Synthesis of Cobalamin Coenzymes by Human Lymphocytes In Vitro and the Effect of Folates and Metabolic Inhibitors

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The uptake of ^{57}Co-cyanocobalamin (CN-Cbl) and its conversion to 5-deoxyadenosylcobalamin (Ado-Cbl), methylcobalamin (Me-Cbl), and hydroxocobalamin (OH-Cbl) has been studied in phytohemagglutinin (PHA)-transformed lymphocytes from normal subjects and patients with pernicious anemia. Uptake and conversion were much greater by PHA-stimulated lymphocytes than by mature non-transformed lymphocytes. In normal cells, uptake of ^{57}Co-CN-Cbl and synthesis of the cobalamin coenzymes were approximately linear between 3 and 48 hr incubation. Ado-Cbl was the major cobalamin formed, and after 72 hr the cells contained about twice as much Ado-Cbl as Me-Cbl. Uptake by lymphocytes from patients with untreated pernicious anemia (PA) was greater than that by normal lymphocytes, but the proportions of Ado-Cbl and Me-Cbl synthesized by each were similar. Folic acid and methyltetrahydrofolate enhanced synthesis of Me-Cbl both in normal and in PA cells, while methotrexate and 5-fluorouracil depressed it. This depression was overcome by 5-formyltetrahydrofolate, suggesting that an uninterrupted folate cycle may play an important role in Me-Cbl synthesis.

MAMMALIAN CELLS already used in studies of vitamin B_{12} metabolism include HeLa cells, Ehrlich ascites carcinoma cells, kidney cells, skin fibroblasts, and various hemopoietic cells, and all have been shown to take up cobalamins in vitro. The immature dividing cells of bone marrow take up cyanocobalamin (CN-Cbl) by an active, calcium-dependent process, requiring cellular respiration, oxidative phosphorylation, and the presence of free sulfhydryl groups,[1] whereas in erythrocytes and reticulocytes uptake is mainly by energy-independent adsorption at the cell surface.[2] In lymphocytes, uptake of CN-Cbl is greater in phytohemagglutinin (PHA)-transformed cells than in mature, nontransformed cells.[3] Lymphocytes stimulated with PHA show increases in RNA, DNA, and protein synthesis[4–7] which precede blastogenesis and mitosis.[8,9] These changes occur in lymphocytes from normal subjects and also in lymphocytes from patients with megaloblastic anemia due to B_{12} or folate deficiency, producing cells which are very similar to megaloblasts. This finding suggests that PHA-transformed lymphocytes provide an excellent model for studies of cobalamin metabolism in proliferating human cells.

Synthesis of cobalamin coenzymes has been studied in HeLa cells[10] and in human fibroblasts,[11,28] but not, so far as we are aware, in any hemopoietic cell in vitro. We have estimated the uptake of ^{57}Co-CN-Cbl and measured the

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cellular synthesis of ^{57}Co-Me-Cbl and ^{57}Co-Ado-Cbl in PHA-transformed lymphocytes from healthy normal subjects and patients with pernicious anemia. Since cobalamins have been implicated in both folate metabolism and DNA synthesis, we have also studied the effects of various folates, antifolate compounds, and other metabolic inhibitors on the synthesis of the cobalamin coenzymes. An abstract of part of these studies has been published.[12]

MATERIALS AND METHODS

Heparinized venous blood (50–60 ml) was taken from healthy adult volunteers and from patients in whom a diagnosis of untreated pernicious anemia (PA) had been established on the basis of megaloblastic anemia, low serum vitamin B_{12} levels, normal serum folate levels, and malabsorption of radioactive B_{12} corrected by intrinsic factor. Lymphocytes were separated on a Triosil–Ficoll gradient[13] and cultures set up as previously described.[14] The lymphocytes were suspended in TC 199 medium (Wellcome) containing 200 μl homologous plasma and 11 μl phytohemagglutinin (PHA-Wellcome) per 1×10^6 cells, in a total volume of 1 ml. Three milliliters of the cell suspension was dispensed into a sterile 5 ml Bijou bottle. ^{57}Co-CN-Cbl (specific activity 100–150 μCi/μg) was diluted to 20 ng/ml, and 100 μl added per 3×10^6 cells. Cells were then incubated in darkness for periods between $\frac{1}{2}$ and 72 hr at 37°C.

^{57}Co-CN-Cbl and folate analogues or metabolic inhibitors were added to further aliquots of cells and incubated in darkness for 72 hr at 37°C. Concentrations were as follows: pteroylglutamic acid (Sigma, PteGlu); 10^{-5} *M*; DL-N^{5}methyltetrahydrofolic acid, barium salt (Sigma, methyl H_4PteGlu), 2×10^{-5} *M*; calcium leucovorin (Lederle, formyl H_4PteGlu), 10^{-5} *M*; methotrexate (Lederle, MTX), 10^{-5} *M*; 5-fluorouracil (Roche, 5-FU), 10^{-5} *M*; hydroxyurea (Squibb, HU), 10^{-3} *M*. After incubation, the cells were centrifuged down, washed three times with approximately 6 ml normal saline, then resuspended in 1 ml glass-distilled water and stored at −20°C.

Total cell uptake of ^{57}Co-CN-Cbl was estimated by measuring the activity of each sample (foil-wrapped to prevent photolytic loss of cobalamin coenzymes) in an auto-γ-spectrometer (Packard). Cobalamins were then extracted with hot ethanol and separated by two-dimensional chromatography and bioautography as previously described.[15,16] The extracts applied to each thin-layer plate were overspotted with markers (50 pg each of Me-Cbl, CN-Cbl, Ado-Cbl, and OH-Cbl), since the *Escherichia coli* mutant used for the later bioautographic location of the separated zones was not sufficiently sensitive to respond to the very small quantities of each labeled cobalamin present in the cell extracts. Bioautogram zones were then excised, and the radioactivity in each was measured in an auto-γ-spectrometer. The amounts of Ado-Cbl, Me-Cbl, and OH-Cbl formed by the lymphocytes were calculated from the total cell uptake, and the proportion of radioactivity recovered in each bioautogram zone. Initially, the whole bioautogram was divided into small areas, and the radioactivity in each area was counted; 87%–94% of the radioactivity applied to the chromatogram was located in zones corresponding to the four cobalamins, while radioactivity recovered at the origin ranged from 0.5%–1.9%.

RESULTS

Effects of PHA Stimulation

A comparison of the total uptake of ^{57}Co-CN-Cbl and synthesis of the coenzymes by lymphocytes with and without PHA stimulation is shown in Table 1. Total uptake of radioactivity after 72-hr incubation was much higher in cells stimulated with PHA, and synthesis of Ado-Cbl and Me-Cbl was approximately five times as great as that in untreated cells from the same subject. In unstimulated lymphocytes, there was very little conversion of CN-Cbl to other cobalamins. More than 80% of the radioactivity remained as CN-Cbl after 72-hr incubation, similar to the proportion recovered as CN-Cbl from chromatograms of aqueous ^{57}Co-CN-Cbl (Table 1). Stimulating "normal"

Table 1. Effect of PHA Stimulation on Uptake and Conversion of ^{57}Co-CN-Cbl After 72 hr by Lymphocytes From a Normal Subject

Chromatogram Zone	Total Radioactivity Recovered From Chromatogram (%)		
	PHA-stimulated Cells	Unstimulated Cells	Aqueous ^{57}Co CN-Cbl*
Me-Cbl	21.4	3.9	0.8
CN-Cbl	37.1	82.8	87.1
Ado-Cbl	26.6	5.8	3.5
OH-Cbl	13.7	6.5	3.7
Origin	1.2	1.0	4.9
Total uptake (pg/10^6 cells)	3.1	1.8	—
Range in 15 normal subjects	2.2–9.4		

*As added to lymphocyte cultures.

lymphocytes with PHA for 18 or 69 hr before adding ^{57}Co-CN-Cbl appeared to decrease rather than to increase CN-Cbl uptake and conversion. By comparison with cells to which PHA was added only half an hour before addition of ^{57}Co-CN-Cbl, total uptake was halved, and the proportion converted to Ado-Cbl and Me-Cbl was slightly reduced (Table 2).

Uptake of radioactivity by PHA-transformed cells from normal subjects was initially rapid, and approximately a quarter of the final uptake at 72 hr occurred within the first 3 hr (Fig. 1). Between 3 and 48 hr the increase was approximately linear, but thereafter uptake virtually ceased. Chromatography revealed that during the first 3 hr unchanged CN-Cbl accounted for much of the uptake of radioactivity (Fig. 1). Between 3 and 72 hr the cellular concentration of this cobalamin altered little, the increase in radioactivity corresponding to increasing amounts of newly formed cobalamins.

Synthesis of the Cobalamin Coenzymes

The results of estimating ^{57}Co-labeled Ado-Cbl, Me-Cbl, and OH-Cbl in lymphocytes following incubation with ^{57}Co-CN-Cbl for 0.5–72 hr are shown in Fig. 2. For much of this time, the rates of interconversion were approximately linear, the proportion of CN-Cbl falling as that of the other cobalamins increased. There was virtually no Me-Cbl, Ado-Cbl, or OH-Cbl formed during the first half hour, since the values obtained for each cobalamin

Table 2. Effect of PHA Stimulation for $\frac{1}{2}$, 18, or 69 hr Prior to Uptake and Conversion of ^{57}Co-CN-Cbl by Lymphocytes From Normal Subjects

PHA Stimulation (hr)*	Total Uptake/ 10^6 Cells†	Total Radioactivity Recovered (%)			
		Me-Cbl	CN-Cbl	Ado-Cbl	OH-Cbl
$\frac{1}{2}$	+77.8	1.9 ± 0.7‡	80 ± 4.9	6.6 ± 1.7	11 ± 3.4
18	+35.7	1.5	90	4.6	3.7
69	+35.9	1.0	84	3.7	11

*Delay between stimulation with PHA and addition of ^{57}Co CN-Cbl.
†Per cent increase in total ^{57}Co CN-Cbl uptake between $\frac{1}{2}$ and 3 hr.
‡Mean ± SEM (n = 4).

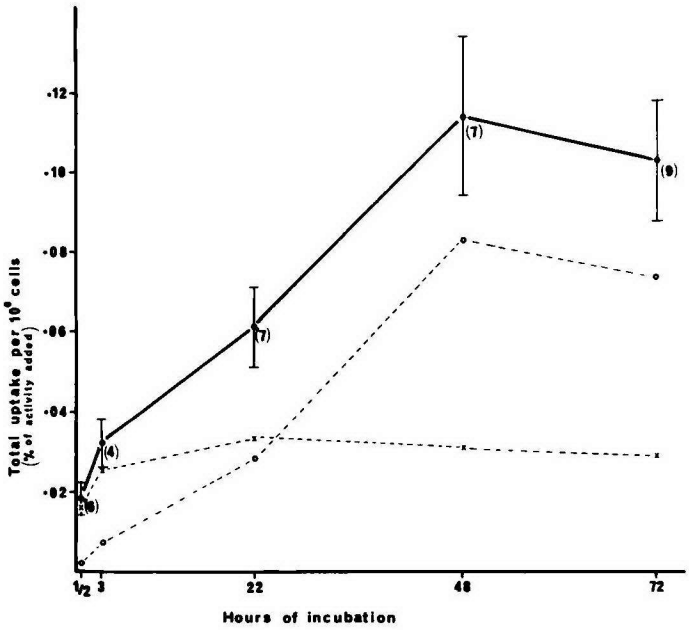

Fig. 1. Total uptake and conversion of ^{57}Co-CN-Cbl between 0.5 and 72 hr by PHA-transformed lymphocytes from normal subjects. The mean ± SEM is shown at each point. The number of experiments is indicated in parentheses. —●—, total uptake of radioactivity; —×—, radioactivity recovered as CN-Cbl; —○—, ^{57}Co-CN-Cbl converted to other cobalamins.

were almost identical to those from chromatograms of the aqueous ^{57}Co-CN-Cbl initially added to the cell cultures. During the early stages of incubation, the formation of OH-Cbl was rapid, and after 3 hr, its proportion was approximately five times that of Me-Cbl, and almost twice that of Ado-Cbl. After 48 hr, Ado-Cbl was the major cobalamin synthesized (mean 31%), though OH-Cbl and CN-Cbl accounted for approximately a quarter of the total labeled cobalamin. Between 48 and 72 hr, the proportions of Ado-Cbl and CN-Cbl remained virtually unchanged, but Me-Cbl synthesis continued, apparently at the expense of OH-Cbl so that after 72 hr the cells contained almost as much Me-Cbl (mean 18%) as OH-Cbl.

The total uptake of ^{57}Co-CN-Cbl after 72 hr incubation was almost twice as high in cells from untreated PA patients (0.2 ± SEM 0.02% of the added radioactivity) as that in cells from normal subjects (0.1 ± 0.01%) (Table 3). Though the proportion of each labeled cobalamin was very similar in PA cells to that in normal cells, the actual amounts of Me-Cbl, Ado-Cbl, and OH-Cbl formed were higher in PA cells, probably as a result of the greater total uptake of ^{57}Co-CN-Cbl.

Effects of Folate Analogues and Antimetabolites

MTX is known to inhibit dihydrofolate reductase and thereby depletes the supply of reduced folates. 5-FU inhibits thymidylate synthetase directly, which leads to a reduction in DNA synthesis. Indirectly, it will interrupt cycling of

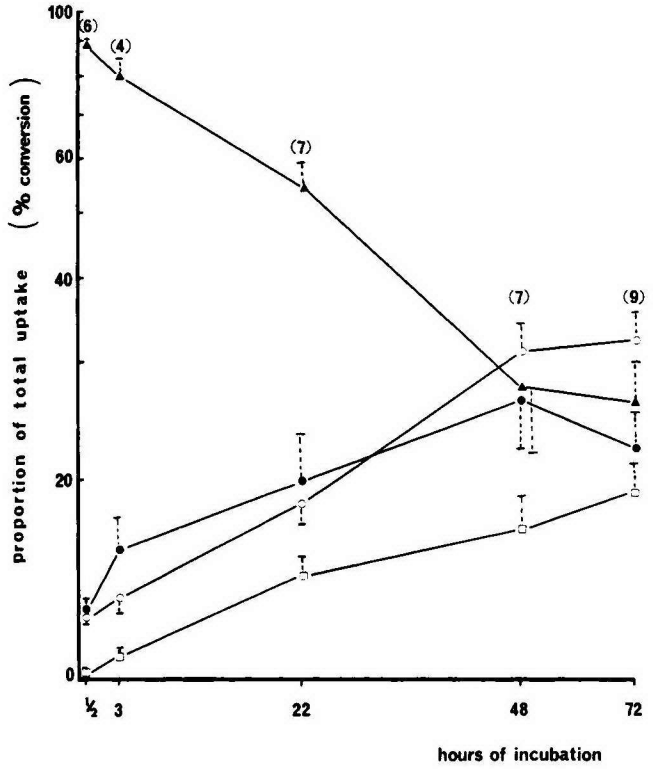

Fig. 2. Formation of ^{57}Co-labeled Ado-Cbl (—○—), Me-Cbl (—□—), and OH-Cbl (—●—) from ^{57}Co-CN-Cbl (—▲—) in PHA-transformed lymphocytes from normal subjects (mean ± SEM). The number of experiments is indicated in parentheses.

the folate coenzymes. We attempted to assess the influence of these inhibitors and folates on cobalamin coenzyme synthesis.

The effect of MTX on the uptake and conversion of ^{57}Co-CN-Cbl in cells from normal subjects is shown in Table 4. Me-Cbl synthesis was significantly reduced by more than a third, but at the concentration used (10^{-5} *M*), MTX had no significant effect either on total uptake or on formation of ^{57}Co-OH-

Table 3. Uptake and Conversion of ^{57}Co-CN-Cbl After 72 hr by PHA-transformed Lymphocytes From Normal Subjects and Patients With Pernicious Anemia

Subjects	Total Uptake/10^6 Cells (pg)	Labeled Cobalamin Formed/10^6 Cells					
		Me-Cbl		Ado-Cbl		OH-Cbl	
		(pg)	(%)	pg	(%)	pg	(%)
Normal controls	5.3*	1.2	18.1†	1.6	27.5	1.2	19.8
(*n* = 15)	±0.6	±0.3	±2.2	±0.3	±2.7	±0.2	±2.1
	$p < 0.01$						
PA patients	9.0	2.0	21.1	2.5	26.5	2.0	22.9
(*n* = 6)	±1.0	±0.4	±2.6	±0.4	±2.0	±0.3	±2.4

*Mean ± SEM.

†Calculated from total activity on chromatogram.

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