECT OF DILUTION OF PERNICIOUS ANEMIA SERUM AND NORMAL SERUM ON DEOXYRIBONUCLEIC ACID SYNTHESIS BY NORMAL MARROW**

Incubation time was five hours.

**FIG. 3B. THE EFFECT OF DILUTION OF PERNICIOUS ANEMIA SERUM AND NORMAL SERUM ON DEOXYRIBONUCLEIC ACID SYNTHESIS BY PERNICIOUS ANEMIA MARROW**

Incubation time was five hours.

**FIG. 3C. THE EFFECT OF VARIOUS MIXTURES OF NORMAL SERUM (N. S.) AND PERNICIOUS ANEMIA SERUM (P. A. S.) ON DEOXYRIBONUCLEIC ACID SYNTHESIS BY PERNICIOUS ANEMIA MARROW AND NORMAL MARROW**

Incubation time was five hours.
FIG. 4. THE EFFECT OF THE TIME OF PREINCUBATION ON DEOXYRIBONUCLEIC ACID SYNTHESIS BY NORMAL BONE MARROW IN VACCINE VIAL CULTURE

After the period of culture shown, C-14-formate was added to the vial. Incubation was then carried on for an additional four hours. In the experiment shown, the triangles represent a nucleated cell concentration of 3,338 per cu. mm. and the open circles represent a concentration of 1,730 per cu. mm.

The action of vitamin B₁₂. The P. A. serum present during incubation has the ability to bind some of the vitamin added in vitro (15). Whether this action is important in the utilization of the vitamin is not known. Experiments have not been conducted in the absence of serum because of the poor preservation and lowered metabolism of the cells in the culture media without serum.

Investigators using the liquid culture-vaccine vial technique have failed to demonstrate a cellular effect of vitamin B₁₂. The work of Swan, Reisner, and Silverman (12) indicated that this failure may be due to the limitations of the technique. They studied the effect of vitamin B₁₂ and folic acid on pernicious anemia marrow in both suspension and solid cultures. Vitamin B₁₂ appeared to stimulate cell growth in cultures grown on clots and to convert megaloblastic hemopoiesis to normoblastic in cultures grown on glass. In suspension cultures, on the other hand, folic acid caused an increase in the number of cells, but vitamin B₁₂ did not. Our studies show that after the first few hours of liquid culture, the rate of DNA synthesis falls off very rapidly, indicating a rapid loss of proliferative ability (Figure 4). Lajtha observed a rapid loss of proliferative power of marrow cells in suspension cultures in vitro (8). Swan, Reisner, and Silverman have also commented on the progressive decrease in the number of “mitotable” cells in suspension cultures (12).

Since vitamin B₁₂ appears to act primarily on DNA synthesis and associated cell proliferation, the rapid loss of these processes in suspension culture makes the interpretation of 48 and 72 hour cultures an unreliable method of studying the action of vitamin B₁₂.

Lajtha (7), Thompson (9), and Bussi, Eridani, Pozza, Fava, and DeMicheli (11) presented data indicating that P. A. serum possesses the property of inhibiting morphological maturation of both P. A. marrow cells and normal marrow cells. Our technique, however, indicates that P. A. serum does not contain an inhibitor of DNA synthesis. The assessment of maturation by morphologic means is dependent primarily on nuclear changes which may not necessarily be associated with the formation of new DNA. Our results do not permit the exclusion of an inhibitor of morphologic maturation in P. A. serum. However, the above-mentioned limitations of the vaccine vial culture technique cast doubt on the existence of a true inhibitor.

One surprising result has been the marked variation in the response of the marrow to added folic acid. In those P. A. marrow cells responding to folic acid, the magnitude of the effect has been much greater than that of vitamin B₁₂. Nieweg, Faber, de Vries, and Kroese (17) measured the folic acid activity of the whole blood in 16 patients with pernicious anemia. Normal values were found in about half the cases, with the remainder being low. In our experiments, the marrows responding to folic acid may have come from patients who were deficient in folic acid as well as vitamin B₁₂. Blood folic acid levels were not measured.

The technique presented here makes it possible to carry out metabolic studies on marrow cells of man. Experiments along these lines, with various radioactive precursors and with various supple-
mented media, may eventually shed light on the mode of action of vitamin $B_{12}$ and folic acid. It is hoped that some of these studies can be carried out, although the decreasing number of patients with untreated pernicious anemia constitutes a major problem to those working in this field.

SUMMARY

Pernicious anemia (P. A.) marrow has been studied in vitro by measuring the rates of oxygen consumption, heme synthesis and deoxyribonucleic acid (DNA) synthesis. From these studies the following conclusions have been drawn:

1. Vitamin $B_{12}$ affects DNA synthesis but not oxygen consumption or heme synthesis.
2. Vitamin $B_{12}$ has a direct effect on DNA synthesis by P. A. marrow cells.
3. Folic acid affects DNA synthesis in different P. A. marrows to a highly variable degree.
4. The liquid suspension-vaccine vial technique is not a suitable method for studies of DNA synthesis by marrow.
5. P. A. serum does not contain an inhibitor of DNA synthesis.

ACKNOWLEDGMENTS

The authors are indebted to the following physicians for their generous cooperation in making available suitable case material: William B. Castle, Robert B. Chodos, Franklin G. Ebaugh, Frank H. Gardner, Gerald F. Parkhurst, Simon Propp, and George D. Vlahides.

REFERENCES