# EFFECT OF VITAMIN B<sub>12</sub> IN VITRO ON INCORPORATION OF NUCLEIC ACID PRECURSORS BY PERNICIOUS ANEMIA BONE MARROW\*

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Glaser and associates (1) reported that the ratios RNA to DNA and uracil to thymine were about 2.5 times higher in bone marrow from pernicious anemia (PA) patients before treatment than in bone marrow from normal subjects. Clinical cures caused these ratios to return to normal. Beck (2) has reviewed the evidence that the major consequence of vitamin B<sub>12</sub> deficiency is impaired deoxyribonucleotide synthesis, resulting in retardation of DNA synthesis and cell division. He suggests that the megaloblast of vitamin B<sub>12</sub> deficiency is in a state of "unbalanced growth" that is analogous to the filamentous forms of Lactobacillus leichmannii produced by vitamin B<sub>10</sub> or deoxyribonucleoside starvation. However, observations of the effect of vitamin B<sub>12</sub> on morphology of megaloblasts in suspension cultures have been contradictory. At least five studies reported no effect, whereas two investigators found some replacement by normoblastic cells (3).

Two investigations have tested the effect of vitamin  $B_{12}$  in vitro on DNA synthesis by PA bone marrow. Thomas and Lochte (4) measured the incorporation of radioactive formate by PA marrows incubated for 5 and 10 hours in PA serum with and without added  $B_{12}$ . Specific activities of DNA thymine were 1.3 to 2.7 times higher with  $B_{12}$ . Lessner and Friedkin (5), on the contrary, were unable to demonstrate a reproducible effect of  $B_{12}$  on incorporation of deoxyuridine-2- $C^{14}$  by washed cell suspensions from  $B_{12}$ -deficient megaloblastic marrows incubated for 3 hours.

The availability of a number of patients with untreated pernicious anemia prompted us to extend these studies to the use of radioactive precursors of both DNA and RNA and of both pyrimidines and purines. We thus measured the effect of vitamin  $B_{12}$  on incorporation of cytidine-2- $C^{14}$  and adenine-8- $C^{14}$  by PA bone marrow. To evaluate the specificity of any stimulation or inhibition, we also measured the effect of  $B_{12}$  on incorporation by nonmegaloblastic, hyperplastic erythroid marrows from hemolytic anemia.

#### METHODS

Seven to 10 ml heparinized bone marrow were removed by sternal aspiration from patients with untreated pernicious anemia or with hemolytic anemia (HA). Marrow from PA Patients 1 to 3 was passed through a wire screen attached to a syringe. This procedure was discontinued because of loss of marrow particles during manipulation. It was found that the marrow could be dispersed with a glass rod after delivery into a flask. Enough Robinson's glucose-salts medium (6) was added to give a total of 12 ml (or 18 ml with PA Patient 5). Samples of 3.0 ml were pipetted into 20-ml Warburg vessels containing 0.1 ml radioactive precursor and 0.05 ml vitamin B<sub>12</sub> (hydroxycobalamin)<sup>1</sup> or saline. Samples in the various experiments contained 1 to  $3 \times 10^8$  nucleated cells. The vessels were shaken for 2 hours at 38° C under an atmosphere of 95% air:5% carbon dioxide. They were then removed to an ice bath, and subsequent operations were carried out at 3° C. Contents of the vessels were centrifuged 10 minutes at  $1,470 \times g$ , and the supernatant fluid was discarded. The cells were washed with 10 vol of cold nonradioactive Robinson's medium, once in cytidine incorporations, and twice in adenine incorporations where cellular acid-soluble adenine was to be isolated. The washes were discarded and the centrifuged cells were extracted with three 3.0-ml portions of 0.2 M perchloric acid.

Purine and pyrimidine bases were isolated, purified, and measured for radioactivity as previously described (6). Differences in the present study were as follows. After separation of RNA and DNA fractions from cytidine incorporations, the RNA supernatant fluid was taken to dryness in a vacuum desiccator and hydrolyzed with 70% perchloric acid as described for the DNA precipitate. After hydrolysis, perchlorate was removed by

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<sup>&</sup>lt;sup>1</sup> Merck & Co., Inc., Rahway, N. J.

TABLE I

Effect of vitamin B<sub>12</sub> on 2-hour incorporations of adenine and cytidine by megaloblastic bone marrow from untreated pernicious anemia patients

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Adenine-8-C <sup>14</sup> , 1.0 µmole 1,350,000 cpm/µmole	DNA adenine	DNA guanine	Cell acid-soluble adenine	RNA adenine	RNA guanine
Patient 1				·-	
SA without $B_{12}^*$ SA with $B_{12}$ , 5 $m\mu g$ $B_{12}$	$3,172 \pm 115 \dagger 5,581 \pm 136$	$511 \pm 40 \\ 853 \pm 28$	$226,700 \pm 1,600$ $235,200 \pm 4,900$	$34,660 \pm 1,340$ $38,160 \pm 980$	$2,891 \pm 54 \\ 3,083 \pm 18$
$\overline{\text{No B}_{12}}$	1.76	1.67	1.04	1.10	1.07
Patient 2					
SA without $B_{12}$ SA with $B_{12}$ , 5 $m\mu g$ $B_{12}$	$4,296 \pm 124$ $5,298 \pm 10$	$528 \pm 32 \\ 564 \pm 46$	$213,700 \pm 7,000$ $217,700 \pm 8,700$	$37,330 \pm 290$ $37,960 \pm 380$	$3,224 \pm 84 \\ 3,111 \pm 95$
$\frac{B_{12}}{\text{No B}_{12}}$	1.23	1.07	1.02	1.02	.96
Patient 3					
SA without $B_{12}$ SA with $B_{12}$ , 5 $m\mu g$	$1,222 \pm 120$ $1,565 \pm 87$		$102,400 \pm 2,500$ $113,100 \pm 5,100$	$18,290 \pm 490$ $18,250 \pm 1,170$	
$\frac{\mathrm{B_{12}}}{\mathrm{No}\;\mathrm{B_{12}}}$	1.28		1.10	1.00	
Cytidine-2-C <sup>14</sup> , 1.0 µmole 662,000 cpm/µmole	DNA thymine	DNA cytosine		RNA uracil	RNA cytosine
Patient 4					
SA without $B_{12}$ SA with $B_{12}$ , 5 $m\mu g$ $B_{12}$	$1,007 \pm 29$ $1,358 \pm 27$	$2,169 \pm 36$ $2,516 \pm 82$		$19,180 \pm 140$ $19,440 \pm 160$	$9,378 \pm 187 \\ 8,867 \pm 170$
$\frac{B_{12}}{\text{No B}_{12}}$	1.35	1.16		1.01	.95
Patient 5					
SA without $B_{12}$ SA with $B_{12}$ , 5 $m\mu g$	$1,558 \pm 33$ $1,946 \pm 59$	$2,860 \pm 10$ $3,184 \pm 125$		$21,940 \pm 120$ $21,140 \pm 690$	$9,137 \pm 113$ $9,626 \pm 57$
$\frac{\mathrm{B_{12}}}{\mathrm{No}\;\mathrm{B_{12}}}$	1.25	1.11		.96	1.05
SA with $B_{12}$ , 50 $m\mu g$ $B_{12}$	$2,256 \pm 10$	$3,488 \pm 19$		$21,540 \pm 450$	$8,640 \pm 99$
$\frac{B_{12}}{\text{No }B_{12}}$	1.45	1.22		.98	.95

<sup>\*</sup> Specific activities are given in counts per minute per micromole.

precipitation with KOH as described. The supernatant fluid was then chromatographed on paper without the extra step of column chromatography.

In experiments employing washed marrow cells, the procedure differed as follows. Aspirated marrow was centrifuged 5 minutes at  $1,470\times g$ , and the plasma was discarded. The cells were stirred gently in 5 vol of Robinson's medium, recentrifuged, and suspended in a fresh portion of the medium. Three-ml portions of this suspension were used in the incubation.

# RESULTS

The data of Table I show that  $5 \text{ m}\mu\text{g B}_{12}$  caused a small but consistent increase in the incorporation of both purine and pyrimidine precursors into DNA of PA marrows. Incorporation of cytidine

into DNA of marrow 5 was higher with 50 mµg  $B_{12}$  than with 5 mµg. Only with marrow 1, where  $B_{12}$  caused the greatest stimulation of incorporation into DNA (1.7 to 1.8 times), was there any increased labeling of RNA (1.1 times). Table II presents data for incorporation of cytidine by three hemolytic anemia marrows. Addition of 5 mµg  $B_{12}$  to these marrows caused no stimulation of incorporation into either RNA or DNA; the largest increase, with DNA thymine of HA marrow 1, was only 1.07 times. With no added  $B_{12}$  (control vessels), the two PA marrows showed a higher incorporation of cytidine into RNA than did the three HA marrows, whereas incorporation into DNA by the two marrow types (incu-

<sup>†</sup> Variation from the mean of duplicate flasks.

TABLE II Effect of vitamin B<sub>12</sub> on 2-hour incorporation of cytidine by hemolytic anemia bone marrow

Cytidine-2-C <sup>14</sup> , 1.0 µmole 662,000 cpm/µmole	DNA thymine	DNA cytosine	RNA uracil	RNA cytosine
Patient 1				
SA without $B_{12}^*$ SA with $B_{12}$ , 5 $m\mu g$ $B_{12}$	$1,093 \pm 73 \dagger 1,169 \pm 51$	$2,013 \pm 13$ $1,935 \pm 20$	$10,740 \pm 401$ $10,870 \pm 200$	$6,410 \pm 529$ $6,491 \pm 44$
No B <sub>12</sub>	1.07	.96	1.01	1.01
Patient 2				
SA without $B_{12}$ SA with $B_{12}$ , 5 $m\mu g$ $B_{12}$	$1,377 \pm 67$ $1,406 \pm 43$	$2,490 \pm 65$ $2,500 \pm 102$	$9,116 \pm 647$ $9,319 \pm 328$	$6,990 \pm 128$ $6,187$
$\frac{S_{12}}{\text{No B}_{12}}$	1.02	1.00	1.02	.89
Patient 3				
SA without $B_{12}$ SA with $B_{12}$ , 5 $m\mu g$ $B_{12}$	$1,321 \pm 58$ $1,279 \pm 28$	$2,781 \pm 30$ $2,873 \pm 28$	$9,249 \pm 29$ $9,421 \pm 19$	$8,489 \pm 354$ $8,691 \pm 243$
$\frac{B_{12}}{\text{No B}_{12}}$	.97	1.03	1.02	1.02

<sup>\*</sup> Specific activities are given in counts per minute per micromole. † Variation from the mean of duplicate flasks.

TABLE III Effect of vitamin  $B_{12}$  on 2-hour incorporations of cytidine by washed suspensions of hemolytic anemia (HA) and pernicious anemia (PA) bone marrow

Cytidine-2-C <sup>14</sup> , 1.0 µmole 662,000 cpm/µmole	DNA thymine	DNA cytosine	RNA uracil	RNA cytosine
Suspended in glucose-salt	s medium			
HA Patient 1				
SA without $B_{12}^*$ SA with $B_{12}$ , 5 $m\mu g$ $B_{12}$	$454 \pm 28 \dagger \\ 452 \pm 14$	$\begin{array}{ccc} 851 \pm & 36 \\ 817 \pm & 46 \end{array}$		
$\frac{B_{12}}{\text{No B}_{12}}$	1.00	.96		
HA Patient 4				
SA without $B_{12}$ SA with $B_{12}$ , 5 $m\mu g$ $B_{12}$	$726 \pm 36$ $714 \pm 63$	$1,128 \pm 16$ $1,054 \pm 21$	$7,835 \pm 304$ $8,020 \pm 201$	$4,510 \pm 65  4,573 \pm 101$
$\frac{B_{12}}{\text{No }B_{12}}$	.98	.93	1.02	1.01
PA Patient 6				
SA without $B_{12}$ SA with $B_{12}$ , $\ddagger 5 m\mu g$ $B_{12}$	$1,506 \pm 84$ $2,172$	$3,094 \pm 153$ $3,570$	$14,450 \pm 580$ $13,540$	$7,607 \pm 463$ $7,337$
$\frac{B_{12}}{\text{No B}_{12}}$	1.44	1.15	.94	.96
Suspended in PA plasma	6			
HA Patient 4				
SA without $B_{12}$ SA with $B_{12}$ , 5 $m\mu g$ $B_{12}$	$\begin{array}{c} 628 \pm 12 \\ 645 \pm 6 \end{array}$	$1,024 \pm 20$ $1,063 \pm 30$	$\begin{array}{c} 10,060 \pm 400 \\ 10,170 \pm 50 \end{array}$	$4,933 \pm 84$ $5,198 \pm 396$
$\frac{B_{12}}{\text{No B}_{12}}$	1.03	1.04	1.01	1.05

<sup>\*</sup> Specific activities are given in counts per minute per micromole. † Variation from the mean of duplicate flasks. ‡ Only one flask containing  $B_{12}$  with this marrow.

bated under the same conditions) was of similar magnitude.

The data of Table II demonstrated that added B<sub>12</sub> did not stimulate incorporation by HA marrow suspended in its own plasma diluted 1-1.2 to 1-1.7 with glucose-salts medium. The amount of  $B_{12}$  added, 1.7 mµg per ml, was well above the level of 0.15 to 0.9 mµg per ml in normal and HA plasma (7). However, PA plasma would contain less than 0.1 mug per ml. Thus, the possibility remained that the extra amount of B<sub>12</sub> in the HA plasma was sufficient to give a maximal effect in vitro, and that the stimulatory effect was not specific for the B<sub>12</sub>-deficient megaloblasts. Another sample of marrow from HA Patient 1 and a sample from a fourth HA patient were treated as follows. Cells were centrifuged, washed with 5 vol of glucose-salts medium, and resuspended in a fresh portion of the medium. procedure would serve to remove intercellular B<sub>12</sub> but not protein-bound intracellular B<sub>12</sub>. shown in Table III, 5 m $\mu$ g B $_{12}$  did not stimulate incorporation by these washed HA marrows.

Lear, Harris, Castle, and Fleming (8) have shown that both PA and normal sera have a factor or factors which bind some of the B<sub>12</sub> added in vitro. In their experiments, 1 ml of serum bound .45 to .66 m $\mu$ g B<sub>12</sub>. To determine if these or other plasma factors were necessary to demonstrate a stimulatory effect of B<sub>12</sub> on PA marrows, the marrow from a sixth PA patient was washed and suspended in glucose-salts medium as described above. With 5 mµg B<sub>12</sub>, incorporation of cytidine into DNA thymine was increased 1.4 times (Table III). This experiment, however, was not entirely satisfactory, since one of the duplicate samples with B<sub>12</sub> was destroyed during a laboratory procedure. Results thus represent two control flasks and one flask with B<sub>12</sub>.

To demonstrate in another way that the failure of  $B_{12}$  to stimulate incorporation by HA cells suspended in glucose-salts medium was not owing to lack of plasma factors, another marrow sample from HA Patient 4 was washed in glucose-salts medium and then suspended in plasma from the marrow aspirate of PA Patient 6. However, 5 mµg vitamin  $B_{12}$  still did not stimulate incorporation by these HA marrow cells (Table III).

#### DISCUSSION

In seven tests with PA marrows incubated in PA serum for 5 and 10 hours, Thomas and Lochte reported that 10 and 100 m $\mu$ g B<sub>12</sub> per ml stimulated the incorporation of formate into DNA 1.3 to 2.7 times. However, in one experiment these workers obtained the same stimulation (1.4 times) with 1, 10, and 100 m $\mu$ g B<sub>12</sub> per ml. Moreover, they reported that the addition of B<sub>12</sub> to PA marrow incubated in normal serum produced no significant increase in DNA synthesis. These data indicated that the level of B<sub>12</sub> in normal sera might exert a maximal effect.

The amount of B<sub>12</sub> added in our experiments, 1.7 mµg per ml, is about twice the highest level to be expected in plasma from normal and HA subjects. With six PA marrows (five suspended in their own plasma and one washed and suspended in glucose-salts medium), incorporation of adenine and cytidine into DNA adenine and thymine, respectively, was increased 1.2 to 1.8 times by this low level of added vitamin. Thus, we have confirmed the stimulation of incorporation into DNA with B<sub>12</sub> reported by the above workers and have shown that this effect occurs with both pyrimidine and purine precursors. Moreover, the present work has demonstrated that incorporation into RNA is not stimulated, at least when measured after the 2-hour incubation period. With the one marrow tested, 17 m $\mu$ g B<sub>12</sub> per ml caused a greater stimulation of incorporation into DNA than did 1.7 mµg per ml, but yet no increased labeling of RNA. We have not attempted to test the hypothesis that vitamin B<sub>12</sub> participates in the conversion of ribonucleosides (or ribonucleotides) to deoxyribonucleosides (or deoxyribonucleotides). Adenine and cytidine were chosen as radioactive precursors because they were readily incorporated into both RNA and DNA of bone marrow incubated under conditions similar to those used in our studies with human white blood cells (6, 9).

Barker (10) has reviewed evidence that cobamide coenzymes are the active forms of vitamin  $B_{12}$  in three enzymatic reactions occurring in some microorganisms. One of these, the methylmalonyl isomerase reaction, also occurs in animals. Either a coenzyme form of  $B_{12}$  is not required to stimulate *in vitro* DNA synthesis by megaloblasts, or these cells can convert hydroxycobalamin to coenzyme  $B_{12}$ . Evidence of the local action of  $B_{12}$  in vivo has been given by Horrigan, Jarrold, and Vilter (11), who demonstrated that direct instillation of cyanocobalamin into the marrow cavity of one iliac crest induced local transformation from megaloblastic to normoblastic proliferation without causing changes in the opposite iliac crest.

Added B<sub>12</sub> did not stimulate incorporation by washed HA cells suspended in a glucose-salts medium or in PA plasma. Evidently, the intracellular protein-bound B<sub>12</sub> in these nonmegaloblastic cells is sufficient for maximal incorporation under our *in vitro* conditions.

The stimulation by B<sub>12</sub> of incorporation into DNA but not into RNA of PA marrows would be in agreement with the clinical findings that the abnormally high RNA to DNA ratio in megaloblasts returns to normal after successful treatment with this vitamin. When B<sub>12</sub>-requiring L. leichmannii is grown in the absence of the vitamin, DNA synthesis and cell division are impaired without concomitant inhibition of RNA and protein synthesis (12, 13). The resulting cytoplasmic growth produces filamentous organisms with an elevated RNA to DNA ratio. It is likely that the increased incorporation into DNA of PA marrows when B<sub>12</sub> is added is related to a similar state of unbalanced growth in the megaloblast.

# SUMMARY

Five pernicious anemia bone marrows were incubated for 2 hours in their own plasma diluted with a glucose-salts medium, and one in glucose-salts medium alone. Added vitamin B<sub>12</sub> (hydroxycobalamin), 1.7 mµg per ml, stimulated the incorporation of adenine-8-C<sup>14</sup> and cytidine-2-C<sup>14</sup> into DNA bases but not into RNA bases. Addition of this level of B<sub>12</sub> to hemolytic anemia marrows, incubated in their own plasma, pernicious anemia plasma, or glucose-salts medium did not stimulate incorporation of cytidine-2-C<sup>14</sup> into either RNA or DNA.

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