The Effect of Various Metabolites on the Growth of Marrow Cells in Vitro

By Harold T. Swan, Edward H. Reisner, Jr., and Morris Silverman

When the cells from megaloblastic bone marrows have been grown in vitro, the conclusions drawn from such experiments have been in apparent conflict in the hands of different workers, and sometimes in disagreement with clinical experience. Most of the published reports have dealt with suspension cultures of the Osgood and Brownlee type, a method of tissue culture which has been criticized by many workers in this field. The present study was undertaken to determine the possibilities and limitations of various methods of culturing marrow cells and to apply these methods to the study of specific factors influencing megaloblast development in erythropoiesis.

Methods and Materials

1. Explants of the buffy coat, prepared from megaloblastic marrow aspirates from patients with pernicious anemia, were grown on chick plasma clots using the double cover slip technique of Maximow and Murray, in a medium of one part of chick embryo extract, and two parts each of balanced salt solution and human serum, obtained from patients with pernicious anemia in relapse, normal adults, and from placental veins. At intervals of 1, 2, 3, 4, and 7 days, the living cultures were examined and subsequently fixed in alcohol and stained with Giemsa stain for more permanent record. Marrows from seven patients with pernicious anemia in relapse were cultured in this manner in normal and autologous pernicious anemia serum, alone and with the addition of vitamin B12 in concentration of 0.005 μg./ml., and in one experiment with folinic acid in concentration of 0.005 mg./ml.

2. Marrow particles from the marrow aspirate of a patient with pernicious anemia in relapse were gently smeared on the surface of glass slivers (dimensions 1 cm. x 3 cm.), which were then carefully submerged in a slanted test tube containing human serum, chick embryo extract and balanced salt solution, in a ratio of 2:1:2. They were left undisturbed for two days to ensure the establishment of good growth on the surface of the glass. After this time they could be air dried and stained with Wright's stain, or the culture medium could be replenished and the culture allowed to grow for a longer period. The media used consisted of pernicious anemia serum or normal human serum, each alone and with added vitamin B12 (0.005 μg./ml.).

3. Suspension cultures were made following the technic devised by Osgood and Brownlee as modified by Lajtha. This modification consisted in the removal of 2 ml. of marrow aspirate which was injected into a siliconized McCartney bottle containing 18 ml. of Gey's or Tyrode's balanced salt solution with 0.005 per cent heparin. The bottle was centrifuged for ten minutes at 1500 r.p.m. and the supernatant fluid discarded. The cells remaining in the bottle were resuspended in 3-4 ml. of balanced salt solution and pumped in and out of a syringe until all the marrow clumps were broken up and the cells evenly distributed throughout the fluid. A smear of the cells was made at this stage to record their appearance and the differential count at the start of the experiment. These cells are hence-
forth referred to as the "stock cells." Enough of this cell suspension was then mixed with the culture medium of human serum and balanced salt solution to give a final suspension of approximately 4000 cells per cu. mm. Serum concentrations used ranged between 66 and 83 per cent. After thorough mixing, a count of the total nucleated cells was made, and designated as the "initial count." Three ml aliquots of the suspension were injected into each of as many McCartney bottles as would be needed for the experiment. In each experiment one bottle was used as a control, while to others various metabolites and antimetabolites were added in the concentrations desired, always in volumes of 0.1 ml. At first the cultures were incubated for from 24 to 96 hours at 37 C. After the initial experiments all cultures were incubated for 48 hours only, since no significant changes had been seen at 96 hours which had not been apparent at 48. At the end of this time each bottle was agitated vigorously for 5 minutes to resuspend the cells evenly, and the total nucleated cell count/cu. mm. determined. In all the experiments the same white cell pipette was used by the same observer (H. T. S.) and all the cells in 1 cu. mm. were counted with the 45 mm. objective lens, always using the same counting chamber. After the count the bottles were centrifuged and most of the supernatant fluid removed so as to concentrate the suspension. The cells were then resuspended in what was left and smeared on glass slides for staining. The smears were stained with Wright's stain and counterstained with Giemsa. The smears from each bottle were made and then coded so that they could not be identified without reference to the key, and at some later time a differential count of the nucleated red cells was made by two observers (E. H. R. and H. T. S.) working independently, until at least 500 cells had been counted from each bottle. From these smears the nucleated red cell count was also computed as a percentage of the total nucleated cell count, based on counting 1000 nucleated cells in the smear.

In the differential count the red cell precursors were classified as megaloblastic or normoblastic according to the criteria in Wintrobe's textbook "Clinical Hematology." They were then classified with respect to their capability to undergo mitosis,* as "mitotable" or maturing cells. The former group consisted of pro- and early basophilic megaloblasts and normoblasts; the latter group contained all cells in which the nucleus appeared to have begun the process of condensation of chromatin, including late basophilic, and the polychromat and orthochromat megaloblasts and normoblasts.

Megaloblastic marrows were obtained from 10 patients with pernicious anemia in relapse, with all the clinical features of that condition, including histamine fast achlorhydria, and subsequent response to parenteral therapy with vitamin B2. Another megaloblastic marrow was obtained from a case of sprue who responded well subsequently to injections of vitamin B12.

Metabolites and anti-metabolites were prepared as follows: therapeutic vitamin B12 preparations (Cobione (Merck) and Rubramin (Squibb)) were diluted to the concentrations desired, usually 0.0007-0.03 µg./ml. of culture. Gastric juice from normal subjects was collected after histamine stimulation, filtered through cheese cloth to remove mucous and solid material, and then filtered through Whatman filter paper, and stored in sterile containers at 4 C. for long enough to ensure autosterilization. It was neutralized before use to a pH of 7.0. An intrinsic factor concentrate of animal origin was supplied through the kindness of Dr. T. B. Jukes of Lederle Laboratories. This material was tested clinically and found to be strongly active. Solutions of this material were made by the addition of NaOH to a pH of 7.0. This was combined with vitamin B12 in a ratio of 5 mg. to 1 µg. of B12. Folinic acid

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* We assume that there is a point in the life history of a cell beyond which mitosis does not normally occur. The logical point for such a division is the time when involution of the nucleus with its accompanying decrease in DNA content begins. (Korson, R. J.: J. Exper. Med. 93: 121, 1951). If division occurs after involution of the nucleus has begun, at least one of the daughter cells must end up with less than a full complement of chromatin, and such abnormal mitoses, are indeed, observable in the marrows of patients with pernicious anemia and other pathologic states. Normally, however, once involution and loss of DNA has begun the cell is no longer "mitotable" (e.g., capable of normal mitosis) and has entered the phase of "maturation."
(Leucovorin (Lederle)) supplied through the courtesy of Dr. J. M. Ruegsegger of Lederle Laboratories, was diluted to concentrations of 0.1-1 μg./ml. of culture. Folic acid was prepared in a strength of 1 and 5 μg./ml. of culture. A-methopterin was supplied in solution by Dr. Ruegsegger and this was diluted to concentrations of 0.05-0.2 μg./ml. of culture. Colchicine (C.P.) in a concentration of 1/100,000 was sterilized by passage through a Berkefeld filter.

OUTLINE OF EXPERIMENTS

Experiment I. Growth of Megaloblastic Marrow in Chick Plasma Clots

Seven samples of megaloblastic marrow were grown by this technic in autologous pernicious anemia serum and in homologous adult normal serum or human placental serum, alone and with added vitamin B₁₂ as described above. In one experiment cultures were also set up containing folinic acid. The medium was changed every two days, and by this method it was possible to keep most of the cultures alive for several weeks. By the end of one week, however, fibroblast "dedifferentiation" was so well advanced that no purpose was served by longer observation.

Results: In general, growth in pernicious anemia serum alone was less vigorous than in normal serum or pernicious anemia serum to which vitamin B₁₂ had been added. In the latter preparations growth was exuberant. The leukocytes in the liquefied area adjacent to the explant had an irregular outline due to pseudopod projection of cytoplasm, and the monocytes had assumed a long spindle shaped contour. Similarly shaped cells could be seen growing out from the edges of the explant of marrow giving the whole culture an areolar appearance visible under the low power of the microscope (fig. 1). In the liquefied area many mature granulocytes and myelocytes and well hemoglobinized erythrocyte precursors could be seen. In contrast, in pernicious anemia serum alone, the culture showed much less areola formation and the cells in the region adjacent to the explant showed much less irregularity due to pseudopod projection, and a larger number of early basophilic red cell precursors with fewer hemoglobinized forms. Differential counts on the red cell precursors are shown in graphic form in figure 2. (Because of the method of fixation employed for these slides it was not possible to apply the criteria of Wintrobe to the identification of megaloblasts and normoblasts in the differential counts.) In general, at four days the staining and preservation of the cells in normal serum or pernicious anemia serum with added vitamin B₁₂ was much better than that of the cells in pernicious anemia serum alone. At seven days fibroblast formation was much more advanced in the cultures containing vitamin B₁₂. (In tissue culture most mesenchymal cells growing on a surface become elongated and spindle-shaped, and similar in appearance to fibroblasts derived from connective tissue. Some authorities refer to all such cells loosely as fibroblasts, although, more properly the term should probably be reserved for those spindle shaped cells derived from connective tissue. However, we have used the term here and elsewhere in this paper in the looser sense.) In the experiment with folinic acid in pernicious anemia serum the same features were observed as described above for vitamin B₁₂. These were somewhat less marked than in the cultures of the same marrow incubated simultaneously with vitamin B₁₂, but were more marked than the changes in the control culture.

In summary, the results of this experiment indicated that better growth
FIG. 1.—Photographs of megaloblastic marrow explants growing on chick plasma clots in pernicious anemia serum with and without added vitamin B₁₂. (1) Control 48 hrs. 90X; (2) Same with added B₁₂ 90X; (3) Same as (1) 250X. Note predominance of large early forms among cells on edge of culture; (4) Same as (2) 250X. Note greater pleomorphism in cells on edge of culture and early "fibroblast" formation; (5) Control 72 hrs. 135X. Early cells are still prominent and there is little evidence of "fibroblast" formation; (6) Outgrowth from the explant is vigorous and "fibroblastic" differentiation is well advanced with added B₁₂, after 72 hrs. 135X.
and more rapid maturation of blood cells were obtained in megaloblastic marrow grown on chick plasma clots in pernicious anemia serum when vitamin B₁₂ was added to the culture medium. Similar results were also observed with folinic acid.

Experiment II. Growth of Megaloblastic Marrow on Glass Cover Slips

A single megaloblastic marrow was grown by this technic as described above in autologous pernicious anemia serum and in normal serum, with and without added vitamin B₁₂.

Results: After two days it could be seen that the marrow particles looked healthy and were actively growing as evidenced by early fibroblast formation. With air dried smears, stained with Wright's stain or Tetrachrome, it was possible to identify megaloblasts and normoblasts (fig. 3). In pernicious anemia serum alone the red cell precursors were predominantly in the basophilic megaloblast stage, fibroblast formation was minimal, and cell staining was poor. With added vitamin B₁₂, and in normal serum, fewer of the earliest red cell forms were present and there were many maturing normoblasts, mostly in the early polychromatric stage. Fibroblast development was more advanced and the cells stained better and appeared to be growing more vigorously.
Fig. 3.—Photographs of megaloblastic marrow grown on glass in pernicious anemia serum for 48 hours. (1) Control 800X. A group of “mitotable” megaloblasts. Note the particularity of the nuclear chromatin and the giant metamyelocyte at the right of the picture. (2) 350X. Same with added vitamin B₁₂. This picture is deliberately taken at a lower magnification in order to show the greater pleomorphism of the cells in the field and the numbers of more mature cells of both normoblast and leukocytic series.
Experiment III. *Influence of Colchicine on the Growth of Cells in Suspension*

Cells from 8 normoblastic marrows were suspended in autologous serum and were distributed into 4–6 bottles in each experiment. One tenth of a milliliter of a 1/100,000 solution of colchicine was added to alternate bottles and the total nucleated cell counts determined in control and experimental bottles for 1, 2 or 3 days.

*Results:* There was a lower nucleated cell count in all of the bottles containing colchicine, the total count being approximately 75 per cent of that of the controls. Similar results were obtained with 4 megaloblastic marrows treated in the same way (table 2). The number of mitotic figures (cells arrested in mitosis) in the smears made from the preparations containing colchicine appeared to be roughly proportionate to the number of "mitotable" cells in the initial count. At 48 hours many disintegrating cells with karyorrhexic nuclei could be seen.

Experiment IV. *The Effect of Vitamin B₁₂, Intrinsic Factor and Folinic Acid on the Growth and Maturation of Cells from Megaloblastic Marrows Grown in Suspension*

Fourteen megaloblastic marrows from patients with pernicious anemia and one with non-tropical sprue, were cultured by the suspension technic in autologous (or homologous) untreated pernicious anemia serum alone, and with added vitamin B₁₂, intrinsic factor, vitamin B₁₂ plus intrinsic factor, and in some, but not all instances, folic and folic acids. Because of variations in the cellularity of the marrows it was not always possible to test all of these metabolites in each case. However, the effect of B₁₂ alone and with added intrinsic factor was tested in all 14 instances. In all but one intrinsic factor alone was used as a control. Folinic acid was tested in eight. Typical results in total nucleated cell counts and total nucleated red and white cell counts are shown in table 1.

*Results:* A consistent and significant increase in the number of nucleated cells per cubic millimeter was obtained in the bottles with added folic acid, compared to the control, and it exceeded that obtained with any other substance added. While, in most experiments there seemed to be an increase in nucleated cells when vitamin B₁₂ was added, the increase was less than when folic acid

| Table 1.—Mean changes at 48 hours in nucleated cell counts per cubic millimeter, taken from consecutive experiments where megaloblastic marrows were grown in suspension culture |
|---------------------------------|---------------|---------------|---------------|---------------|
|                                | Control       | Substance added to culture medium |               |               |
|                                |               | B₁₂           | B₁₂ plus Int. Factor | Int. Factor | Folinic Acid |
| Total nucleated cells/cu. mm. (6 marrows) | -283 | -49.4   | -419  | -247  | 1184* |
| Total nucl. red cells/cu. mm. (6 marrows) | 308 | 561     | 490.3 | 282.7 | 1027* |
| Total nucl. white cells/cu. mm. (10 marrows) | -264.2 | -264.6 | -540.4* | -418.9 |

* = statistically significant difference (p less than .05).

For all the experiments taken together is less than .001 for the red cell counts and less than .01 for the white cell counts.
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o = stock cells

\( i = \text{intrinsic factor} \)

\( b = \text{B-12} \)

\( \text{bi} = \text{B-12} + \text{int. factor} \)

\( f = \text{folinic acid} \)

All cultures at 48h unless otherwise noted

All Normoblasts

Maturing Megaloblasts

Mitotable Megaloblasts

Fig. 4.—Differential counts of nucleated red cells in Romanowsky stained smears of megaloblastic marrows cultured in suspension with pernicious anemia serum alone and with various added metabolites.

was added and was not statistically significant for the number of experiments performed. It was noteworthy that intrinsic factor substance added to vitamin B\(_{12}\) did not enhance the increase in cells caused by vitamin B\(_{12}\) alone.

Differential counts showing the ratio of megaloblasts to normoblasts in the cultures are shown graphically in figure 4. It is apparent from them that the proportion of megaloblasts decreased at a variable rate in the control bottles but that the decrease was more marked in the presence of vitamin B\(_{12}\) or folinic acid. There was no enhancement of the maturing effect of vitamin B\(_{12}\) by the addition of normal gastric juice or intrinsic factor concentrate. Indeed, in a majority of the cultures there were more megaloblasts remaining at 48 hours when an intrinsic factor source was added to the vitamin B\(_{12}\) than with B\(_{12}\) alone. The differences in rate of maturation as shown by the differential counts were not statistically significant owing to the width of variation encountered in the differential counts.

The absolute white cell counts per cubic millimeter were lower after 48 hours in all of the cultures compared with the initial count, and this decrease in cells was more marked in the presence of intrinsic factor. This last difference was statistically significant (p = <.05). (See Appendix.)
Table 2.—Mean change in total nucleated cells per cubic millimeter in suspension cultures after 48 hours

<table>
<thead>
<tr>
<th>Type &amp; Number of Marrows</th>
<th>Cultured in serum from</th>
<th>Substance added to culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Folinic Acid</td>
</tr>
<tr>
<td>Normoblastic (7)</td>
<td>Normal</td>
<td>436</td>
</tr>
<tr>
<td></td>
<td>P.A.</td>
<td>283</td>
</tr>
<tr>
<td>Megaloblastic (7)</td>
<td>Normal</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>P.A.</td>
<td>70</td>
</tr>
</tbody>
</table>

a, b, c, and d represent concentrations of .05, .5, 5.0 and 50.0 μg. of folic acid per ml. of medium, respectively.

Experiment V. Effect of Colchicine on the Response to Folinic Acid

To a suspension of a megaloblastic bone marrow in pernicious anemia serum, folinic acid and colchicine were added separately and together. In 48 hours folinic acid produced an increase of 1800 nucleated cells/cu. mm. (table 2) over the control, while in the bottle containing colchicine alone and colchicine plus folinic acid there were respectively 1300 and 1340 fewer cells per cubic millimeter than in the control.

Experiment VI. Effect of Folinic and Folic Acids on Normoblastic Marrows in Normal Serum

In suspension cultures of 7 normoblastic marrows in normal serum treated with folinic acid, and 3 normoblastic marrows in normal serum treated with folic acid there was no significant increase in the absolute nucleated cell count over the control at 48 hours (table 2).

Experiment VII. Effect of Amethopterin on Marrow Growth in Suspension

A normoblastic marrow was grown in serum from a patient who had a megaloblastic marrow due to non-tropical sprue (chosen because it was presumably low in folic and folinic acid). This marrow was cultured both by itself and with added folic acid (5 μg./ml.), or folinic acid (0.1 μg./ml.). Amethopterin (0.05 μg./ml.) was also added alone and together with the same amounts of folic and folinic acids used in the other bottles. In the control and folinic acid bottles there was a significant increase of nucleated cells after 48 hours (table 2) and a slight increase in the folic acid bottle. In the bottles containing amethopterin alone or with folic acid there were fewer cells than were observed initially.
However, when folinic acid was added with the amethopterin an increase of cells comparable to that observed with the control or folinic acid alone was obtained.

Another normoblastic marrow was cultured in normal serum with folic acid in concentrations of 0.05, 0.5, 5.0 and 50 μg./ml. alone and together with 0.05 μg./ml. of amethopterin. The control bottle and the folic acid bottles showed an increase of cells (table 2) that was absent in the bottles containing amethopterin. A few megaloblasts were found in the smears made from the latter cultures (fig. 5).

A megaloblastic marrow was grown in pernicious anemia serum with 1 μg./ml. of folic acid and 0.2 μg./ml. of amethopterin, separately and together. With folic acid alone the same amount of cell multiplication was observed as in the
TABLE 3.—Percentages of Megaloblasts Found on Differential Counting of Nucleated Red Blood Cells in Smears of Normoblastic Marrows Cultured for 72 Hours in Different Concentrations of Pernicious Anemia Serum and Balanced Salt Solution

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>90%</th>
<th>83%</th>
<th>75%</th>
<th>50%</th>
<th>25% (Bal. Saline Sol.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>3</td>
<td>7</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td></td>
<td></td>
<td>6</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>2</td>
<td></td>
<td></td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>0</td>
<td></td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>(c)</td>
<td></td>
<td></td>
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</table>

(a) Culture medium contained 17% Normal Serum added.
(b) Culture medium contained vitamin B₁₂ (10⁻¹⁰).
(c) Culture medium contained vitamin B₁₂ (10⁻⁸).

Control (table 2) but in the presence of amethopterin the count after 48 hours was less than the initial count.

Experiment VIII. Conversion of Normoblasts to Megaloblasts in Suspension

Normoblastic marrows from four patients with recent blood loss were cultured for 72 hours in pernicious anemia serum diluted with balanced salt solution to concentrations of 90, 83, 75, 50 and 25 per cent. In two experiments parallel bottles were set up with added vitamin B₁₂ in concentrations of 10⁻⁸ and 10⁻¹⁰ Gm./ml. of culture. Differential counts of nucleated red cell precursors were done on the stained smears. The percentages of megaloblasts which developed are shown in table 3. Dilution of the pernicious anemia serum led to increasing numbers of megaloblasts, up to the lowest dilutions in which the state of cell preservation was poor and the counts are considered unreliable. The addition of vitamin B₁₂ appeared to be protective until the lower dilutions were attained. Pictures of the type of cells seen in these cultures are shown in figure 5.

Discussion

In their original report on the suspension culture method Osgood and Brownlee reported that the “anti-pernicious anemia principle” definitely “seemed to favor growth” as used in the form of liver extract. Astaldi and Baldini observed that liver extract stimulated the migration of cells from explants of megaloblastic marrows and stimulated a return of normoblastic hematopoiesis. Lajtha and Callender observed that when megaloblastic marrows were grown in suspension there was little or no “ripening” (conversion of megaloblasts to normoblasts) in pernicious anemia serum alone or with added vitamin B₁₂. When, however, folic acid, folinic acid or vitamin B₁₂ plus normal gastric juice was added, or normal serum alone was used, there was rapid replacement of megaloblasts by normoblasts. When normoblastic marrows were grown for 72 hours in serum or spinal fluid from pernicious anemia patients in relapse, megaloblasts appeared where there had been none before. Lajtha suggested that there was a thermostable inhibitory factor in the blood of patients with pernicious anemia which could be overcome by folic acid, folinic acid and liver extract in vitro. Thompson using the same technic as Lajtha observed more rapid maturation of erythroblasts in normal serum than in pernicious anemia serum and claimed that as the latter serum was diluted so was there an acceleration of maturation. In these experiments he did not dif-
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ferentiate between megaloblasts and normoblasts. From his evidence he inferred that an "inhibitor" in the pernicious anemia serum was being diluted out. Subsequently he reported\textsuperscript{11} that vitamin B\textsubscript{12}, liver extract, and thymine exerted no maturing effect on megaloblastic marrows grown in pernicious anemia serum, but that such an effect was observed with added folic acid. Feinman et al.\textsuperscript{11} using a similar method also showed that megaloblasts ripened to normoblasts in normal serum and did so only slowly in pernicious anemia serum but in contrast to the work of Lajtha and Thompson, they observed no significant differences between 60 per cent and 40 per cent pernicious anemia serum in this respect. They were unable to induce megaloblast formation in normoblastic marrows cultured in pernicious anemia serum. Nieweg\textsuperscript{12} using a suspension method found that vitamin B\textsubscript{12} was ineffective in causing megaloblasts to mature in pernicious anemia serum but the maturation was observed in cultures with each of two different liver preparations. One of these was asserted to be free of both folic and folinic acid from which he postulated an unknown factor in liver that was effective in bringing about maturation in vitro. Sachetti and Parreira\textsuperscript{13} cultured megaloblastic marrow in normal plasma with embryo extract and found that vitamin B\textsubscript{12} stimulated proliferation of cells and promoted a normoblastic maturation of the early erythropoietic elements.

It was difficult to reconcile the observations of Callender and Lajtha with respect to the need for a source of 'extra-gastric intrinsic factor' to activate vitamin B\textsubscript{12}, with the work of Horrigan and Vilter\textsuperscript{14} who had shown that vitamin B\textsubscript{12} injected directly into the marrow cavity of patients with pernicious anemia in relapse induced normoblastic blood formation at the site of injection. If such an 'extra-gastric intrinsic factor' was lacking from the serum of patients with pernicious anemia as Callender and Lajtha suggested, it must at least have been present in the marrow if their hypothesis was to remain valid. The observations of Callender and Lajtha were surprising in view of the clinical effectiveness of parenteral vitamin B\textsubscript{12} in curing pernicious anemia, and the lack of need for any intrinsic factor supplement at all if large enough doses of vitamin B\textsubscript{12} are administered by mouth,\textsuperscript{15} or are inhaled as snuff.\textsuperscript{16} Wallerstein et al.\textsuperscript{17} failed to demonstrate any enhancement of the effect of suboptimal intravenous doses of vitamin B\textsubscript{12} by the addition to them prior to injection, of normal gastric juice.

Furthermore, the concept proposed by Ungley\textsuperscript{18} that the role of vitamin B\textsubscript{12} in pernicious anemia was to neutralize an antagonist of folic acid (the inhibitor) to perform which act the B\textsubscript{12} had to be activated by an "intrinsic factor-like substance" seemed to overlook a mass of biochemical evidence linking vitamin B\textsubscript{12} to methylation reactions involved in the synthesis of deoxyribose nucleic acid,\textsuperscript{19} and would necessitate the supposition that in pernicious anemia, vitamin B\textsubscript{12} had an additional physiologic role peculiar to that disease.

It appeared likely that some of the conflicting reports at least, might have been due to differences in the techniques of marrow culture employed, and that others might be resolved in differences in interpretation of results. In any event further study of the whole problem was clearly indicated and the present investigations were begun in an effort to study the possibilities and limitations of various methods of marrow culture in their application to the megaloblast-normoblast problem.

Preliminary studies with pernicious anemia marrow cultured on clots or glass surfaces indicated that the marrow grew well in normal serum and less well in pernicious anemia serum unless vitamin B\textsubscript{12} was added. In the double cover slip preparations differential counts on the cells adjacent to the explant (figs. 1 and 2), indicated a larger number of maturing cells in the B\textsubscript{12} containing preparations, but it was impossible to distinguish megaloblasts from normoblasts by the criteria selected, in the alcohol fixed smears.

On glass surfaces the marrow particles remained alive and resembled good aspirates of marrow which stained well enough with Romanowsky stains to distinguish megaloblasts and normoblasts from each other. In these preparations (fig. 3) the addition of vitamin B\textsubscript{12} brought about normoblastic hematopoiesis. The similarity between the appearance of these preparations and those shown by Horrigan and Vilter to illustrate their in vivo observations was great.

It was obvious that while the presence or absence of vitamin B\textsubscript{12} in these cultures appeared to be the deciding factor influencing growth and maturation, it could not be excluded
that unknown factors present in the chick plasma or embryo extract might have enhanced its action, thus accounting for the discrepancy between these results and those reported by the observers in England. In order to answer this question it was necessary to repeat their work with suspension cultures, following their technic as closely as possible.*

In turning to the suspension technics a question that was foremost in our minds was to what extent cells would grow by this method. Most experts in tissue culture were agreed that in suspension cells grew very poorly and that they seemed to require a supporting structure and the proximity of other cells in order to multiply. More recent observations indicate that this is not so* with proper technical procedures, but the criticism is valid with respect to the suspension method of Osgood and Brownlee. The limitations of the suspension method in this respect were acknowledged by Lajtha, but, as he pointed out, they were offset by the fact that this was the only method that permitted a quantitative estimation of growth. Also, as had been stressed by Osgood and Brownlee, the method permitted the staining of the cells by the same techniques applicable to blood smears, and the identification of blood cells with ease and accuracy. After three or four days the cells in suspension culture show increasing signs of degeneration. This can be postponed to some extent by meticulous attention to sterile technique and by using only chemically clean glassware. Since, however, the conversion of megaloblasts to normoblasts takes place in vivo in less than twenty-four hours, the short life of suspension cultures need not limit their use in the study of this problem.

Of much greater consequence was the question of the amount of mitotic activity in such cultures, since we consider megaloblastosis as arising from a defect in mitosis. By arresting mitosis in metaphase with colchicine (experiments III and V) we were able to satisfy ourselves that some mitotic activity did take place in suspension, and that the increase in cells in megaloblastic marrows cultured with folinic acid, was due to increased cell division, since this increase was abolished by colchicine, in a concentration that was not, in itself, toxic for cells not dividing. We believe that the amount of mitosis that will occur in suspensions under conditions optimal for cell division is limited by the number of cells that enter the suspension in a "mitotable" state (i.e., cells whose nuclei have not yet begun to involute, and which are therefore capable of undergoing a normal mitotic division) although none of our experiments have directly proved this point. In experiment VI, folinic acid, which had been found to produce a significant increase of cells in cultures of megaloblastic marrows was found to have no discernible effect on normoblastic marrows. A possible explanation for this difference lies in the much larger number of "mitotable" cells in megaloblastic marrows. The progressive decrease in the numbers of "mitotable" cells from day to day in a suspension, offers no basis for supposing that there is a second cell generation of any magnitude able to undergo another division, in such cultures. This fact has direct bearing on the question of the formation of megaloblasts from normoblasts in suspension cultures since only "mitotable" cells could undergo the inhibition of mitosis that results in the formation of megaloblasts. It would follow that the formation of megaloblasts in suspension cultures could only be performed on normoblastic marrows with significant numbers of "mitotable" normoblasts present at the onset of the experiment.

A factor influencing the ability to detect the effects of metabolites on cell growth in suspension is the rate of cell maturation, which varies from one serum to the next. We were unable to detect any consistent differences in growth rate in autologous versus homologous serum (as has been claimed) and attribute any variations in rate of growth to individual variations in the concentration of undefined growth factors in the sera. Nevertheless, the rate of growth of cells in the control bottle has a serious effect on the ease of detection of differences in the experimental bottles. If growth is rapid in the control it may be impossible to detect stimulatory effects, while inhibitory effects will be rendered more apparent, and vice versa.

* One of us (I. T. S.) personally visited the laboratories of Dr. Lajtha and that of Dr. C. C. Ungley where Thompson had done his work. Both Dr. Lajtha and Dr. Ungley were most generous of their time and knowledge in helping us to attain our objective in this respect, and saved us much time and effort for which we are most grateful, and would here like to express our thanks.
Our observations (table 1) indicate a slight increase of total nucleated cells due to an increment of nucleated red cells in the bottles containing vitamin B$_{12}$ (alone or with intrinsic factor) which was not statistically significant (p only slightly less than .2) and a highly significant increase in the bottles containing folinic acid (p less than .05). The percentages of megaloblasts show the same general trends (fig. 4) but the error in the differential counts is too great to permit a valid statistical analysis. In neither the absolute cell counts nor the percentage counts of megaloblasts could any enhancing effect of intrinsic factor on B$_{12}$ be seen. In fact, if any trend is discernible it would appear that in a majority of the cultures there were more megaloblasts present after 48 hours in the presence of intrinsic factor alone or in combination with vitamin B$_{12}$, than with B$_{12}$ alone. Further evidence of the possible inhibitory effect of intrinsic factor is seen in the comparison of absolute white cell counts. The decrease in white cells seen with B$_{12}$ and intrinsic factor is statistically significant (p less than .05) but it is impossible to say whether this is due to less white cell formation or increased white cell destruction. It appears that the effect of intrinsic factor on vitamin B$_{12}$ in relation to the growth of blood cells in vitro is similar to that observed with bacteria, where the intrinsic factor substance binds the B$_{12}$ making it unavailable for bacterial growth.

The belief that the increase of cells observed with folinic acid was due to an effect on mitosis is supported by its inhibition with colchicine (experiment V) and the ability of folinic acid to reverse the inhibitory effects of a folic acid antagonist (experiment VII).

In contrast to the situation in the suspensions, marrow cells grown on clots or glass surfaces continue to multiply as long as the medium is replenished, although the cells rapidly lose the capacity to differentiate into the different lines of blood elements. In these cultures mitotic activity is extensive and comparable to that occurring in vivo, and this activity appeared to be stimulated by vitamin B$_{12}$ alone. When folinic acid was used in the chick plasma clot experiment it was found to stimulate growth but not as markedly as B$_{12}$.

It is difficult to reconcile this seeming paradoxical behavior of vitamin B$_{12}$ and folinic acid. The close interrelationship between the two substances in many different chemical reactions involving the transfer of single carbon units is well established. Since the requirements in pernicious anemia for folic acid and folinic acid on a weight basis, are about a thousand times as great as those for vitamin B$_{12}$, it has been suggested by some that their relationship may be that of substrate to enzyme or catalyst. It has been shown that folinic acid can carry formate in a labile form to supply carbon atoms, and it may be that the transfer of these atoms is catalyzed by B$_{12}$. Whatever the exact mechanism, for the reaction to proceed both substances must be present in a minimal concentration. If this is so it appears possible that the amounts of B$_{12}$ present in relapsed pernicious anemia serum are inadequate to meet the requirements for nucleoprotein synthesis in actively dividing tissue in vivo or as it exists in cultures on clots or glass surfaces, and additional B$_{12}$ is necessary to stimulate growth. In the suspension, however, the limited amount of cell division taking place can be adequately supplied by even the minute amounts of vitamin B$_{12}$ present in relapsed pernicious anemia serum. In such a case additional B$_{12}$ would have very little enhancing effect and the limiting factor would become the availability of folinic acid, which would be stimulatory under these circumstances.

An alternate explanation might be based on the hypothesis that vitamin B$_{12}$ plays some role in the conversion of folic to folinic acid. There is equally convincing evidence both for...
and against such a concept. The results in the suspension experiments could then be explained on the basis that in the absence of adequate $\text{B}_{12}$ there was inadequate folic acid available for nucleoprotein synthesis. This deficiency could be directly corrected by the addition of folic acid, but the effect of added $\text{B}_{12}$ would be determined by the availability of the precursor substance, folic acid, on which the $\text{B}_{12}$ might work. With this hypothesis one would have to assume that in the chick plasma clots and on the glass cover slips the accompanying media contained ample supplies of folic and folic acids, to permit a full $\text{B}_{12}$ effect to become apparent. It would be helpful to know just what the $\text{B}_{12}$, folic acid and folic acid content was of each constituent of every culture medium employed, a procedure however, fraught with obvious practical difficulties in attainment.

A final word should be said about the conversion of normoblasts to megaloblasts in suspension cultures. The pathogenesis of megaloblasts has been discussed by one of us in another paper.24 These cells are considered as arising wherever there is a prolongation of interphase between mitoses which allows a more thorough dispersion of the nuclear chromatin into smaller particles producing the characteristic appearance of the “mitotable” megaloblast. This cell, if it matures without undergoing mitosis develops into a hypochromatic and then an orthochromatic megaloblast and eventually into the oval macrocyte of the peripheral blood in pernicious anemia. The condition which most commonly brings about this sequence of events is an absence of the enzymes essential for rapid nucleoprotein synthesis, e.g., folic acid (or folic acid) and vitamin $\text{B}_{12}$. Any “mitotable” normoblast entering an environment deficient in either one of these substances, or containing any factor interfering with their action such as a folic acid antagonist, might be expected to undergo a prolonged interphase leading to amitotic maturation, and develop megaloblastic characteristics. We observed this to occur in pernicious anemia serum in various concentrations, and in normal serum in the presence of Amethopterin. The numbers of such cells produced in any suspension was small and was probably limited by the numbers of “mitotable” normoblasts in the original marrows. As can be seen from table 3, the number of megaloblasts produced was also affected by the concentration of the serum, in which dilution increased the number of megaloblasts. That this was due to dilution of available $\text{B}_{12}$ was suggested by the fact that addition of the vitamin appeared to prevent the appearance of as many megaloblasts until a lower concentration of serum was reached. These experiments do not provide any support for the hypothesis of an “inhibitor” substance in pernicious anemia serum that can be diluted out.

**Summary**

Marrow was cultured on chick plasma clots, glass surfaces and in suspension and the influence of various metabolites and antimetabolites on the growth and differentiation of red cell precursors was studied.

Vitamin $\text{B}_{12}$ appeared to stimulate cell growth in cultures grown on clots and to convert megaloblastic hematopoiesis to normoblastic in cultures grown on glass.

In suspension cultures of megaloblastic marrows in pernicious anemia serum significant cell increases were obtained with the addition of folic acid, but not with vitamin $\text{B}_{12}$. Both vitamin $\text{B}_{12}$ and folic acid appeared to accelerate the conversion of megaloblasts to normoblasts in differential counts of stained
EFFECT OF METABOLITES ON GROWTH OF MEGALOBLASTS

Smears of such cultures. No enhancing effect of intrinsic factor upon the activity of $B_{12}$ in suspension cultures of megaloblasts was observed. The effect of folinic acid could be blocked by colchicine and folinic acid could reverse the inhibitory effects of Amethopterin. These facts indicated that folinic acid acted in suspension cultures by stimulating mitosis.

Megaloblasts could be produced from normoblastic marrows by culturing them in pernicious anemia serum, or in normal serum in the presence of Amethopterin.

**SUMMARIO IN INTERLINGUA**

Specimens de medulla human esseva culturate (1) super coagulos de plasma ab gallinas embryonic, (2) super superficies vitrose, e (3) in suspension, e le influentia de varie metabolitos e antimetabolitos super le crescentia e differentiation del precursores de erythrocytos in le medulla esseva studiate.

Vitamina $B_{12}$ pareva stimular le crescentia cellular in culturas super coagulos; in culturas super vitro illo pareva transformar le hematopoiese megaloblastic in un hematopoiese normoblastic.

In culturas suspensional de medulla megaloblastic in sero a anemia perniciose, le addition de acido folinic—sed non le addition de vitamina $B_{12}$—resultava in significative augmentos cellular. Ambe ille agentes—i.e. acido folinic e vitamina $B_{12}$—pareva accelerar le conversion de megaloblastos in normoblastos secundo contos differential de tincturate frottis de tal culturas. Nulle effecto promovente del factor intrinsec super le activitate de $B_{12}$ in culturas suspensional de megaloblastos esseva observate. Le effecto de acido folinic poteva esser blocate per colchicina, e acido folinic se monstrava capace a reverter le effecto inhibitori de Amethopterina. Iste factos indicava que acido folinic ageva in culturas suspensional per exercer un influentia stimulante super le processo mitotic.

Il esseva possibile producer megaloblastos ab medullas normoblastic per culturar iste medullas in sero a anemia perniciose o in sero normal in le presentia de Amethopterina.

**APPENDIX**

*Statistical Analysis of Data From Two Sets of Experimental Data*

1. Total Nucleated Red Cells/cu.mm. in Suspension Culture Minus Initial Count, 48-Hour Cultures.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Control</th>
<th>$B_{12}$</th>
<th>I.F.</th>
<th>$B_{12}$ &amp; I.F.</th>
<th>Folinic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>567</td>
<td>369</td>
<td>762</td>
<td>452</td>
<td>354</td>
<td>2504</td>
</tr>
<tr>
<td>18A</td>
<td>126</td>
<td>-226</td>
<td>-132</td>
<td>138</td>
<td>1086</td>
<td>992</td>
</tr>
<tr>
<td>26</td>
<td>352</td>
<td>941</td>
<td>721</td>
<td>681</td>
<td>1157</td>
<td>3852</td>
</tr>
<tr>
<td>27</td>
<td>265</td>
<td>548</td>
<td>187</td>
<td>387</td>
<td>1193</td>
<td>2580</td>
</tr>
<tr>
<td>28A</td>
<td>-172</td>
<td>395</td>
<td>-81</td>
<td>-154</td>
<td>247</td>
<td>235</td>
</tr>
<tr>
<td>30</td>
<td>710</td>
<td>1339</td>
<td>239</td>
<td>1438</td>
<td>2128</td>
<td>5854</td>
</tr>
</tbody>
</table>

Total: 1848 | 3366 | 1696 | 2942 | 6165 | 16017
Mean: 308.0 | 561.0 | 282.7 | 490.3 | 1027.5 | 533.9
**Analysis of Variance**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of Freedom</th>
<th>Mean Square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments</td>
<td>4,063,108.70</td>
<td>5</td>
<td>812,621.74</td>
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<tr>
<td>Treatments</td>
<td>2,162,534.53</td>
<td>4</td>
<td>540,633.63</td>
</tr>
<tr>
<td>Treatments vs. control</td>
<td>382,731.07</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4 treatments</td>
<td>1,779,803.46</td>
<td>3</td>
<td>593,267.82</td>
</tr>
<tr>
<td>Interaction</td>
<td>2,411,027.47</td>
<td>20</td>
<td>120,551.37</td>
</tr>
<tr>
<td>Treatments vs. control</td>
<td>252,245.68</td>
<td>5</td>
<td>50,449.14</td>
</tr>
<tr>
<td>4 treatments</td>
<td>2,158,781.79</td>
<td>15</td>
<td>143,918.79</td>
</tr>
<tr>
<td>Total</td>
<td>8,666,707.70</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>

\[ F_{\text{Exp.}} = \frac{812,731.07}{120,551.37} = 6.74 \quad \text{p less than .001} \]

\[ F_{\text{Treat. vs. control}} = \frac{382,731.07}{50,449.14} = 7.50 \quad \text{p less than .05} \]

\[ F_{\text{Treat.}} = \frac{593,267.82}{143,918.79} = 4.12 \quad \text{p less than .05} \]

2. Total Nucleated White Cells/cu.mm. in Suspension Culture Minus Initial Count. 48-Hour Cultures.

Data from ten experiments chosen in chronologic order.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Control</th>
<th>B₁₁</th>
<th>I.F.</th>
<th>B₁₁ &amp; I.F.</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>-172</td>
<td>306</td>
<td>-392</td>
<td>-362</td>
<td>-620</td>
</tr>
<tr>
<td>18A</td>
<td>-1096</td>
<td>-474</td>
<td>-1028</td>
<td>-1208</td>
<td>-3806</td>
</tr>
<tr>
<td>26</td>
<td>-282</td>
<td>-631</td>
<td>-171</td>
<td>-581</td>
<td>-1665</td>
</tr>
<tr>
<td>27</td>
<td>-1305</td>
<td>-908</td>
<td>-807</td>
<td>-1007</td>
<td>-4117</td>
</tr>
<tr>
<td>28A</td>
<td>-228</td>
<td>-505</td>
<td>-1199</td>
<td>-1116</td>
<td>-3048</td>
</tr>
<tr>
<td>30</td>
<td>-490</td>
<td>-500</td>
<td>131</td>
<td>-538</td>
<td>-1406</td>
</tr>
<tr>
<td>10</td>
<td>688</td>
<td>-32</td>
<td>11</td>
<td>0</td>
<td>667</td>
</tr>
<tr>
<td>24</td>
<td>-487</td>
<td>-443</td>
<td>-468</td>
<td>-816</td>
<td>-2214</td>
</tr>
<tr>
<td>25</td>
<td>426</td>
<td>388</td>
<td>409</td>
<td>907</td>
<td>2130</td>
</tr>
<tr>
<td>32</td>
<td>304</td>
<td>162</td>
<td>-594</td>
<td>-683</td>
<td>-811</td>
</tr>
</tbody>
</table>

Total................. | -2642   | -264.6| -4198| -5404      | -14800|
Mean.................... | -264.2  | -264.6| -419.8| -540.4     | -372.2|

**Analysis of Variance**

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Sum of squares</th>
<th>Degrees of freedom</th>
<th>Mean square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments</td>
<td>8,557,296.50</td>
<td>9</td>
<td>950,810.72</td>
</tr>
<tr>
<td>Treatments</td>
<td>537,987.50</td>
<td>3</td>
<td>179,329.17</td>
</tr>
<tr>
<td>B₁₁ vs. No B₁₁</td>
<td>36,602.50</td>
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</tr>
<tr>
<td>I.F. vs. No I.F.</td>
<td>465,264.90</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td>36,120.10</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Interaction (error)</td>
<td>2,750,989.50</td>
<td>27</td>
<td>101,888.50</td>
</tr>
<tr>
<td>Total</td>
<td>11,846,273.50</td>
<td>39</td>
<td></td>
</tr>
</tbody>
</table>

\[ F_{\text{Exp.}} = \frac{950,810.78}{101,888.48} = 9.83 \quad \text{p less than .01} \]

\[ F_{\text{I.F. vs. No I.F.}} = \frac{465,264.9}{101,888.48} = 4.57 \quad \text{p less than .05} \]
REFERENCES


The Effect of Various Metabolites on the Growth of Marrow Cells in Vitro

HAROLD T. SWAN, EDWARD H. REISNER, JR. and MORRIS SILVERMAN

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