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Some Aspects of the Role of Vitamin B_{12} in Nucleic Acid Synthesis in a Neoplastic C3H Mouse Cell Strain. I. Growth Studies [1]

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SUMMARY—Which of certain selected nucleic acid precursors were required to sustain continuous proliferation of the neoplastic C3H mouse cell strain, NCTC 3681, was found to be related as follows to the vitamin B_{12} and folate supplementation of the medium: In protein-free medium containing B_{12} and 0.25 μg/ml (5.7×10^{-7}M) of folate, 3681 cells proliferated continuously in the absence of added purines and pyrimidines or their nucleosides. However, when the folate content of medium that contained B_{12} was dropped to 10% of this value and other components were kept at the same level, it became necessary to add thymidine or deoxycytidine. If B_{12} was omitted from the "low" folate medium there was an additional requirement for adenine, deoxyadenosine, or deoxyguanosine. When B_{12} was left out of the "high" folate medium, thymidine and deoxyadenosine were again required, but the thymidine could then be replaced by deoxycytidine, cytidine, uridine, or deoxyuridine. With thymidine, the deoxyadenosine could be replaced by adenosine, adenine, or guanine. With cytidine or uridine, the deoxyadenosine could be replaced by adenine.—J Nat Cancer Inst 44: 85–90, 1970.

THE STUDIES described in this paper and in (1) were carried out to discover which, if any, of certain pathways of nucleic acid synthesis in mammalian cells in culture require the participation of vitamin B_{12}. For these studies we used a cell strain derived from the C3H/HeN mouse, NCTC 3681 (2). This cell strain has undergone neoplastic conversion in culture and proliferates continuously in protein-free medium. We could examine the effect of the vitamin on certain metabolic sequences in nucleic acid synthesis by substituting other nucleic acid precursors for those previously found to be needed to sustain cell proliferation when B_{12} was absent from the culture medium (3, 4). Also, additional data were obtained extending our earlier findings concerning some metabolic characteristics of this cell strain. These descriptive data may be useful in the future as a basis for comparison with similar data for related non-neoplastic cells.

Previously we had found that proliferation of 3681 cells was limited in medium of "low" folate content (.025 μg/ml, 5.7×10^{-8}M), even in the presence of vitamin B_{12} when thymidine was

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absent (*3*). This limitation was overcome by the addition of thymidine, deoxycytidine, or 5-methyldeoxycytidine, but not by addition of deoxyuridine, cytidine, uridine, or orotic acid. Purines were omitted from the medium. Also, if thymidine or deoxycytidine was present and both B_{12} and purines were left out of the medium, cell division was limited at the "low" folate level (*3*). However, when the folate content of the medium that contained B_{12} was increased to 0.25 μg/ml, a higher rate of proliferation was sustained in the absence of any added pyrimidine nucleosides and purines (*4*). Additional data concerning some interrelationships between folic acid, B_{12}, and nucleic acid precursor requirements for proliferation of NCTC 3681 cells are given here.

MATERIALS AND METHODS

The cells were derived from minced 12-day C3H/HeN mouse embryos and designated 3681 (*2*). Stock cultures growing in protein-free medium NCTC 135 (table 1) were carried by the methods described (*2*). At the time of these experiments the cells had been in the characterized medium for 7 years. To obtain experimental cultures, stocks were grown for 5 days after the last subculture; then the experimental media were added to prime, confluent cultures. After 48 hours and every 7 days thereafter, we subcultured the cells by dividing each culture into two. The cultures were maintained in 3 ml of the test medium through 10 transplant generations (70 days) with medium renewal on the 2d and 5th day after subculturing. In those experimental media that were deficient, the population of healthy, dividing cells decreased after subculture to a greater or lesser degree in different media. A deficient medium was defined as one which, before the tenth generation, gave an insufficient number of healthy cells to serve as an inoculum for the subsequent culture generation. In nondeficient media, cells proliferated during each 7-day growth period to form a continuous sheet of cells comparable in cell number to that of the parent culture. Cultures were periodically assayed for bacterial and mycoplasma contamination by the use of thioglycolate broth and sheep blood agar plates (NIH media room, Bethesda, Md.) and mycoplasma broth and agar medium (Grand Island Biological Co., Grand Island, N.Y.), respectively.

A basal experimental medium, NCTC 141 (*a*, footnote, table 1), containing folic acid (at 0.002 mg/100 ml, 5.7×10^{-8}M) was devised by omission from medium NCTC 135 (table 1) of the coenzymes, sodium acetate, nucleic acid derivatives, vitamin B_{12}, and phenol red. To this basal medium, test components were added individually, or in groups, as the experiments dictated, in the following concentrations: 1 mg/100 ml (3.7–4.4×10^{-5}M) of thymidine, deoxycytidine, cytidine, uridine, deoxyuridine, deoxyadenosine, deoxyguanosine, or adenosine; or 1 mg/100 ml (6.4×10^{-5}M) of orotic acid; 0.5 mg/100 ml (3.3–4.5×10^{-5}M) of guanine, adenine, thymine, uracil, or deoxyribose; and folic acid (0.025 mg/100 ml, 5.7×10^{-7}M) and vitamin B_{12} (1 mg/100 ml, 7.3×10^{-8}M). These components were put in solution in the balanced saline component of the original medium. All solutions were passed through Selas 0.03 bacteriological filters and stored at 4°C.

The extent of contamination of the cytidine, uridine, uracil, and thymine with deoxyribosides was less than 1 mg/5000 mg as determined by the *Lactobacillus acidophilus* R-26 microbiological assay. There was no B_{12} contamination of medium 141, and the cytidine, deoxycytidine, and deoxyuridine stock solutions were devoid of B_{12}. The uridine contained about 2×10^{-6} μg B_{12}/10 μg as determined microbiologically by the method of Hutner *et al.* (*5*) modified by Bonelli and Rinaldini (*6*).

RESULTS

The effects of the tested components on the survival of the cultures are shown in tables 2–4. For the purposes of this study, survival of the cultures was defined as continuous proliferation of the cells for 10 transplant generations (*see* "Materials and Methods").

In the B_{12}-containing medium of higher folate content (0.25 μg/ml) cell proliferation was sustained in the absence of any added pyrimidine nucleosides and purines, whereas in medium of lower folate content thymidine or deoxycytidine, but not deoxyadenosine, was required (table 2, #1–3). In medium of "high" or "low" folate content, cell proliferation was sustained in the

absence of B_{12} when thymidine or deoxycytidine and deoxyadenosine were present (table 2, #4 and 5). The medium of higher folate content was deficient, however, when B_{12} was left out unless both thymidine or deoxycytidine and deoxyadenosine were added (table 2, #6 and 7). Deoxyguanosine and deoxyadenosine were equivalent for survival of cultures in #4, table 2.

Cytidine, uridine, or deoxyuridine could be substituted for the thymidine or deoxycytidine requirement in "high" folate medium that contained deoxyadenosine but no B_{12} (table 3, #3–5). Cell division was limited, however, if this substitution was made in the "low" folate medium containing deoxyadenosine, whether or not B_{12} was added (table 3, #7–12). When deoxycytidine was added

TABLE 1.—Composition of NCTC medium 135*

Component	Molar concentration	Concentration (mg/100 ml)
Amino acids		
L-alanine	36×10^{-5}	3.146
L-amino-n-butyric acid	5.3×10^{-5}	0.551
L-arginine-HCl	15×10^{-5}	3.116
L-asparagine-H_2O	7.0×10^{-5}	0.919
L-aspartic acid	7.5×10^{-5}	0.991
L-cystine	4.4×10^{-5}	1.049
L-glutamic acid	5.6×10^{-5}	0.826
L-glutamine	93×10^{-5}	13.573
Glycine	18×10^{-5}	1.351
L-histidine-HCl-H_2O	13×10^{-5}	2.665
Hydroxy-L-proline	31×10^{-5}	0.409
L-isoleucine	14×10^{-5}	1.804
L-leucine	16×10^{-5}	2.044
L-lysine-HCl	21×10^{-5}	3.843
L-methionine	3.0×10^{-5}	0.444
L-ornithine-HCl	7.1×10^{-5}	0.941
L-phenylalanine	10×10^{-5}	1.653
L-proline	5.3×10^{-5}	0.613
L-serine	10×10^{-5}	1.075
L-taurine	3.3×10^{-5}	0.418
L-threonine	16×10^{-5}	1.893
L-tryptophan	8.6×10^{-5}	1.750
L-tyrosine	9.1×10^{-5}	1.644
L-valine	21×10^{-5}	2.500
Coenzymes		
Diphosphopyridine nucleotide (coenzyme I)	$.095 \times 10^{-5}$	0.70
Triphosphopyridine nucleotide sodium salt	$.012 \times 10^{-5}$	0.10
Coenzyme A	0.33×10^{-5}	0.25
Cocarboxylase	0.21×10^{-5}	0.10
Flavin adenine dinucleotide	0.13×10^{-5}	0.10
Uridine triphosphate sodium salt	0.20×10^{-5}	0.10
Nucleic acid derivatives		
Deoxyadenosine	4.0×10^{-5}	1.00
Deoxycytidine-HCl	3.8×10^{-5}	1.00
Deoxyguanosine	3.7×10^{-5}	1.00
Thymidine	4.1×10^{-5}	1.00
5-Methylcytosine	$.080 \times 10^{-5}$	0.01
Glucose derivatives and acetate		
D-glucuronolactone	1.0×10^{-5}	0.18
Glucose derivatives and acetate—Continued		
Sodium glucuronate-H_2O	1.0×10^{-4}	0.195
D-glucosamine-HCl	1.8×10^{-4}	0.385
Sodium acetate-$3H_2O$	25×10^{-5}	5.00
Balanced saline		
NaCl	12×10^{-4}	680.00
KCl	$.54 \times 10^{-3}$	40.00
$CaCl_2$	$.18 \times 10^{-3}$	20.00
$MgSO_4$	$.083 \times 10^{-2}$	10.00
NaH_2PO_4-H_2O	$.10 \times 10^{-2}$	14.00
$NaHCO_3$	2.6×10^{-3}	220.00
Dextrose	$.56 \times 10^{-4}$	100.00
Other compounds		
Phenol red		2.00
Tween 80		1.25
Ethyl alcohol	$.087 \times 10^{-4}$	4.00
Vitamins		
Thiamine-HCl	$.0074 \times 10^{-5}$	0.0025
Riboflavin	$.0067 \times 10^{-5}$	0.0025
Pyridoxine-HCl	$.0030 \times 10^{-5}$	0.00625
Pyridoxal-HCl	$.0031 \times 10^{-5}$	0.00625
Niacin	$.0051 \times 10^{-5}$	0.00625
Niacinamide	$.0051 \times 10^{-5}$	0.00625
D-pantothenate, calcium salt	$.011 \times 10^{-5}$	0.0025
D-biotin	$.010 \times 10^{-5}$	0.0025
Folic acid	$.0057 \times 10^{-5}$	0.0025
Choline chloride	0.90×10^{-5}	0.125
Vitamin B_{12}	0.73×10^{-5}	1.00
I-inositol	0.009×10^{-3}	0.0125
p-Aminobenzoic acid	$.091 \times 10^{-5}$	0.0125
Vitamin A (crystalline alcohol)	$.087 \times 10^{-5}$	0.025
Calciferol (vitamin D_2)	$.063 \times 10^{-5}$	0.025
Menadione (vitamin K)	$.0055 \times 10^{-5}$	0.0025
α-Tocopherol phosphate disodium salt (vitamin E)	$.0058 \times 10^{-5}$	0.0025
Reducing agents		
Glutathione, monosodium salt	3.0×10^{-4}	1.00
Ascorbic acid	28×10^{-4}	5.00

*The composition of NCTC medium 141 was the same as NCTC 135 except that the nucleic acid derivatives, vitamin B_{12}, the coenzymes, sodium lactate, and phenol red were omitted.

TABLE 2.—Effects of vitamin B_{12}, folate concentration, and deoxynucleosides on the proliferation of NCTC 3681 cells

Experimental medium No.	Folic acid content μg/ml	Vitamin B_{12}	Thymidine or deoxycytidine	Deoxyadenosine	Survival of cultures for 10 generations
1	0.250(5.7×10^{-7}M)	+	−	−	+
2	0.025(5.7×10^{-8}M)	+	+	−	+*
3	0.025	+	−	−	−
4	.025	−	+	+	+
5	.250	−	+	+	+
6	.250	−	+	−	−
7	.250	−	−	+	−

*The same result was obtained when the vitamin B_{12} concentration was reduced from 10 μg/ml to 1.0 μg/ml (7.3×10^{-10}M).

to experimental medium #9, table 3, the cultures survived for 10 generations. There was no change in time of survival of the cultures when deoxyguanosine as well as deoxyadenosine was added to media #2–6, table 3. Orotic acid could not be substituted for thymidine in medium #1, table 3.

In "high" folate medium containing thymidine, cell division was limited in the absence of deoxyadenosine and vitamin B_{12} (table 4, #1). In medium of "high" or "low" folate content this limitation was overcome in the presence of thymidine by addition of adenosine or adenine but not by deoxyribose (table 4, #2–4). In "high" folate medium #7, adenine and guanine were equivalent (table 4). Uridine or cytidine together with adenine were adequate substitutes for thymidine plus adenine (table 4, #8 and 9). Media containing 0.25 μg/ml folate, thymine or uracil, as well as deoxyadenosine or deoxyribose plus adenine (table 4, #10-14) were deficient.

The B_{12} content of 3681 cells grown in medium NCTC 135 containing B_{12} was 4.4×10^{-3} μmole/μmole DNA-P, and after subculture for 3 transplant generations in medium containing no B_{12}, this dropped to 1.4×10^{-7} μmole/μmole DNA-P (*5, 6*).

DISCUSSION

For continuous proliferation of NCTC 3681 cells it was unnecessary to add any pyrimidine nucleosides or purines to the "high" folate (0.25 μg/ml) medium containing B_{12}. When the folate content of this medium was reduced to 0.025 μg/ml, thymidine or deoxycytidine was required. If B_{12} was omitted from the "low" folate medium, the medium was deficient, but the B_{12} could be replaced by adenine. If B_{12} was left out of the "high" folate medium, cell division was limited. In the "high" folate medium, B_{12} could be re-

TABLE 3.—Effects of vitamin B_{12} and folate concentration on the requirements for pyrimidine nucleosides in the presence of deoxyadenosine, for the proliferation of NCTC 3681 cells

Experimental medium No.	Folic acid content μg/ml	Vitamin B_{12}	Cytidine	Uridine	Deoxyuridine	Thymidine or deoxycytidine	Survival of cultures for 10 generations
1	0.250(5.7×10^{-7}M)	−	−	−	−	+	+
2	0.250	−	−	−	−	−	−
3	.250	−	+	−	−	−	+
4	.250	−	−	+	−	−	+
5	.250	−	−	−	+	−	+
6	.025	−	−	−	−	+	+
7	.025	−	+	−	−	−	−
8	.025	−	−	+	−	−	−
9	.025	−	−	−	+	−	−
10	.025	+	+	−	−	−	−
11	.025	+	−	+	−	−	−
12	.025	+	−	−	+	−	−

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placed by thymidine, deoxycytidine, cytidine, uridine, or deoxyuridine together with deoxyadenosine. With thymidine the deoxyadenosine could be replaced by adenosine, adenine, or guanine. Uridine and adenine together also met this requirement for a purine and a pyrimidine nucleoside which together substituted for B_{12} in the "high" folate medium.

A number of tentative conclusions can be drawn from the observations made in these growth studies. It appears that nucleic acid precursors were synthesized *de novo* by 3681 cells at rates sufficient for continuous cell proliferation when vitamin B_{12} and an adequate level of folate were made available. In "low" folate medium containing B_{12}, proliferation seemed to be limited by insufficient synthesis of pyrimidine deoxynucleosides, but when either thymidine or deoxycytidine was added, it was unnecessary to add the other pyrimidine nucleoside or ribonucleosides. In "low" folate medium lacking B_{12}, the synthesis of purines and pyrimidine deoxynucleosides seemed limited and in the "high" folate medium unsupplemented with B_{12}, purine and pyrimidine ring synthesis seemed to be insufficient. In the presence of the higher concentration of folate, if B_{12} was absent, cytidine and adenine or uridine and adenine had to be supplied to sustain continuous proliferation.

Since continuous cell proliferation was sustained in the "high" folate medium when adenine together with cytidine or uridine were substituted for B_{12} or when deoxyadenosine with deoxyuridine were substituted for B_{12}, possibly B_{12} was not required for reduction or methylation of these precursors of DNA-thymidine. This explanation seems consistent with results we have obtained in precursor studies of a cell strain, NCTC 5905, derived from NCTC 3681 (*1*). We concluded, however, from some exploratory data, that in two cell strains derived from 3681 cells there were two cell types. One, when grown in medium containing 0.25 μg/ml folate, required vitamin B_{12} for the utilization of cytidine and deoxyuridine for DNA synthesis, and the other did not.

Whether the effects of B_{12} on nucleic acid precursor requirements for cell proliferation observed in this study are due to its function as a carrier in hydrogen or methyl transfer in the formation of folate coenzymes or in reactions not involving folate remains to be demonstrated. Perhaps at still higher levels of folate it would be unnecessary to add either B_{12} or a pyrimidine nucleoside and a purine. Data reported from studies by other investigators concerning possible coenzyme functions of B_{12} in nucleic acid synthesis and interrelationships in the formation of folate coenzymes have been discussed in reviews by Jacoicke (*7*), Blakley and Vitols (*8*), Stahlberg (*9*), O'Brien (*10*), and Reichard (*11*).

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TABLE 4.—Purine and pyrimidine requirements for proliferation of NCTC 3681 cells in the absence of vitamin B_{12}

Experimental medium No.	Folate content μg/ml	Pyrimidine	Purine	Deoxyribose	Survival of cultures for 10 generations
1	0.025 or 0.25	Thymidine	—	−	−
2	.025 or 0.25	Thymidine	Adenine	−	+
3	.025 or 0.25	Thymidine	Adenosine	−	+
4	.025 or 0.25	Thymidine	—	+	−
5	0.25 (5.7×10^{-7}M)	—	Adenine	−	−
6	0.25	—	Adenosine	−	−
7	0.25	Thymidine	Guanine	−	+
8	0.25	Uridine	Adenine	−	+
9	0.25	Cytidine	Adenine	−	+
10	0.25	Thymine	Adenine	+	−
11	0.25	Uracil	Adenine	+	−
12	0.25	Thymine	Deoxyadenosine	−	−
13	0.25	Uracil	Deoxyadenosine	−	−
14	0.25	Thymine	Adenine	−	−

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Vitamin B_{12} Content of Cells of the Blood, Bone Marrow and Liver Of the Rat.* (30961)

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It was reported(1) that $Co^{60}B_{12}$ injected intraperitoneally into rats can be recovered in the liver, kidneys and also the bone marrow of the limbs, spinal column and skull. In animals rendered anemic through loss of blood there was a fall in radioactive vitamin B_{12} in the liver, kidneys and other viscera, and an increased radioactivity could be demonstrated in the bone marrow of the vertebrae and ribs. As an extension of this study, the vit B_{12} concentration was determined microbiologically in the cellular elements of the bone marrow, and compared with that of red cells, leukocytes, lymphocytes and liver cells. The results are reported here.

Material and methods. Male albino rats of the Hebrew University strain, weighing 145-165 g, fed Purina laboratory chow, were used. The animals were bled from the vena cava under ether anesthesia and killed. The bone marrow cell suspensions were prepared as follows: the tibial bone marrow was removed with the aid of a needle and suspended in cold phosphate-buffered saline (PBS) pH 7.0 and dispersed gently by drawing the fluid up and down in a Pasteur pipette. The bones of the lumbar vertebrae were minced with scissors in a small volume of PBS and homogenized in a loosely fitting glass homogenizer with a teflon pestle. The suspension was then left to settle and the supernatant fluid was removed and centrifuged. The cells were resuspended in 1-2 ml of PBS, and aliquots of cell suspensions were taken for counting the number of cells and for determination of vitamin B_{12}. Cells were counted in a hemocytometer in duplicate. To hemolyze the erythrocytes, the cell suspension was treated with dilute acetic acid and the cell count repeated after treatment. Liver cell suspensions were prepared from livers perfused with ethylenediaminetetra-acetic acid(2). Leukocyte suspensions were prepared using the method described by Nelken *et al*(3). Lymphocyte suspensions were prepared from mesenteric lymph nodes. Vit B_{12} was also estimated in red cell suspensions and in the serum.

Vit B_{12} was determined microbiologically with a mutant of *Escherichia coli*(4), with the turbidity measurements carried out in a Beckman DB Spectrophotometer at wavelength 550 mμ. Release of the bound vit B_{12} in the serum and different cell types was achieved by diluting the serum samples or cell suspensions 1:10 with 1 N acetate buffer, pH 4.5, and heating for 30 minutes at 100°C in the presence of cyanide (0.2 mg KCN per 0.5 ml of undiluted serum or cell suspension). The supernatant was assayed.

Results. The vit B_{12} concentration in the bone marrow of the tibit and vertebrae was 16.04 and 20.94 pg/10^6 nucleated cells, respectively, (Table I). A higher vit B_{12} concentration was found in the nucleated cells of the skull, *i.e.*, 39.48 pg/10^6 cells. The average vit B_{12} content in the circulating leukocytes was 19.02 pg/10^6 cells (Table II). Somewhat higher vit B_{12} concentration was found in the lymphocytes of the mesenteric lymph nodes, *i.e.*, 31.1 pg/10^6 cells (Table III). The vit B_{12} content in the nucleated cells of the bone marrow and the circulating leukocytes differed markedly from that found in the red blood cells, the latter being 0.11 pg/10^6 cells (Table II). The highest vit B_{12} concentration was present in liver cell suspensions, averaging 560 pg/10^6 cells (Table III).

Comment. Few data exist on the vit B_{12} content of the bone marrow. According to Halsted(5) vit B_{12} concentration in human marrow varies from 7 to 29 ng/g. Using Halsted's results, the ratio of vit B_{12} content in the liver to that in the bone marrow is 30:1, which is similar to that observed by us when the ratio was calculated not on the

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TABLE I. Vitamin B_{12} Content of Bone Marrow Cells from Different Sites.

Bone marrow site	No. of rats	Erythrocytes (%)	Vit B_{12} content pg/10^6 nucleated cells mean ± S.E.	Statistical significance
Tibia	13	33	16.04 1.77 (A)	A vs B $t = 1.36$ $P > .1$
Vertebrae	13	28	20.94 3.14 (B)	B vs C $t = 6.11$ $P < .01$
Skull	13	37	30.48 0.04 (C)	A vs C $t = 8.40$ $P < .01$

basis of weight but per million cells. The estimation of vit B_{12} content of the rat marrow in a given number of nucleated bone marrow cells has the advantage of enabling a more accurate quantitative evaluation of changes in vit B_{12} concentration of bone marrow cells under different experimental conditions.

Vit B_{12} concentration of the nucleated bone marrow cells is similar in the tibia and vertebrae. A significantly higher vit B_{12} concentration was found in the nucleated cells of the skull. Although the cellularity of the skull was much less than that of the vertebrae and tibia, the cell composition did not differ essentially in the various marrow sites to explain the difference in vit B_{12} content. Vit B_{12} content of the circulating leukocytes was found to be similar to that of nucleated cells of the vertebrae and tibia. Kidd and Thomas (6) found lower concentrations in human leukocytes (3.84 pg/10^6 cells). Our findings of vit B_{12} concentrations in human leukocytes of 5 normal subjects did not differ significantly from those obtained in rat leukocytes. The reason for the discrepancy between our findings and those of Kidd and Thomas is not clear; it may be due to differences in technique and bioassays employed.

TABLE II. Vitamin B_{12} Content of Leukocytes, Erythrocytes and Serum.

Exp No.	Vit B_{12} content: Leukocytes,* pg/10^6 cells	Erythrocytes, pg/10^6 cells	Serum, pg/ml
1	23.4	.10	510
2	21.4	.12	1179
3	17.9	.15	960
4	15.8	.11	790

* In each experiment the leukocytes were separated from blood derived from 3-5 rats.

TABLE III. Vitamin B_{12} Content of Liver Cells and Lymph Node Cells.

Exp No.	Vit B_{12} content: Liver cells, pg/10^6 cells	Lymphocytes, pg/10^6 cells	Serum, pg/ml
1	593	27.0	740
2	629	30.5	780
3	726	36.5	1280
4	794	—	—
5	364	—	1040

In striking contrast to the high concentration of vit B_{12} in the leukocytes and bone marrow cells is the very low concentration found in erythrocytes. It appears that all the cellular elements containing nuclei have a high vit B_{12} content. It is not surprising that the highest concentration of vit B_{12} was found in the liver cells which contain about 20 times more vit B_{12} than the leukocytes, since the liver represents the main storage organ for vit B_{12}.

Summary. Vit B_{12} content was determined microbiologically in different cellular elements of the rat. The highest concentration was found in liver cells. The vitamin B_{12} content of nucleated bone marrow cells and circulating leukocytes was of the same magnitude, except for the nucleated cells of the skull which had a higher concentration than the other marrow sites. The lowest concentration of vit B_{12} was found in the red cells.

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Serum Vitamin B_{12} in Leukemias and Malignant Lymphomas

By M. Rachmilewitz, G. Izak, A. Hochman, J. Aronovitch and N. Grossowicz

RECENTLY developed microbiologic methods for the determination of vitamin B_{12} concentrations in body fluids[1,2] have established that the vitamin B_{12} levels in the serum are low in patients with pernicious anemia in relapse and other macrocytic anemias.[1,3] Normal blood levels of vitamin B_{12} were found in a wide variety of diseases including the sera of patients with lymphatic leukemia, undifferentiated stem cell leukemia and some types of malignant lymphomas[1,3] while extremely high vitamin B_{12} values were reported in the sera of patients with myeloid leukemia.[4-7]

In this communication our findings on B_{12} concentrations in various types of leukemias and malignant lymphomas are reported. An attempt has been made to use the serum B_{12} concentrations as additional means of differentiation between the various types of malignant conditions of the blood forming organs. In addition the effect of treatment in chronic leukemia on serum vitamin B_{12} concentrations was studied.

Methods and Material

The modified *E. coli* assay described previously[8] was employed. Serum vitamin B_{12} values ranged from 200-500 μμg/ml. with a mean of 340 μμg/ml. in 25 healthy individuals. The serum samples were diluted 1:8 with distilled water. For estimation of the total vitamin B_{12} content the diluted serum (1:8) was heated in a boiling water bath for 30 minutes and added aseptically to the medium. In an alternative procedure the vitamin B_{12} was liberated from the serum proteins by precipitation with acetate buffer (pH 5.5), according to Spray;[9] nearly the same results were obtained as without precipitation. The free vitamin B_{12} was determined in the unheated sample. The bound vitamin was calculated as the difference between the total and free values.

To determine the maximum binding capacity of serum for B_{12} (M.B.C.) 1 ml. of serum was mixed with 0.5 ml. of distilled water containing known amounts of crystalline vitamin B_{12} and incubated at 4 C. for 24 hours. The serum was then re-assayed for bound and free vitamin B_{12}. The M.B.C. was determined using at least two different concentrations of vitamin B_{12}, usually 1,500 and 3,000 μμg/ml. In 10 normal individuals in whom the total B_{12} ranged from 200-500 μμg/ml., the M.B.C. was in the range of 1,200-2,000 μμg/ml.; the recovery was 80-110 per cent of the added vitamin. When the serum with added B_{12} was incubated for one hour instead of twenty-four hours, the M.B.C. was found to be lower, approximately 1,000 μμg/ml.

Since the *E. coli* responds to methionine as well as to vitamin B_{12}, control assays were done on serum treated with sodium hydroxide (final concentration 0.4N), to destroy the B_{12}. Thereafter the samples were steamed for one hour, neutralized and re-assayed. Practically no growth promoting activity was found in the sera after this treatment.

Sixty-seven patients are included in this study.

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	Patients
Acute, subacute and chronic myeloid leukemia	20
Chronic lymphatic leukemia	8
Undifferentiated leukemia	9
Hodgkin's disease	11
Generalized and localized lymphosarcoma	6
Myelosclerosis	3
Primary polycythemia	8
Multiple myeloma	2

In some of the patients with myeloid leukemia who showed elevated serum levels of vitamin B_{12}, repeated determinations were performed after various therapeutic procedures.

Results

Table 1 shows the vitamin B_{12} concentrations in patients with myeloid leukemia. It may be seen that all but one patient had very high serum vitamin B_{12} values ranging from 1,100 to 25,000 $\mu\mu$g/ml. This group includes one patient with subacute (case 1, table 1) and another with acute (case 6, table 1) myeloid leukemia, with myeloid differentiation; in both these cases the serum B_{12} was high. In the one patient with chronic myeloid leukemia with normal B_{12} concentrations the assay was performed shortly before death at the time when the disease was complicated by erysipelas and septicemia for which large amounts of antibiotics had been administered.

Generally, there was no correlation between the total W.B.C. count and the serum vitamin B_{12} concentrations; the highest B_{12} value of about 25,000 $\mu\mu$g/ml. was found in a case where at the time the W.B.C. was 14,000/cu.mm. On the other hand, the serum of a patient with 106,000 W.B.C./cu.mm. had a serum B_{12} level of 2,500 $\mu\mu$g/ml.

There also appeared to be no correlation between the duration of the illness, the extent of hepato-splenomegaly and the serum vitamin B_{12} concentrations.

Serial examinations of B_{12} in 4 cases (1, 2, 5, 10, table 1) of myeloid leukemia illustrate the effect of treatment on the B_{12} level. In case no. 2 the initial B_{12} level of 3,350 $\mu\mu$g/ml. fell to 2,750 $\mu\mu$g/ml. within 10 days following x-ray therapy and then dropped to 720 $\mu\mu$g/ml. 4 weeks later. At the same time the leukocyte count had dropped gradually from 135,000/cu.mm. to 68,000/cu.mm. after 10 days and down to 5,900/cu.mm. after 4 weeks. Two months later, the B_{12} rose slightly to a level of 1,120 $\mu\mu$g/ml., while the leukocyte count was 23,400/cu.mm. At this time x-ray therapy was reinstituted, following which the B_{12} level dropped to 680 $\mu\mu$g/ml. in spite of a high leukocyte count (45,300) (fig. 1).

In another patient (case 1, table 1) a woman aged 32, with subacute myeloid leukemia and a W.B.C. count of 3,350/cu.mm., the B_{12} value was found to be 2,700 $\mu\mu$g/ml. After cortisone therapy and several blood transfusions the patient's condition improved, the vitamin B_{12} concentrations fell to 950 $\mu\mu$g/ml. 2 weeks later, but it soon rose to the pre-treatment level in spite of continuous therapy (fig. 2). In 2 other cases of chronic myeloid leukemia (cases 5 and 10) the drop in white cell count following x-ray therapy preceded the decrease in the serum B_{12} concentration (fig. 3).

In a case of myeloid leukemia which developed in the course of a long-standing

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TABLE 1.—*Myeloid Leukemia*

Case no.	Date	Hb Gm%	W.B.C./cu.mm.	Serum B_{12} μμg/ml.	Treatment
1	11.18.54	5.0	3,350	2,700	ACTH + Blood
	12. 3.54	14.3	5,350	950	ACTH
	12.19.54		15,800	3,000	ACTH
	1.13.55	5.3	21,000	2,400	ACTH + Blood
	1.27.55	6.6	19,400	1,120	ACTH + Blood
2	4.21.55	11.0	135,000	3,350	X-ray
	5. 2.55	10.5	68,000	2,750	X-ray
	6.29.55	12.0	5,900	720	
	8.24.55	12.6	23,400	1,120	X-ray
	11.10.55		45,300	680	
3	11.16.55	11.7	22,900	4,500	X-ray
	12. 8.55	10.0	20,600	1,650	X-ray
4	3.24.55	12.8	126,000	4,600	Myleran
	10.10.55	13.6	80,000	3,000	X-ray
	4.13.56	9.8	108,000	8,000	Mercaptopurine
5	12.28.54	12.4	166,200	2,500	Mercaptopurine +
	6.26.56	11.5	38,200	6,590	X-ray
	7. 1.56	11.2	21,000	6,400	
	7. 8.56	12.0	9,700	6,270	Mercaptopurine +
	7.28.56	10.6	7,600	3,850	X-ray
	8. 2.56		7,500	2,100	
6	7.15.55	4.8	87,000	2,270	Mercaptopurine
7	8. 7.55	9.0	32,000	1,600	ACTH Mercaptopurine
8	3.29.55	10.0	64,000	1,100	X-ray
9	4.12.55	13.8	42,000	1,920	X-ray
10	10.12.55	9.8	131,000	1,100	Cortisone
11	12.28.54	7.3	61,500	160	X-ray
12	1.12.56	8.0	14,000	26,500	Meticorten
13	4.21.56	12.0	18,500	2,000	Mercaptopurine
14	5.18.56	12.2	97,000	1,400	
15	6. 8.55	5.2	52,000	1,700	ACTH + Blood Radium
16	2. 3.55	10.0	28,000	1,100	Urethan
17	2.22.56	8.0	27,000	14,600	Mercaptopurine
18	3. 4.56	7.6	97,000	1,000	Urethan
19	7. 5.56		116,000	5,200	X-ray
	7.10.56		75,400	—	X-ray
	7.19.56		31,300	5,350	X-ray
	8. 6.56			2,400	X-ray
20	6.20.56	7.0	45,000	5,000	

polycythemia (case 7, table 3) x-ray treatment had no effect on the W.B.C. and on the size of liver and spleen; the B_{12} level was very high (6,250 μμg/ml.) and remained so despite treatment.

Acute and Chronic Undifferentiated Leukemia

This group includes 9 patients of whom 7 were suffering from acute stem cell leukemia, 1 from chronic stem cell leukemia and 1 from chronic reticulum cell leukemia. The diagnosis in these cases was established on the basis of the morphology of the peripheral blood and bone marrow. In 4 of the cases post mortem

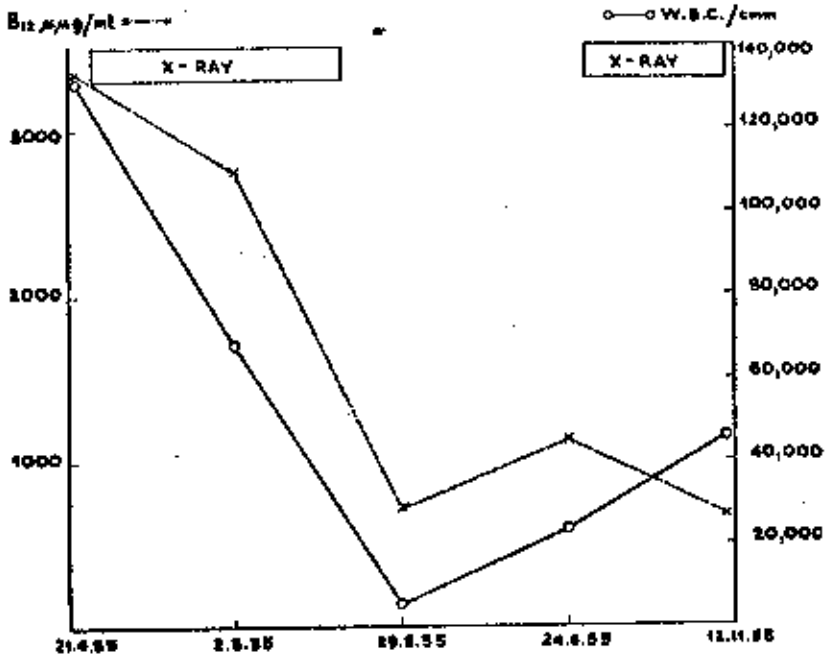

FIG. 1

examinations confirmed the clinical diagnosis. In all of these patients the serum vitamin B_{12} levels were normal and ranged from 130 to 600 µµg/ml. (table 3.)

In 8 patients with chronic lymphatic leukemia the serum vitamin B_{12} fell within the normal range (from 50 to 650 µµg/ml.).

Hodgkin's Disease

In 11 cases the serum vitamin B_{12} examined at different stages of the disease showed normal values (from 120 to 650 µµg/ml.) (table 2).

Lymphosarcoma

Four cases with generalized lymphosarcoma with systemic lymph node involvement, without abnormal cells in the peripheral blood and bone marrow were studied. The diagnosis in these cases was established by biopsy. In 2 of them the serum B_{12} was normal (380, 450 µµg/ml.) while in the other 2 cases the serum B_{12} was increased (700 and 1,450 µµg/ml.). Two cases with localized lymphosarcoma were examined. In one case with round cell sarcoma involving the spleen and abdominal lymph nodes, which was verified at autopsy, the serum B_{12} value was moderately increased (780 µµg/ml.). In the sixth case with lymphosarcoma of the spleen infiltrating the surrounding tissue, the B_{12} was normal (table 2).

Myeloproliferative Disorders: Polycythemia Vera

Eight patients were examined and the results are given in table 3. One patient was found to have very high B_{12} serum levels (case 7, table 3) at the time when she developed clinical and hematologic signs of myeloid leukemia. In this patient

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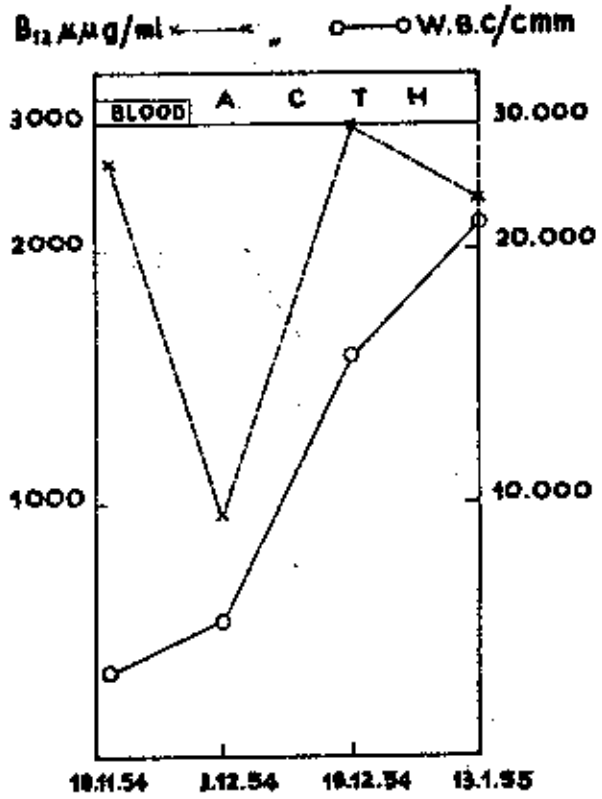

Fig. 2

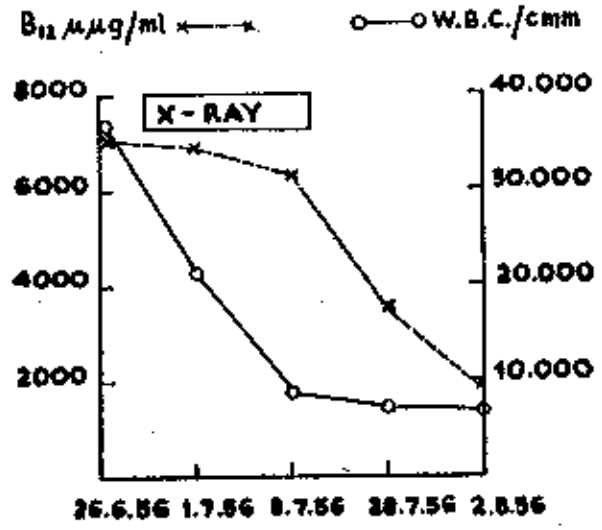

Fig. 3

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the serum B_{12} was found to be high on three occasions during x-ray therapy and remained high following the treatment. The remaining 7 patients had serum vitamin B_{12} concentrations within the normal range with leukocyte counts as high as 60,000/cu.mm., but with a normal differential count.

In another patient (case 8, table 3) with polycythemia of 5 years duration which had been treated by venesection, at the time of the serum vitamin B_{12} examination, the leukocyte count was 36,000 with a differential count of 1 per cent blasts, 3 per cent myelocytes, 29 per cent metamyelocytes, 54 per cent polymorphonuclears, thus suggesting myeloid leukemia. The serum vitamin B_{12} was in the upper range of normal.

Myelosclerosis

Three patients were examined. In one case of myelosclerosis (case 1, table 4) with myeloid infiltration of kidneys, liver and spleen, which was later verified at autopsy, the total serum vitamin B_{12} was at first slightly increased (970 μg/ml.). The patient had marked leukopenia without abnormal cells in the peripheral blood. Repeated blood transfusions were followed by the lowering of the serum vitamin B_{12} level (480 and 300 μg/ml.). Shortly before death the serum vitamin

TABLE 2

Diagnosis	Number of cases	Serum B_{12} μg/ml	
		Minimum	Maximum
Lymphosarcoma			
(a) Generalized	4	340	1,450
(b) Localized	2	360	780
Multiple myeloma	2	250	280
Stem cell leukemia	9	130	600
Lymphatic leukemia	8	50	650
Hodgkin's disease	11	120	650

TABLE 3.—*Polycythemia Vera*

No.	Date	Hb Gm%	R.B.C./cu.mm. (million)	W.B.C./cu.mm.	Thromb./cu.mm.	Serum B_{12} μg/ml
1	12.13.54	17.1	6.06	11,000	320,000	150
2	3. 6.55	18.0	7.5	60,000	280,000	260
3	5. 4.55	16.8	5.6	15,700	288,000	400
4	12.18.54	16.7	5.6	11,600	350,000	190
5	12.16.54	17.0	6.2	14,000	280,000	350
6	5. 9.55	21.7	7.1	10,300	168,000	320
7	1.18.56	9.5	3.2	10,500	160,000	3,000
	3. 4.56	9.5	2.36	9,150	188,000	3,000
	6.10.56	8.5		20,000	—	2,800
	7.15.56	8.0	2.4	65,200	120,000	6,250
	7.30.56					6,000
8	4.20.56	17.2	7.65	36,000		600

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TABLE 1.—*Myelosclerosis*

No.	Date	Hb Gm%	R.B.C./cu.mm (million)	W.B.C./cu.mm.	Serum B_{12} μμg/ml
1	12.16.54	8.4	3.3	2,400	970
	12.28.54	10.5	4.0	3,700	480
	1.18.55	9.0	3.5	3,600	300
	2.23.55	5.2	2.1	3,150	400
	5. 2.55	4.8	1.6	3,300	400
	5.22.55	5.4	—	21,000	2,000
2	5.31.55	—	—	—	700
	6. 6.55	6.2	2.2	3,100	400
	11.11.55	7.4	2.8	3,700	900
3	6.18.56	—	—	16,500	300
	7.24.56	—	—	15,400	400

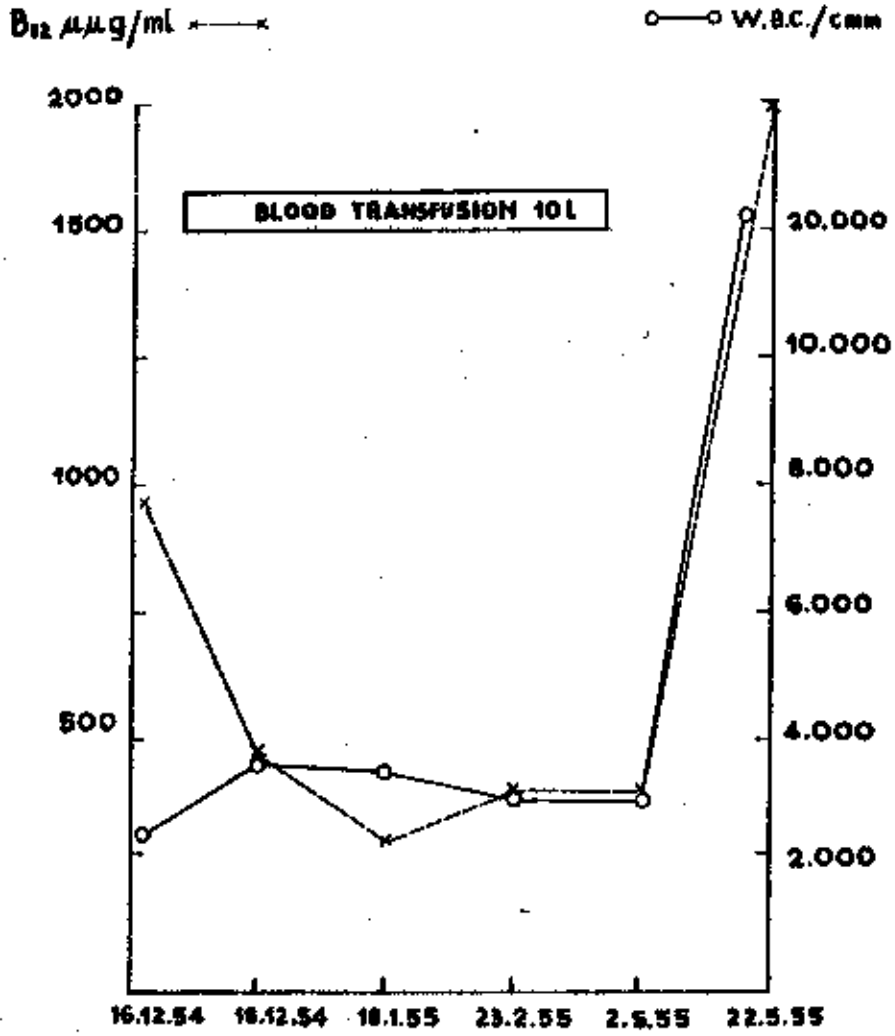

FIG. 4

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B_{12} concentration suddenly rose to 2,000 μμg/ml., together with the appearance of leukocytosis and young myeloid cells in the peripheral blood (fig. 4).

In the second case, which ran a chronic course, the vitamin B_{12} concentration in the serum was slightly elevated on two occasions (700, 900 μμg/ml.). Seven months later, the patient developed a full-blown picture of myeloid leukemia and died shortly afterwards. In the third case the serum vitamin B_{12} was normal.

In 2 cases of multiple myeloma the serum vitamin B_{12} concentrations were normal (table 2).

Serum Binding Capacity of Vitamin B_{12}

In 6 patients with chronic myeloid leukemia with high serum vitamin B_{12} concentrations, free and total B_{12} were examined. In 5 of them practically no free B_{12} was present and all the B_{12} was bound to the proteins of the serum. In the sixth patient, free vitamin B_{12} constituted 20 per cent of the total. The B_{12} binding capacity was examined in 5 patients of this group. The values found were rather high: 3,500, 4,300, 5,000, 5000 and 8,000 μμg/ml. respectively.

In 6 patients with Hodgkin's disease, with normal vitamin B_{12} concentrations, the binding capacity was within the normal range (900–1,400 μμg/ml.).

In 2 cases with acute undifferentiated leukemia and in 1 case of lymphosarcoma, the binding capacity was normal (750, 750, and 1,050 μμg/ml. respectively).

In 1 case of myelosclerosis, with normal serum vitamin B_{12}, the binding capacity of the serum was increased (1,900 and 3,100 μμg/ml.). When the serum vitamin B_{12} increased to 2,000 μμg/ml. the binding capacity of the serum rose to 6,000 μμg/ml.

Discussion

Our findings of high serum vitamin B_{12} concentrations in chronic myeloid leukemia are in agreement with those reported by Beard et al.[5, 8] Killander[7] and Mollin and Ross.[4] In 2 cases of acute myeloid leukemia with differentiation beyond the myeloblast stage, high serum vitamin B_{12} values were found. On the other hand, acute and chronic stem cell leukemia and lymphatic leukemia were associated with normal serum vitamin B_{12} levels. These results indicate that the serum vitamin B_{12} determinations may be used as an additional means of differentiation between the various types of leukemia, particularly in those cases where the classification on a morphologic basis is difficult or impossible.

It seems that the myeloid proliferation is responsible for the elevation of serum vitamin B_{12}. There is, however, no correlation between the number of leukocytes in the peripheral blood and serum vitamin B_{12} concentrations. It was also impossible to correlate the differences in the serum vitamin B_{12} concentration with differences in the numbers of mature or immature cells in the peripheral blood and bone marrow, an observation made also by Mollin and Ross.[4]

It might be concluded, therefore that the degree of increase of serum vitamin B_{12} is dependent upon the extent of the myeloid proliferation in the bone marrow and in the extramedullary sites of hematopoiesis, and not on the degree of release of myeloid cells into the peripheral blood. This point is also borne out by the observation in myelosclerosis, where the serum B_{12} level seems to rise with

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myeloid proliferation in the extramedullary organs, in the absence of abnormal cells in the peripheral blood.

The cause of the increase in serum vitamin B_{12} is not known. The increased binding capacity of serum protein for vitamin B_{12} reported,[8] and confirmed in this study, suggests that in the process of myeloid proliferation vitamin B_{12} binding proteins are liberated in large amounts. In view of our findings of increased serum vitamin B_{12} values in liver disease as well as in experimental liver damage[8, 10] the possibility of liver cell disintegration in myeloid leukemia as the cause of the high serum vitamin B_{12} concentrations must be considered, more so since in liver cell necrosis the binding capacity of serum for vitamin B_{12} is also increased, together with elevation of serum vitamin B_{12}. However, it is questionable whether the extent of liver cell injury in myeloid leukemia could account for the increase of serum vitamin B_{12}.

It is possible that the moderate elevation of serum vitamin B_{12} in 2 cases of generalized lymphosarcoma was due to invasion of the liver. This assumption gains support by the findings of high B_{12} values in the sera of patients with malignant tumors with extensive metastases to the liver.[11] The role of the liver as a possible cause of elevation of serum vitamin B_{12} in myeloproliferative diseases needs further elucidation.

Our findings in polycythemia suggest that leukocytosis alone is not associated with elevated serum vitamin B_{12}. In 1 case with a leukocyte count as high as 60,000 the serum vitamin B_{12} concentration was found to be normal. In another case, however, in which the full-blown clinical and hematologic picture of leukemia developed in spite of only a moderate leukocytosis, a very high B_{12} concentration was present and the values increased steadily despite x-ray therapy. Thus the serum vitamin B_{12} determination may possibly help to differentiate between leukemoid reactions and real leukemia in the course of polycythemia.

It is also possible that the serum vitamin B_{12} may be used in evaluating the extent of myeloid metaplasia in myelosclerosis.

Summary

High serum vitamin B_{12} levels were found in chronic myeloid leukemia and in acute leukemia with myeloid differentiation. Following x-ray therapy and repeated blood transfusions, a drop of serum vitamin B_{12} was found.

In chronic lymphatic leukemia, undifferentiated stem cell leukemia, Hodgkin's disease, and multiple myeloma, the serum vitamin B_{12} concentrations were normal.

In polycythemia with marked leukocytosis the serum B_{12} was normal.

In myelosclerosis high B_{12} values may be found.

The serum vitamin B_{12} in chronic myeloid leukemia is in a bound form and the binding capacity for added B_{12} is increased.

Serum vitamin B_{12} determination may be of some value in differentiating various types of leukemia and other myelo-proliferative disorders.

Summário in Interlingua

Alte nivellos seral de vitamina B_{12} esseva trovate in chronic leucemia myeloide e in acute leucemia con differentiation myeloide. Post roentgenotherapia e

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repetite transfusiones de sanguine, un reduction del nivello seral de vitamina B_{12} esseva constatate.

In chronic leucemia lymphatic, leucemia a cellulas indifferentiate, morbo de Hodgkin, e myeloma multiple, le concentrationes seral de vitamina B_{12} esseva normal.

In polycythemia con marcate grados de leucocytosis le nivello seral de vitamina B_{12} esseva normal.

In myelosclerosis, alte nivellos seral de vitamina B_{12} esseva trovate.

In leucemia myeloide, le vitamina B_{12} del sero es presente in forma ligate, e le capacitate ligatori pro B_{12} additional es augmentate.

Determinationes del nivellos seral de vitamina B_{12} va possibilemente esser de valor in le differentiation de varie typos de leucemia e de altere disordines myelo-proliferative.

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THE EFFECTS IN RATS OF VITAMIN B_{12}, WITH AND WITHOUT ETHYL ALCOHOL, ON NITROGEN BALANCE, SERUM ALBUMIN, LIVER NITROGEN AND FAT [1]

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In addition to its role in stimulating the regeneration of red blood cells, vitamin B_{12} has been implicated in a wide variety of metabolic processes. Particularly in animals on low-protein intakes, vitamin B_{12} has been reported to increase growth and improve protein utilization (Bosshardt et al., '46; Ershoff, '47; Hartman et al., '49; Luecke, '49; Emerson, '49; Cunha et al., '50). Many investigators have noted that the administration of vitamin B_{12} is usually associated with an increased appetite and food intake and that this effect may play an important role in the growth response observed (Ershoff, '47; Luecke, '49; Wetzel et al., '50; Anderson and Hogan, '50; Bosshardt et al., '50; Rupp et al., '51). Vitamin B_{12} is also involved in some manner in the synthesis of methyl groups (Bennett, '50; Stekol and Weiss, '50) and has a lipotropic effect in animals on diets deficient in choline and methionine (Drill and McCormick, '49; McCormick and Drill, '50; György and Rose, '50; Dumm et al., '52). In the following experiments, animals were placed on a diet deficient in vitamin B_{12} and the effects of the vitamin and of ethyl alcohol on growth, nitrogen retention, the nitrogen and fat content of the liver and the electrophoretic pattern of the plasma pro-

[1] These studies were supported by a grant from Merck Sharp and Dohme.

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teins were studied. Alcohol was added in certain experiments because of its association with liver damage and to note the effect of vitamin B_{12} under these circumstances.

PROCEDURES

Female rats bred in the laboratory were maintained on a dog chow [2] diet from weaning until the start of the experiment. They were started on a diet deficient in vitamin B_{12} at 42 days of age.[3] After 14 days on the deficient diet, the animals were divided at random into two groups. One group (control) was continued on the diet alone. The other group was given daily subcutaneous injections of 7.5 μg of vitamin B_{12}. After 21 days of vitamin B_{12} administration, each group of animals was again divided at random into two subgroups, one of which was given a drinking solution of 15% ethyl alcohol instead of tap water for the remainder of the experiment. This regimen was continued for 28 days, at which time the animals were sacrificed. A total of 42 animals was studied in these experiments.

At approximately weekly intervals throughout the experiment, food and fluid intake and urinary nitrogen were determined for 48-hour periods. At the start of the experiment, and before each change in regime, blood samples were taken from the tail for the determination of the distribution of the plasma proteins. At the termination of the experiments, the animals were sacrificed and blood specimens were taken both from the tail and from the abdominal aorta. The livers were removed, weighed and samples taken for microscopic sections and for

[2] Gaines Dog Chow is used in our breeding and stock rat colony.

[3] Composition of vitamin B_{12}-deficient diet (per 100 gm diet): 25 gm heated soybean meal, 30 gm sucrose, 37 gm "Primex" (hydrogenated cottonseed oil), 2 ml cod liver oil, 6 gm mineral mixture, 0.15 gm thiamine, 0.15 mg pyridoxine, 0.25 mg riboflavin, 0.05 mg folic acid, 0.025 mg biotin, 0.5 mg Ca pantothenate, 0.5 mg niacin, 2.5 mg vitamin K ("Synkavit"). By analysis the diet contained 12% protein. Composition of mineral mixture: 17% NaCl, 2% ferric citrate ($FeC_6H_5O_7 \cdot 3H_2O$) 36% calcium phosphate ($CaHPO_4 \cdot 2H_2O$), 35% potassium citrate ($K_3C_6H_5O_7 \cdot H_2O$), 10% magnesium citrate [$Mg_3(C_6H_5O_7)_2$], 0.08% $CuSO_4$, 0.004% KI. All chemicals were "reagent" grade.

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determinations of nitrogen and fat. The adrenals were weighed and their cholesterol content determined.

In the experiments just described, the animals were allowed to eat the diet ad libitum. In addition, a paired-feeding experiment was done. In this experiment rats were started on the deficient diet at 42 days of age and allowed to eat ad libitum. During this period, their food intake was recorded and after 14 days they were divided into pairs on the basis of body weight and ad libitum food intake. One rat in each pair, selected at random, was continued on the deficient diet alone; the other rat in each pair was injected with 7.5 μg of vitamin B_{12} daily and its food intake limited to that of the deficient rat. This experiment was continued for 28 days and was completed on 9 pairs of rats. At that time the food intake of the deficient rats had decreased to the point where it was necessary to discontinue the experiment. The animals were sacrificed and the same determinations on blood, liver and adrenals were performed as in the ad libitum experiments.

The total protein in the blood and in the liver was determined by a micro Kjeldahl method. The distribution of the plasma proteins was determined by paper electrophoresis (barbiturate buffer, pH 8.6; $\mu = 0.1$). The papers were stained with bromphenol blue and the density of the stain read in a Spinco Analytrol. The fat in the liver was determined gravimetrically and the adrenal cholesterol by a modification of the Schoenheimer-Sperry method (Clarke and Marney, '45).

RESULTS

The administration of vitamin B_{12}, which was started at 56 days of age, was associated with an increased rate of growth which was very marked by the end of the experiment compared to that of the control rats (60 gm total gain for vitamin B_{12}-treated, 25 gm total gain for control rats; period of time, 40 days). The administration of 15% alcohol, which was started at 77 days of age, was associated with a decreased rate of growth in the rats receiving vitamin B_{12} and a complete cessation of growth in the deficient rats not receiving vitamin

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TABLE 1

Effect of vitamin B_{12} and alcohol on food intake and nitrogen balance

AGE OF RATS	DAYS ON DEFICIENT DIET	Control rats	B_{12}-treated rats		Alcohol-treated rats		B_{12} + Alcohol-treated rats		
		Food intake	Days on B_{12}	Food intake	Days on alcohol	Food intake	Days on B_{12}	Days on alcohol	Food intake
		FOOD INTAKE							
days		*gm/day*		*gm/day*		*gm/day*			*gm/day*
63	21	8.10 ± 0.29 [1]	7	7.00 ± 1.15					
71	29	7.96 ± 0.36	15	14.0 ± 1.36					
84	42	6.50 ± 1.10	28	9.50 ± 1.05	8	3.90 ± 1.16	28	8	6.60 ± 0.97
		NITROGEN BALANCE							
		mg N retained/day		mg N retained/day		mg N retained/day			mg N retained/day
63	21	98.9 ± 7.1	7	97.9 ± 18.3					
71	29	91.7 ± 5.4	15	179.2 ± 26.8					
84	42	77.8 ± 7.6	28	105.4 ± 27.1	8	35.4 ± 20.8	28	8	84.4 ± 16.6

[1] Mean ± standard error.

TABLE 2

Nitrogen balance of pair-fed rats treated with vitamin B_{12}
(7.5 μg injected daily)

AGE	DAYS ON B_{12}-DEFICIENT DIET	DAYS ON VITAMIN B_{12}	CONTROL RATS		VITAMIN B_{12}-TREATED RATS	
			Food intake	Nitrogen balance	Food intake	Nitrogen balance
			gm/day	*mg/day*	*gm/day*	*mg/day*
63	21	7	7.16	74.7 ± 7.33	6.99	86.8 ± 9.37
71	29	15	8.36	85.4 ± 11.6	8.49	91.8 ± 15.45
77	35	21	6.70	66.3 ± 5.47	6.70	67.0 ± 5.57
84	42	28	6.35	54.9 ± 9.53	6.73	70.8 ± 10.10
63-84	21-42	7-28	7.14	69.0 ± 4.52 [1]	7.23	78.7 ± 5.26 [1]

[1] (Fisher's "t" = 1.143; $p = 0.1$ to 0.2).
Data are given as mean ± standard error.
Nine pairs of rats in this experiment.

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B_{12}. The rats on alcohol plus vitamin B_{12} continued to grow at a rate about the same as that of the vitamin B_{12}-deficient rats receiving tap water.

In the pair-fed rats, vitamin B_{12} was associated with a small increase in the rate of growth, which was not statistically significant ($p = 0.1 - 0.2$).

The food, caloric intake and nitrogen balance of the ad libitum fed rats are summarized in table 1. Vitamin B_{12} administration was associated with a marked increase in food intake, as has been reported by other investigators (Ershoff, '47; Luecke, '49; Anderson and Hogan, '50). This was also true when the rats received alcohol. The total caloric intake was about the same for the alcohol-treated rats (33.3 ± 7.06 Cal./day) as for the deficient rats not receiving alcohol (36.6 ± 6.19 Cal./day) when the calories from alcohol are included. In the rats treated with vitamin B_{12}, the total caloric intake also was about the same whether or not they received alcohol (53.5 ± 5.91 and 54.8 ± 7.60 Cal./day). The nitrogen balance in the rats fed ad libitum was greater after vitamin B_{12} administration both for the rats on tap water compared to their controls and for the rats on alcohol compared to their controls. Alcohol administration was associated with decreased nitrogen retention both in animals treated with vitamin B_{12} compared to vitamin B_{12}-treated rats on tap water and in deficient rats compared to deficient rats on tap water.

In table 2, the data on nitrogen balance of the pair-fed rats treated with vitamin B_{12} are summarized. Throughout the experiment, the nitrogen balance of the rats treated with vitamin B_{12} was slightly greater than for their pair-fed controls. However, the differences are small and do not meet the usual criteria of statistical significance, even when the data for the whole period of the experiment are combined.

In figure 1, the distribution of the plasma proteins is shown for the pair-feeding and for the ad libitum experiments. Since the determinations during the course of the experiment were done on small volumes of tail blood, the albumin is presented as percentage of the total protein. At the time of the sacrifice,

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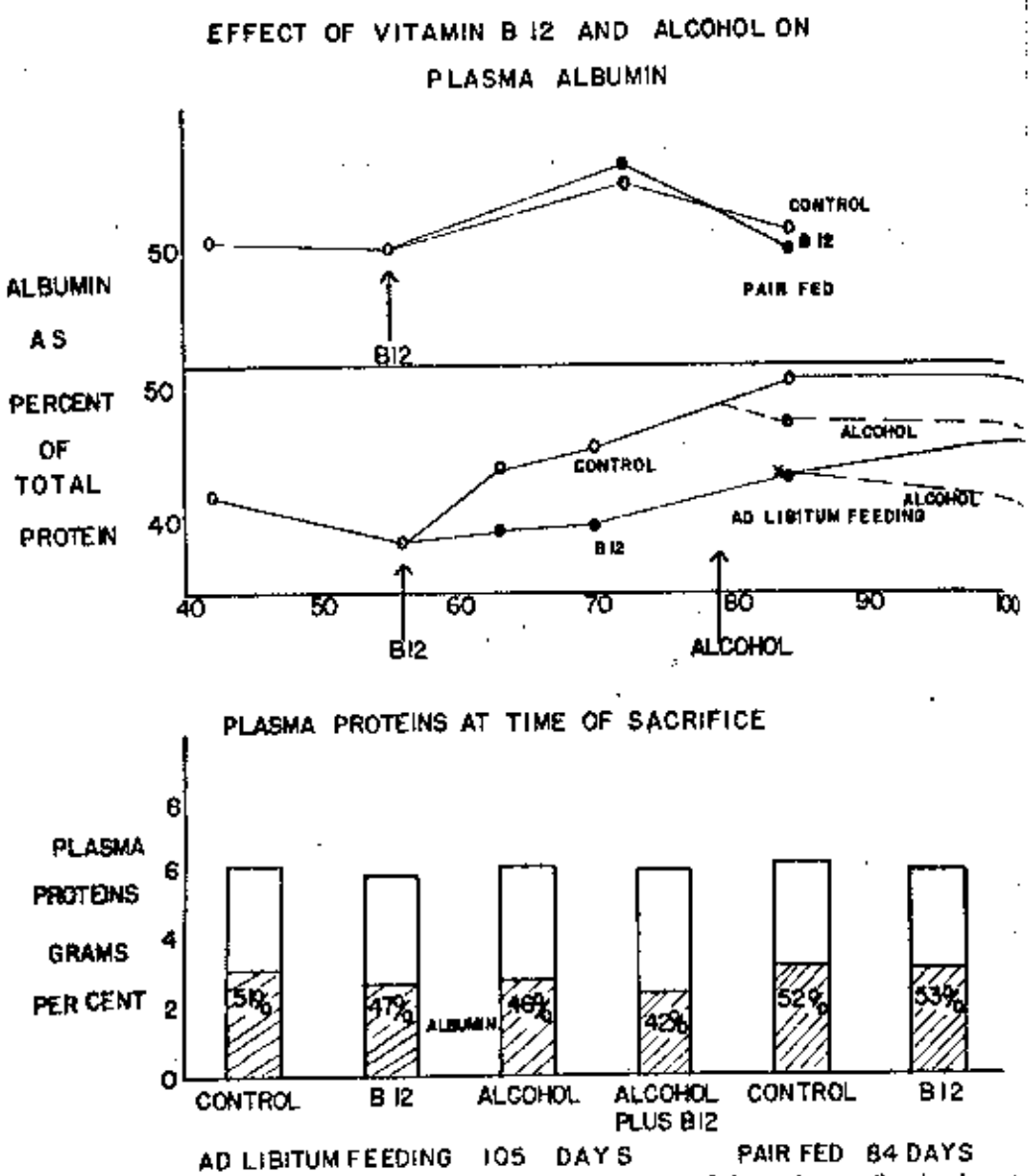

Fig. 1 Effect of vitamin B_{12} (7.5 μg daily) and of 15% ethyl alcohol on plasma albumin shown a cent of total plasma protein. From top to bottom; top — pair-fed rats treated with vitamin B_{12} (●— on diet alone (○——○); middle — ad libitum-fed rats on diet alone (○——○), on diet plus 15% e (⊗——⊗), treated with vitamin B_{12} (●——●), treated with vitamin B_{12} plus 15% ethyl alcohol (×— bottom — total plasma proteins in grams per cent at sacrifice and albumin as per cent of total.

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the total proteins were determined on the aorta blood and are shown at the bottom of the figure. In the paired-feeding experiment, the administration of vitamin B_{12} had no significant effect on the percentage of plasma albumin. However, in the ad libitum-feeding experiment, vitamin B_{12} administration was associated with a consistent decrease in the percentage of albumin. The administration of alcohol was also associated with a further decrease in the percentage of albumin. For any given point, the difference in the plasma albumin associated with the administration of vitamin B_{12} is not large,

TABLE 3

Effect of vitamin B_{12} and of alcohol on the nitrogen and fat content of the liver
(All values based on weight of fresh tissue)

NUMBER OF RATS	INJECTED SUPPLEMENT	DRINKING SOLUTION	NITROGEN	FAT
			gm %	*gm %*
		Ad libitum experiments		
13	None	Water	2.11 ± 0.081 [1]	30.1 ± 3.46
8	Vitamin B_{12}	Water	2.76 ± 0.098	13.6 ± 4.92
10	None	15% alcohol	2.21 ± 0.012	30.6 ± 1.35
11	Vitamin B_{12}	15% alcohol	2.85 ± 0.062	11.8 ± 1.62
		Pair-fed experiments		
9	None	Water	2.39 ± 0.186	19.1 ± 8.06
9	Vitamin B_{12}	Water	3.37 ± 0.195	7.6 ± 1.64

[1] Mean ± standard error.

but the fact that this change was observed consistently, suggests that this effect of vitamin B_{12} administration on the percentage of albumin is probably a real one. The concentration of total protein was not affected by any of the dietary regimes employed, as is shown in the bottom of the figure.

In table 3, the data on the effect of vitamin B_{12} and of alcohol on the nitrogen and fat content of the liver are summarized for the ad libitum and pair-fed rats. In the ad libitum experiments, the deficient diet was associated with low liver nitrogen (2.11 gm%) and elevated liver fat (30.1 gm%). The administration of vitamin B_{12} resulted in a significant increase in liver nitrogen (2.76 gm%, $p < 0.001$) and decrease in liver fat (13.6 gm%, $p < 0.01$). The composition of the livers of

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the rats receiving alcohol was not significantly different from the rats on water (2.21% nitrogen, 30.8% fat). Vitamin B_{12} was associated with a significant increase in liver nitrogen (2.85%, $p < 0.001$) and decrease in liver fat (11.8%, $p < 0.001$) in the rats receiving alcohol.

In the pair-fed rats treated with vitamin B_{12}, the liver nitrogen averaged 3.37 gm% compared to 2.39 gm% for the deficient rats not receiving vitamin B_{12} ($p < 0.01$). The fat content of the livers of the vitamin B_{12}-treated rats averaged 7.6 gm% compared to 19.1 gm% for the deficient rats, ($p < 0.01$).

The data on adrenal weight and cholesterol are summarized in table 4. For comparison, values for adrenal weight and cholesterol in a group of rats receiving a 12% casein diet are also included.[4] The adrenal weight in rats on the soy bean diet expressed per 100 gm of body weight did not differ significantly in any group. However, in the ad libitum-fed rats, vitamin B_{12} was associated with lower adrenal cholesterol levels expressed either as percentage of adrenal tissue or as milligrams per 100 gm body weight of the rats. No significant changes in the level of adrenal cholesterol were observed, either in the pair-fed rats on the soy bean diet or in the ad libitum-fed rats on the casein diet.

The effects of the various dietary situations on the microscopic appearance of the liver are shown in figures 2, 3 and 4. Rats on tap water and the deficient diet ad libitum showed profound fatty infiltration of the liver at the time of sacrifice (fig. 2a). In some areas, early fibrosis of the liver tissue was also seen. The staining capacity of the cells was reduced. In the rats receiving vitamin B_{12} and eating the diet ad libitum, fatty infiltration was at a minimum and the staining capacity of the cells was within normal limits (fig. 2b). In figure 3 are sections from the livers of rats on the deficient and supplemented diet fed ad libitum and with 15% ethanol as drinking solution. As the data in table 4 also show, the lipotropic effect of vitamin B_{12} was demonstrated in spite of alcohol administration. In figure 4 are sections from the livers of

[4] Ralli, E. P., and M. E. Dumm, unpublished data.

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TABLE 4

Adrenal weight and cholesterol

DRINKING SOLUTION	SUPPLEMENT	ADRENAL WEIGHT		ADRENAL CHOLESTEROL	
		mg	*mg/100 gm*	*gm %*	*mg/100 gm*
		(Soybean meal diet — ad libitum)			
Water	None	31.9 ± 1.9 [1]	21.6 ± 1.2	5.99 ± 0.36 [2]	1.32 ± 0.14
Water	Vitamin B_{12}	38.5 ± 1.6	20.4 ± 1.0	4.77 ± 0.42 [2]	0.99 ± 0.13 [2]
Alcohol	None	26.4 ± 2.0	21.7 ± 1.5	6.20 ± 0.47 [2]	1.36 ± 0.15 [2]
Alcohol	Vitamin B_{12}	30.6 ± 2.0	20.2 ± 0.2	4.99 ± 0.10 [2]	1.01 ± 0.08 [2]
		(Soybean meal diet — pair-fed)			
Water	None	32.8 ± 2.5	21.4 ± 1.8	5.66 ± 0.56	1.23 ± 0.19
Water	Vitamin B_{12}	36.2 ± 2.8	22.6 ± 1.2	5.11 ± 0.71	1.11 ± 0.15
		(12% casein diet — ad libitum)			
Water	None	28.8 ± 2.0	22.1 ± 2.0	5.10 ± 0.27	1.12 ± 0.11
Water	Vitamin B_{12}	25.5 ± 1.5	19.9 ± 1.3	5.75 ± 0.45	1.11 ± 0.08

[1] Mean ± standard error.

[2] Vitamin B_{12}-treated compared to controls. Differences significant at 0.05 level.

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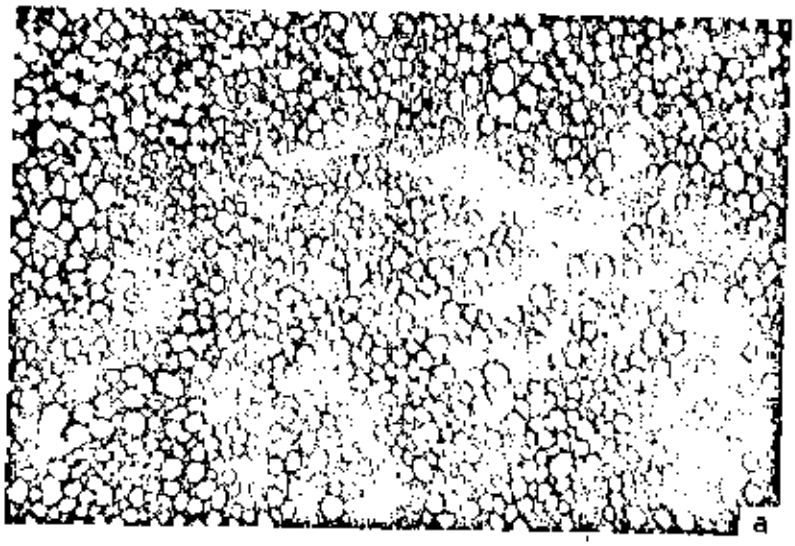
pair-fed rats. In spite of the limited food intake, vitamin B_{12} still exerted a lipotropic effect (fig. 4b) and prevented the fatty infiltration which occurred in the deficient rats (fig. 4a).



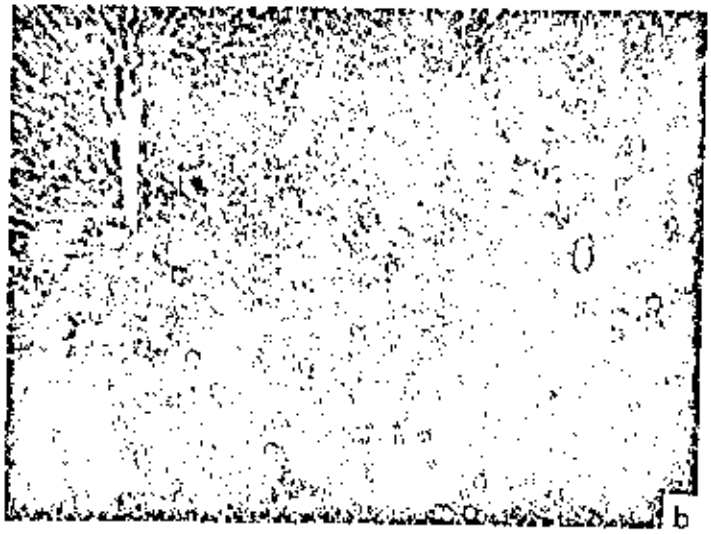


Fig. 2 Microscopic sections of livers of rats fed soybean meal diet ad libitum with tap water as drinking solution: a, Control; b, 7.5 μg vitamin B_{12} daily.

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Fig. 3 Microscopic sections of livers of rats fed soybean meal diet ad libitum with 15% ethyl alcohol as only drinking solution; a, Control; b, 7.5 μg vitamin B_{12} daily.

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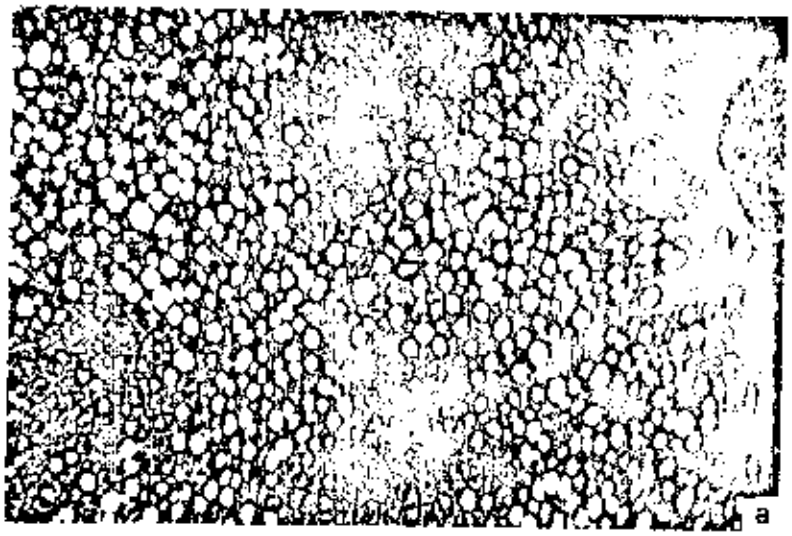

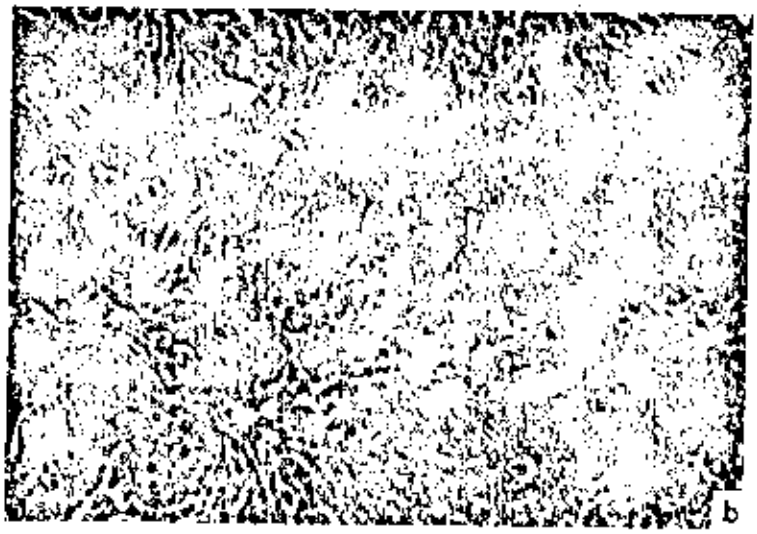

Fig. 4 Microscopic sections of livers of pair-fed rats on soybean meal diet; a, Control; b, vitamin B_6 treated.

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DISCUSSION

In earlier unpublished experiments from this laboratory, the effect of vitamin B_{12} on the liver, adrenals and plasma proteins was studied in young female rats on a 12% casein, 72% carbohydrate, 11% fat diet to which no choline or vitamin B_{12} was added.[3] When this diet was fed ad libitum, the injection of 5 μg of vitamin B_{12} daily was without significant effect on growth, liver nitrogen or fat, on the distribution of the plasma proteins or on adrenal weight or cholesterol, as was shown in table 4.

In the present experiments on rats fed a soy meal diet containing 12% protein and 39% fat, the growth-promoting effect of vitamin B_{12} was obvious when the rats were allowed to eat ad libitum. However, in the pair-fed rats on this diet, vitamin B_{12} had little or no effect on growth or on nitrogen balance. These observations confirm the results of other observers, that the growth-promoting effect of vitamin B_{12} depends on food intake and does not occur in pair-fed animals (Bosshardt et al., '50; Rupp et al., '51). The effect of vitamin B_{12} in rats on a 12% protein soybean meal diet and the lack of effect in animals on a 12% casein diet is presumably related both to the presence of vitamin B_{12} in the casein diet and also to the higher methionine content of casein (3.0 to 3.3 gm/100 gm) compared to soybean meal protein (1.6 to 2.0 gm). Block and Bolling ('45).

The administration of ethyl alcohol decreased food intake, growth and nitrogen retention, in spite of the fact that the total caloric intake was unaltered. When vitamin B_{12} was given along with alcohol, food intake, growth and nitrogen retention were improved and were about equal to the values observed on deficient rats not receiving alcohol.

There is no evidence from either the nitrogen or fat content of the liver or in the histological appearance of the tissue that the ingestion of alcohol was associated with any increase in liver damage beyond that attributable to the diet alone. When

[3] See footnote 3, page 42.

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rats receiving alcohol were treated with vitamin B_{12}, liver nitrogen increased and liver fat decreased to the same extent as in rats not receiving alcohol.

The "lipotropic" effect of vitamin B_{12} occurred in both the paired-feeding and the ad libitum experiments and apparently does not have any relation to increased food intake or growth. The fact that this effect was not observed in the casein experiments in which the total protein content of the diet was the same although the methionine content was higher, suggests that the critical factor was the role of vitamin B_{12} in the synthesis of methyl groups (Bennett, '50; Stekol and Weiss, '50).

One of the more intriguing effects of vitamin B_{12} in the ad libitum experiments was the consistent tendency for a decrease in the albumin concentration of the plasma. The fact that this did not occur in the paired-feeding experiment or in experiments where a 12% casein diet was used, suggests that this is related to the increased food intake and growth and is probably associated with an increased deposition of protein in other body tissues.

Adrenal weight was not affected by the dietary situation in any group of animals. However, adrenal cholesterol was lower in the ad libitum-fed rats treated with vitamin B_{12} than in their controls. This effect was not observed in the pair-fed rats on the soybean meal diet or in the ad libitum fed rats on the casein diet, suggesting that the lower adrenal cholesterol is related in some way to increased growth and food intake. Apparently it is not simply a result of the manipulations associated with the daily injection of vitamin B_{12}, since it did not occur in the pair-fed or casein-fed rats. The data suggest that the adrenals of the ad libitum-fed rats on the soybean meal diet with no vitamin B_{12} supplementation either were less intensively stimulated to release adrenal steroids or were somewhat refractory to stimulation compared to the animals treated with vitamin B_{12}. We have previously observed that rats on low protein diets have elevated levels of adrenal cholesterol which are less affected by stress than are the

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adrenal cholesterol levels of normal animals. It was suggested that the secretion of protein hormones by the pituitary may be decreased in animals suffering from a general protein deficiency (Gershberg and Ralli, '52). In the present experiments, the synthesis of the protein hormones of the anterior pituitary may have been increased in the vitamin B_{12}-treated rats. If so, this increase was probably associated with the increased food intake, growth and protein synthesis of these animals.

SUMMARY

Female rats were maintained on a soybean meal diet containing 12% of protein. The effect of supplementing the diet by injecting 7.5 μg of vitamin B_{12} daily was studied in both ad libitum and paired-feeding experiments. A 15% solution of ethyl alcohol was the only drinking solution for sub-groups of both the vitamin B_{12}-treated and the control rats fed ad libitum.

The rats receiving vitamin B_{12} and eating the deficient diet ad libitum grew more rapidly, ate more of the diet and retained more nitrogen than their controls. Vitamin B_{12} was also associated with increased growth, food intake and nitrogen balance in the alcohol treated rats. In the pair-fed rats, vitamin B_{12} did not significantly increase either growth or nitrogen retention. Caloric intake was essentially the same for the ad libitum rats on the diet alone as for rats on diet plus alcohol. In the rats receiving vitamin B_{12}, the total caloric intake was considerably greater than in the deficient rats and did not differ significantly between the group on vitamin B_{12} plus water and the group on vitamin B_{12} plus alcohol. Alcohol *per se* was associated with decreased growth, food intake and nitrogen balance. In both the paired feeding and the ad libitum experiments, the administration of vitamin B_{12} was associated with lower levels of liver fat and higher levels of liver nitrogen. Drinking 15% ethanol for the period of the experiment did not further alter the liver composition with respect to total fat and nitrogen. Administration of vitamin B_{12} led to a decrease in the plasma albumin expressed

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as percentage of the total plasma proteins in the ad libitum-fed rats. Alcohol further decreased plasma albumin. Total plasma proteins at sacrifice were not affected by any regimen tried; vitamin B_{12} had no effect on plasma albumin levels of the pair-fed rats.

Adrenal weight per 100 gm of body weight was not affected by the administration of vitamin B_{12} or alcohol. Adrenal cholesterol was decreased in the ad libitum-fed rats on vitamin B_{12} regardless of whether or not they had ingested ethyl alcohol. Vitamin B_{12} did not affect the adrenal cholesterol concentration of the pair-fed rats.

Microscopic sections of the livers from animals treated with vitamin B_{12} showed much less fat than did the sections from deficient rats. This was also the case when alcohol and vitamin B_{12} were given.

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J. Nutr. 71: 411-415

The Interrelationship Between Vitamin B_6 and B_{12} Deficiencies in Rats[1]

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Although the biochemical changes accompanying severe deficiencies of vitamins B_6 and B_{12} have been subjects of many studies, few reports are available on the effect of deficiencies of one of these two vitamins leading to the deficiency of the other vitamin. Recently, it was demonstrated that vitamin B_6 deficiency brings about a partial impairment of the absorption of vitamin B_{12} by adult rats (Hsu and Chow, '57). If this phenomenon were true, it might be expected that prolonged vitamin B_6 deficiency in rats may result in the development of vitamin B_{12} deficiency. The purpose of this communication is to present our data on the effect of prolonged feeding of a vitamin B_6-deficient diet on the vitamin B_{12} reserves as measured by the plasma and hepatic B_{12} levels of the vitamin. Conversely, data will be presented on the effect of vitamin B_{12} deficiency on transaminase concentrations, an enzyme believed to be related to vitamin B_6 deficiency.

METHODS

Preparation of animals

With vitamin B_{12} deficiency. Male and female progenies of rats fed a soybean diet (Ling and Chow, '52) low in vitamin B_{12} content during pregnancy and lactation, were placed in individual cages with large screen bottoms, and offered the same soybean diet. For comparison, litter mates were fed the same basal diet supplemented with vitamin B_{12} (100 µg/kg of diet). These diets were offered to the two groups of animals for 6 to 8 months, to be certain that severe deficiencies of vitamin B_{12} developed in the group deprived of this vitamin.

With vitamin B_6 deficiency. Male weanling rats of the McCollum strain, as well as of the Wistar strain, were offered, ad libitum, the vitamin B_6-deficient diet consisting of casein, sucrose, minerals and all known vitamins except vitamin B_6, (Tulpule et al., '55). These animals were housed in screen bottom cages. For comparison, the litter mates were supplied daily (1) by the injection of 50 µg of pyridoxine hydrochloride, and (2) by the supplementation of the basal diet with pyridoxine (100 µg/kg). The animals were placed under these dietary regimens for 6 months, and the usual symptoms of vitamin B_6 deficiency such as acrodynia and loss of weight and appetite developed in the group deprived of this vitamin.

Methods of analysis

Serum glutamic oxalacetic acid transaminase activity. The serum glutamic oxalacetic acid transaminase activity (SGOT) was measured by the procedure of Steinberg et al. ('56) and was taken as a measure of vitamin B_6 deficiency.

Xanthurenic acid test. The normal metabolism of tryptophan is impaired as the result of vitamin B_6 deficiency, with a large increase in the excretion of xanthurenic acid. To perform a tryptophan load test, 50 mg of D,L-tryptophan per 100 gm of body weight of animals deficient in vitamin B_6 or those treated with it were given by mouth. Urine collection was made for 24 hours for the measurement of xanthurenic acid, according to the procedure of Rosen and Sprince ('51).

Vitamin B_{12} determinations. Vitamin B_{12} concentrations in sera or plasma were determined by the modified procedures of

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Okuda et al.,[5] the details of which have been published elsewhere (Gaffney et al., '57). Livers from experimental animals were homogenized in Potter-Elvehjem glass homogenizers with 10 times their weight of distilled water, treated with potassium cyanide and assayed microbiologically for vitamin B_{12} activity.

RESULTS

The effect of vitamin B_6 deficiency on vitamin B_{12} absorption. Fifty millimicrograms of radioactive vitamin B_{12} labeled with Co^{60} (B^*_{12}) were given by mouth to vitamin B_6-deficient and B_6-injected animals. The radioactivities in the 4 days' feces and various target organs are shown in table 1. These results demonstrate that deficient and treated animals absorbed 32 and 64% of the administered dose, respectively, and, therefore, confirm our previous findings that vitamin B_{12} absorption is impaired by vitamin B_6 deficiency.

The effect of vitamin B_6 deficiency on vitamin B_{12} reserves. Four groups of weanling rats were offered a basal diet deficient in vitamin B_6. Group A received the basal diet alone. To group B, only vitamin B_{12} was given as the supplement to the basal diet (50 µg/kg). Group C received the basal diet supplemented with both vitamin B_6 (5 mg/kg) and vitamin B_{12} (50 µg/kg). Group D received both vitamins B_{12} (50 µg/kg) and B_6 (100 mg/kg). Thus, group D diet contained 20 times more vitamin B_6 than the group C diet. After three months' feeding, the vitamin B_6-deficient animals (both groups A and B) had lower mean body weights than those animals (groups C and D) receiving the vitamin B_6 supplement (table 2). However, there was no statistically significant difference in favor of the higher dose of vitamin B_6. The vitamin B_{12} serum level of rats in group A was lower than that of those in group B which received supplementary vitamin B_{12}, showing that the ability of these animals to absorb was not completely destroyed. Despite supplementation with such a large dose of vitamin B_{12}, the serum levels of vitamin B_{12} in the latter group (B) was still considerably below those of groups C and D. Similarly, animals in group A deprived of both vitamins B_6 and B_{12} had the lowest concentration of B_{12} in the liver. The hepatic storage was increased when vitamin B_{12} was added to the diet in group A and was further increased when a moderate amount of vitamin B_6 was also provided in the

[5] Okuda, K., R. D. Wood, C. A. Lang and B. F. Chow 1954 Serum levels of vitamin B_{12} in man. Federation Proc., *13*: 471 (abstract).

TABLE 1

Effect of vitamin B_6 deficiency on vitamin B_{12} absorption

Treatment	Feces	B_{12} absorbed	Liver vitamin B^*_{12}[1]	Kidney vitamin B^*_{12}[1]
	mµg	mµg	mµg	mµg
Vitamin B_6-injected (6)[2]	18	32 ± 2.6	4.4 ± 0.2	3.3 ± 0.1
Vitamin B_6-deficient (6)	34	16 ± 2.4	2.9 ± 0.3	2.7 ± 0.2

[1] Fifty millimicrograms of vitamin B^*_{12} (radioactive B_{12} labeled with Co^{60}) was given by mouth.

[2] Numbers in parentheses denote number of rats used.

TABLE 2

Effect of vitamin B_6 deficiency on vitamin B_{12} levels in serum and in liver

Group	Treatment		Body weight	Vitamin B_{12} in serum	Vitamin B_{12} in liver
	Vitamin B_6	Vitamin B_{12}			
			gm	µµg/ml	mµg/gm
B (10)[1]	—	+	164 ± 8	656 ± 51	335 ± 11
C (10)	+	+	268 ± 15	1020 ± 68	464 ± 30
A (10)	—	—	165 ± 8	132 ± 20	196 ± 27
D (10)	++	+	281 ± 16	1105 ± 129	262 ± 25

[1] Numbers in parentheses denote number of rats used.

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ame diet (compare groups B and C). However, in contrast the hepatic level of vitamin B_{12} was considerably reduced when a large amount of vitamin B_6 was incorporated in the diet in group D. The interplay between concentrations of vitamins B_6 and B_{12} can also be seen from the results to be described later. Since vitamin B_6 deficiency could affect the absorption of vitamin B_{12}, one might inquire conversely whether vitamin B_{12} deficiency would likewise result in some abnormalities of enzyme systems associated with vitamin B_6 deficiency. Therefore, the plasma SGOT level of vitamin B_{12}-deficient animals was determined.

The effect of vitamin B_{12} deficiency on SGOT. Male and female rats were made deficient in vitamin B_{12} by offering them the basal soybean diet deficient in vitamin B_{12} for 6 months. The results shown in table 3 demonstrate the degree of deficiency by the low serum and hepatic levels. It is interesting to note that the animals deficient in vitamin B_{12} showed considerably higher SGOT concentration, which is probably due to some damage to transaminase-rich organs such as liver. The elevation of this enzyme is particularly marked in the male animals which are more susceptible to vitamin B_{12} deprivation than the females, as judged by retardation of growth rates.

The effect of vitamin B_{12} injection on SGOT of vitamin B_6-deficient rats. Since the above data demonstrate interrelationship between vitamin B_{12} and B_6 deficiencies, it was also of interest to determine whether the administration of vitamin B_{12} to animals deficient in vitamin B_6 would alter the SGOT level. To this end, vitamin B_{12} (5 μg/day/rat) was injected into vitamin B_6-deficient and B_6-treated animals. Their SGOT levels were compared with those receiving neither vitamins B_6 nor B_{12}. It can be seen from table 4 that the injection of vitamin B_{12} had no effect on the SGOT level; whereas, supplementation with vitamin B_6, as one would expect, increased the serum level of this enzyme.

Interplay between vitamin B_{12} and B_6. Data presented in table 2 indicate that supplying vitamin B_6 in greater abundance brought about a marked decrease in the

TABLE 3

The SGOT[1] level of vitamin B_{12}-deficient animals

Group	Body weight	Liver	Serum B_{12}	Liver B_{12}	Total liver B_{12}	SGOT
	gm	gm % body weight	mμg/ml	mμg/gm liver	mμg/% body weight	units/ml
Vitamin B_{12}-deficient (12 males)[2]	211.3 ± 18[3]	5.20 ± 0.33	114.6 ± 10.1	24.5 ± 6.4	121.9 ± 12.7	116.6 ± 8.8
Vitamin B_{12}-supplemented (12 males)	326.0 ± 20	3.43 ± 0.01	1372.0 ± 50.3	150.8 ± 14.2	542.1 ± 59.3	77.2 ± 2.9
Vitamin B_{12}-deficient (10 females)	233.8 ± 4	3.13 ± 0.10	65.3 ± 7.0	56.9 ± 4.8	176.9 ± 14.1	84.0 ± 3.7
Vitamin B_{12}-supplemented (10 females)	231.5 ± 6	3.06 ± 0.14	954.1 ± 37.5	328.7 ± 19.3	1009.7 ± 79.2	62.1 ± 2.5

[1] Serum glutamic oxaloacetic acid transaminase activity.
[2] Numbers in parentheses denote number of rats used.
[3] Standard error of the mean.

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TABLE 4
Injection of vitamin B_{12} and SGOT[1] levels in serum

Group	Treatment		SGOT
	B_6	B_{12}	
			units/ml
A (12)[2]	—	—	23.9 ± 0.6
B (12)	—	+	26.8 ± 2.2
C (10)	+	+	68.5 ± 5.3

[1] Serum glutamic oxalacetic acid transaminase activity.
[2] Numbers in parentheses denote number of rats used.

hepatic vitamin B_{12} level, as expressed both in the amount of B_{12}/gm of liver, or total B_{12} in the entire organ, per 100 gm of body weight. Data given in table 5 demonstrate further that a larger supply of vitamin B_6 did not increase the SGOT level, and as a matter of fact brought about a significant decrease of this enzyme and a slight increase in xanthurenic acid upon a tryptophan load test (compare C and D groups).

DISCUSSION

The procedure often employed to study the effect of acute vitamin deficiency is to feed the experimental animals a diet containing all the known vitamins in sufficiently large quantities, except the one under investigation. It is assumed that the vitamins provided in the diet will meet the so-called normal requirements on the premises that deficient animals will have a normal dietary intake, with no impairment of absorption. Unfortunately, neither assumption is true since the deficient animals usually consume lesser amounts of the experimental diet. In this communication, we have brought forth the fact that in addition to the lower intake, deficiency of one vitamin, e.g., B_1, may bring about poorer absorption of a second one (vitamin B_{12}). If the poorer absorption is allowed to take place for a prolonged period, one may expect animals to become deficient in vitamin B_{12}. This was found to occur in the vitamin B_6-deficient animals, which were offered for a prolonged period a diet low in vitamin B_1, even though the diet contained unusually large amounts of vitamin B_{12}. Also noteworthy is that in the animals receiving excessive amounts of vitamin B_6, that is, a quantity considered far above the daily requirement for rats, a marked decrease in vitamin B_{12} serum level in liver and plasma was observed. Furthermore, the supplementation with a large amount of vitamin B_6 will decrease the transaminase level in plasma. These observations are suggestive of the importance of balanced intake of the vitamins.

The present study was not intended to throw additional light on the mechanism of vitamin B_6 deficiency which brings about the impairment of absorption. It might be worthwhile to mention the work of Yeh et al. ('59), who demonstrated that vitamin B_6 deficiency brought about a decrease in the secretion of gastric juice and intrinsic factor as well as an increase in the gastric acidity. However, these investigators pointed out further that vitamin B_6 deficiency also causes dysfunction of adrenals. These findings merely emphasize our thesis that deficiency of one vitamin can create disturbances in endocrines, and abnormal utilizaton of other vitamins besides the one being studied.

TABLE 5
The intake of vitamin B_{12} and B_6 on SGOT[1] levels

Group	Treatment		SGOT	Xanthurenic acid
	Vitamin B_6	Vitamin B_{12}		
			units/ml	mg % / body weight
B (10)[2]	—	+	32.1 ± 4.1	12.5 ± 1.4
C (10)	+	+	86.5 ± 5.0	0.5 ± 0.2
A (10)	—	—	23.9 ± 0.8	10.9 ± 1.1
D (10)	++	+	56.0 ± 4.2	1.17 ± 0.2

[1] Serum glutamic oxalacetic acid transaminase activity.
[2] Numbers in parentheses denote number of rats used.

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SUMMARY

Data are presented to show that feeding rats a diet deficient in vitamin B_6 will result in the lowering of vitamin B_{12} reserve as manifested by the low B_{12} serum level and hepatic storage. Oversupply of vitamin B_6 likewise lowers the vitamin B_{12} reserve in liver, perhaps due to increased metabolic requirement. Vitamin B_{12} deficiency in rats brings about an elevation of transaminase activity in serum, a measure commonly used to estimate the state of sufficiency of vitamin B_6. The significance of the interplay between vitamins is discussed.

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Effect of Vitamin B_{12} on the Formation of Collagen and Nucleic Acids in the Albino Rat Skins and Granulomas[1]

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With a view to study the changes in collagen and nucleic acids content of skins and granulomas due to vitamin B_{12} deficiency, two groups of weanling albino rats were fed for six weeks with the vitamin B_{12} deficient and supplemented diets respectively and another two groups were raised under similar conditions for inducing granulomas by cotton sponge implantation technique. The analyses of skins, granulomas, urine and plasma showed that neutral salt-soluble collagen, insoluble collagen and RNA and DNA contents of skins and granulomas, the urinary excretion of both bound and free hydroxyproline and the free hydroxyproline content of plasma were all significantly decreased in the vitamin B_{12} deficient animals compared to the supplemented group. These results collectively show that the synthesis of collagen in skins and granulomas was appreciably decreased in the vitamin B_{12} deficient group. The analyses of skins further showed that the percent reversibility of gel and α_1- and α_2-chains of neutral salt-soluble collagen appreciably increased, β_{11}- and β_{12}-chains and aldehyde content of the neutral salt-soluble collagen significantly decreased in the vitamin B_{12} deficient animals, which indicated that the intra-molecular cross-linking of collagen was also impaired.

It is well established that vitamin C is essential for the formation and maintenance of collagen in animal body (1). Natarajan and Bose (2) have observed significant reduction of the hydroxyproline content of the skins in vitamin A, thiamine and riboflavin deficiency and also reduction of RNA and DNA contents of the skins in vitamin A deficiency. Brown and Smith (3) and Brown *et al.* (4) have suggested that vitamin E plays a role in altering or preventing the cross-linking of collagen.

Vitamin B_{12} has been reported to promote the growth and protein utilization in animals fed with low protein diets (5, 6). It has also been reported that vitamin B_{12} participates in the nucleic acids synthesis in microorganisms (7-9). Vitamin B_{12} plays an important role in the RNA and protein synthesis in animal tissues (10-19). It is observed that vitamin B_{12} functions *in vivo* and *in vitro* in the incorporation of amino acids into proteins (20-28). Serum proteins *viz.*, albumin, α_1-, α_2- and β-globulins are considerably decreased owing to vitamin B_{12} deficiency (29-31).

So far no study has been made on the influence of vitamin B_{12} on the formation of collagen and nucleic acids in skins. In the present investigation, therefore, a systematic study has been undertaken on the effect of vitamin B_{12} on the synthesis or breakdown of

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collagen and RNA and DNA contents of skin and granuloma by measuring the different collagen fractions, the urinary hydroxyproline excretion and the plasma hydroxyproline level. Data have also been collected on the influence of vitamin B_{12} on the formation of cross-links in collagen by studying the reversibility of neutral salt-soluble collagen gel, separation of collagen subunits by carboxymethyl cellulose (CM-cellulose) chromatography and aldehyde measurements.

Materials and Methods

Twenty one days old weanling albino rats were fed with the basal diet for five days and then divided into two groups of twentyfour each, keeping equal number of litter mates in each group. Then both the groups were given for six weeks vitamin B_{12} deficient diet (32) composed of soybean meal 60 %; sucrose 32 %, groundnut oil 4 % and salt mixture[2] 4 %. Along with the required vitamins, the first group was administered orally a daily dose of vitamin B_{12} (0.5 μg/rat). The second group was given all the required vitamins except vitamin B_{12}. Food and water were supplied *ad libitum*. Daily records for food consumption were kept and the body weights were taken weekly. On the last day of the experimental period, the urine from each group was collected under toluene for a period of 24 hours. Then the rats were killed by decapitation and the blood sample from each group was collected and the skin was removed from each rat immediately for analysis.

1. Analysis of Skin

The skins were unhaired with a razor blade, fleshed and pooled together for each group. An aliquot of the skins from each group was analysed for total hydroxyproline content by the method of Prockop and Udenfriend (33) and the collagen content was calculated by multiplying the hydroxyproline content by the factor 7.46 (34).

Fractionation of Collagen — An aliquot of the skins was finely minced in a meat mincer and the macerated material was extracted (35) thrice by shaking for 24 hours at 4° with 5 volumes (W/V) of 1 M NaCl at pH 7.4 using octane-2-ol as a preservative. The residue left after NaCl extraction was again extracted thrice with 0.5 M citrate buffer (pH 3.7) under similar conditions. The pooled extract obtained after 1 M NaCl or 0.5 M citrate buffer extractions was centrifuged and the collagen content of the clear supernatant was calculated after estimating the hydroxyproline content as mentioned before. The insoluble collagen content was calculated by subtracting the sum of soluble collagen from the total collagen content of the whole skin (36). The results obtained on neutral salt-soluble, acid soluble and insoluble collagen contents of skins are presented in Table 1.

TABLE 1

Influence of vitamin B_{12} on neutral salt-soluble, acid soluble and insoluble collagen contents of albino rat skins

Group	1 M NaCl soluble collagen	Acid soluble collagen	Insoluble collagen
B_{12} supplemented	3.60	1.34	76.03
B_{12} deficient	1.38	1.27	56.02

Each value is expressed as g/100 g dry defatted skins.

Reversibility of Neutral Salt-Soluble Collagen — Another aliquot of the skins was finely minced and extracted thrice with 1 M NaCl under the same conditions as described in the previous section. The combined extract was centrifuged and the supernatant fluid was filtered with suction through Whatman No. 42 filter paper. A portion of filtrate was purified by the method of Jackson *et al.* (37) and the reversibility of gel was determined by the method of Hausmann (38) measuring the optical density at 540 mμ in Beckman DU Spectrophotometer. The results were expressed as percent reversibility of gel and are presented in Table 2.

CM-Cellulose Chromatography — Another portion of the filtrate containing the neutral salt-soluble collagen was purified by the method of Piez *et al.* (39) as described by Page and Benditt (40) and a suitable portion (40 mg. of collagen) of the purified neutral salt-soluble collagen was analysed for subunits

TABLE 2

Influence of vitamin B_{12} on the reversibility of 1 M neutral salt soluble collagen gel prepared from albino rat skins

Group	Percent reversibility of gel
B_{12} supplemented	46.4
B_{12} deficient	69.6

[2] Hawk-Oser salt mixture No. 4 Hawk's Physiological Chemistry, 14th edition, edited by B. L. Oser (McGraw-Hill Book Company, New York) 1965, p 1376

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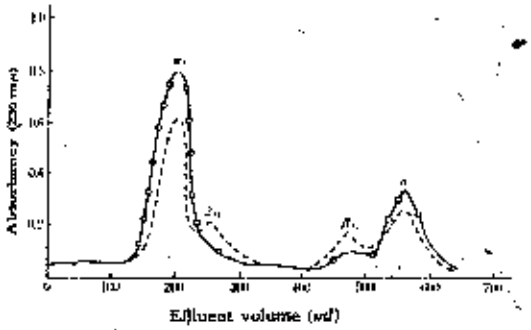

FIG. 1 *CM-Cellulose chromatography of 1 M NaCl extracted denatured rat skin collagens*

Vitamin B_{12} supplemented ----, and vitamin B_{12} deficient ○—○.

pattern by CM-cellulose chromatography on a 2.2×18 cm. column by the technique of Piez *et al.* (39) as adopted by Tanzer (41). The results obtained are illustrated in Fig. 1.

Aldehyde Measurements — The remaining portion of the purified 1 M neutral salt-soluble collagen was analysed for the aldehyde content by the method of Paz *et al.* (42) and the results are presented in Table 3.

RNA and DNA Contents of Skin — The RNA and DNA contents of the skins were calculated from the ribose and deoxyribose contents which were estimated from another aliquot of the skins by the methods of Ceriotti (43, 44) as adopted by Hanumanthu Rao and Bose (45). The results obtained on RNA and DNA contents of skins are presented in Table 4.

TABLE 3

Influence of Vitamin B_{12} on aldehyde content of dermal neutral salt-soluble collagen

Group	Aldehyde content
B_{12} supplemented	0.65
B_{12} deficient	0.44

Values are expressed as μmoles acetaldehyde equivalents/100 mg of collagen.

TABLE 4

Influence of vitamin B_{12} on nucleic acids contents of albino rat skins

Group	RNA	DNA
B_{12} supplemented	539	148
B_{12} deficient	218	106

Each value is expressed as mg/100 g dry defatted skin.

2. Analysis of Urine and Plasma

The total and free hydroxyproline contents of urine or plasma were determined upon hydrolysed and unhydrolysed samples respectively by the method of Prockop and Udenfriend (33) and the bound hydroxyproline content was calculated in each case. In the case of plasma, the free hydroxyproline content was determined after it was deproteinized with trichloroacetic acid. The data obtained on the urinary hydroxyproline excretion and plasma hydroxyproline content in rats are presented in Table 5.

3. Analysis of Granuloma

For this study, two groups of rats of thirty six each were raised according to the same plan as described in the previous section. Five days before the termination of six weeks experimental period, two granulomas were induced in each rat by bilateral implantation of weighed sterilized cotton sponges subcutaneously in the dorsum by the method of Meier (46) as adopted by Sekhara Varma and Bachhawat (47). On the fifth day after the implantation the rats were killed by decapitation and the granulomas were removed by carefully dissecting the surrounding connective tissue.

Fractionation of Collagen — The granulomas were homogenized and an aliquot of the homogenized material was analysed for total hydroxyproline. The remaining material was extracted serially with 1 M

TABLE 5

Influence of vitamin B_{12} on urinary hydroxyproline excretion and plasma hydroxyproline content in albino rats

Group	Urinary hydroxyproline		Hydroxyproline content of plasma	
	Free	Bound	Free	Bound
	μg/24 hours/rat		μg/ml	
B_{12} supplemented	66.2	381.5	11.2	18.6
B_{12} deficient	49.7	189.2	8.3	18.3

TABLE 6

Influence of vitamin B_{12} on neutral salt-soluble acid soluble and insoluble collagen contents of granulomas in albino rats

Group	1 M NaCl soluble collagen	Acid soluble collagen	Insoluble collagen
B_{12} supplemented	0.674	0.385	8.564
B_{12} deficient	0.201	0.363	5.823

Each value is expressed as g/100 g dry defatted granulomas.

TABLE 7

Influence of vitamin B_{12} on nucleic acid contents of granulomas in albino rats.

Group	RNA	DNA
B_{12} supplemented	2.4	3.3
B_{12} deficient	0.9	1.4

Each value is expressed as g/100 g dry defatted granulomas.

NaCl and 0.5 M citrate buffer, the hydroxyproline content of each was estimated, the collagen content in each case was calculated and the insoluble collagen content was calculated by difference as described in the previous section. The results obtained on neutral salt-soluble, acid soluble and insoluble collagen contents of granulomas are presented in Table 6.

RNA and DNA Contents of Granulomas — The RNA and DNA contents of the granulomas were determined by the methods as adopted for skins in the previous section. The results obtained on RNA and DNA contents of granulomas are presented in Table 7.

RESULTS AND DISCUSSION

Table 1 shows that compared to the vitamin B_{12} supplemented group, the neutral salt-soluble and insoluble collagen contents of the skins of vitamin B_{12} deficient group significantly decreased by 66.3 and 26.3 % respectively. Only slight decrease (6.2 %) in the acid soluble collagen content of skins of the vitamin B_{12} deficient group was observed. The results obtained on the analysis of granulomas (Table 6) show that the trend of changes in different collagen fractions due to the influence of vitamin B_{12} is comparable to the changes in different collagen fractions of skins under the same conditions. Compared to the vitamin B_{12} supplemented granulomas, the neutral salt-soluble, insoluble and acid soluble collagen contents of vitamin B_{12} deficient granulomas were found to decrease by 70.2, 32.0 and 5.8 % respectively. Jackson and Bentley (48) reported that the fraction extracted with neutral salt solutions contains the most recently synthesized collagen molecules. Peacock (49) suggested that the level of neutral salt-soluble collagen present in the healing wound is a good measure of the rate of collagen synthesis. Recent studies indicated that acid soluble collagen represents rather a degradation product of insoluble collagen than its precurser (50-52). Only slight decrease in the acid soluble collagen content of skins and granulomas, which was observed in the vitamin B_{12} deficient animals, indicates that the catabolism of collagen did not decrease appreciably.

Table 5 shows that compared to the vitamin B_{12} supplemented group, the urinary excretion of bound and free hydroxyproline decreased by 50.4 and 24.9 % respectively in the vitamin deficient animals. The analysis of plasma, however, showed that only the free hydroxyproline content significantly decreased by 21.8 % in the vitamin B_{12} deficient group. No change was observed in the bound hydroxyproline content of plasma in vitamin B_{12} deficiency. Significant reduction in the urinary excretion of both bound and free hydroxyproline and the free hydroxyproline content of plasma in the vitamin B_{12} deficient group may not be due to collagen breakdown, as collagen catabolism would increase the urinary excretion of hydroxyproline (53-58).

Table 4 shows that compared to the vitamin B_{12} supplemented group, both RNA and DNA contents of skins of vitamin B_{12} deficient group significantly decreased by 59.5 and 28.4 % respectively. Similarly the RNA and DNA contents of granulomas were found to decrease by 62.5 and 57.6 % respectively in the vitamin B_{12} deficient animals (Table 7). It has been suggested that for the synthesis of protein new RNA also is synthesized concomitantly (59, 60). An intimate relationship between soluble collagen and nucleic acids in the regenerating skin wound has been reported by Hosoda (61).

Compared to the vitamin B_{12} supplemented group the percent reversibility of gel from dermal neutral salt-soluble collagen increased by 50.0 % in the vitamin B_{12} deficient animals (Table 2). The formation of gels from neutral salt-soluble collagen and their stability to cold have been found to be related to the degree of cross-linkage in collagen (62).

The subunits of the neutral salt-soluble

collagen from vitamin B_{12} supplemented group by CM-cellulose chromatography revealed the presence of four major components designated as α_1-, α_2-, β_{11}- and β_{12}-chains. The α_1- and α_2- chains were considerably increased whereas the β_{11}- and β_{12}-chains were significantly reduced in the vitamin B_{12} deficient group compared to the supplemented group (Fig. 1). 0.14 M NaCl soluble collagen which represents the most recently synthesized collagen molecules (48) was found to consist of only α-chains (63) while the fraction extracted with NaCl solutions above 0.3 M consisted of mainly α-chains with a small proportion of β-chains (39, 63-67). Some of these studies with labelled glycine also revealed that the β-chains are formed from pairs of α-chains by intra-molecular cross-linking.

Table 3 shows that compared to the vitamin B_{12} supplemented group the aldehyde content of dermal neutral salt-soluble collagen decreased by 32.3 % due to the influence of vitamin B_{12} deficiency. Aldehyde containing peptides were isolated from ichthycol collagen and rat skin collagen (68-72). Blumenfeld and Gallop (73) and Schneider *et al.* (74) reported that the amino aldehydes are derived from lysine by reduction of the carboxyl group and a non-oxidative deamination of α-amino group. Bornstein and Piez (72) and Rojkind *et al.* (70), however, reported that the aldehydes are formed by oxidative deamination of ϵ-amino group of lysine and the intra-molecular cross-links are formed by an aldol condensation of the aldehydes.

The significant reduction in the neutral salt-soluble collagen, insoluble collagen, RNA and DNA contents of skins and granulomas, in the urinary excretion of both bound and free hydroxyproline and the free hydroxyproline content of the plasma in the vitamin B_{12} deficient animals shows that the synthesis of collagen was appreciably decreased compared to the vitamin B_{12} supplemented animals. The significant increase in the percent reversibility of dermal neutral salt-soluble collagen gel and the decrease in the aldehyde content and the changes in subunits pattern of dermal neutral salt-soluble collagen collectively show that the intramolecular cross-linking of collagen was also appreciably decreased in vitamin B_{12} deficiency.

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Transport Function of Transcobalamin II

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ABSTRACT The uptake of free and bound $^{57}CoB_{12}$, principally to transcobalamin II (TC II), was studied in isolated, perfused liver and kidney of the dog. (*1*) There was good uptake of canine TC II-B_{12} by both organs. (*2*) In the liver TC II enhanced uptake over that of free B-12. (*3*) Renal uptake of free B-12 was greater than that of TC II-B_{12}. Free B-12 was neither lost in the urine nor returned to the circulation. (*4*) On a per gram tissue basis, renal uptake of TC II-B_{12} was greater than hepatic. (*5*) There was renal release or production of TC II (*6*). Some TC II but more of a larger molecular size binder came from the liver. (*7*) Passing free B-12 through the kidney enhanced its uptake by the liver. (*8*) Passing free B-12 through the liver depressed its uptake by the kidney. (*9*) It is postulated that the distribution of B-12 can be modified by (*a*) different responses of tissue to TC II-B_{12}, (*b*) synthesis of TC II by an organ, and (*c*) the effects of B-12 passing through one organ to another.

INTRODUCTION

The plasma transport of vitamin B_{12} (B-12)[1] has been reviewed (1). Radioactive B-12 bound to one of the transport proteins, transcobalamin II (TC II), is cleared rapidly from plasma (2, 3), carries B-12 from the ileum to tissues (2, 4), and is necessary for life (5). This in vivo function is supported by the promotion by TC II of B-12 uptake by human cells in suspension (4, 6). The hepatic uptake of TC II-B_{12} has been studied by external counting in man (4). Tan (7) showed murine TC II-B_{12} to be taken up by the liver and kidney of the mouse. Tissues vary widely in their B-12 content (8) and possibly distribution could be influenced by the reaction between TC II and its receptors in different tissues.

Transcobalamin I (TC I) is the dominant carrier of B-12 in plasma after it has been in the body for hours or days (1, 2). The need for two transport proteins has never been explained. Radioactive B-12 bound to TC I is cleared slowly from plasma (2, 3) and is not well taken up by cells in suspension (4, 6). Clinical experience demonstrates that B-12 can move from stores to other tissues but TC I seems to be an unlikely transport protein for this role. Transport between organs by TC II has not been studied.

The above questions were approached by injection of free B-12 and B-12 bound to canine transport proteins into isolated, perfused organs of the dog. This model was chosen because the dog has a B-12 transport protein chemically and functionally like that of human TC II (9). It was considered vital that the proteins and organs come from the same species.

METHODS

Isolated canine kidney and liver were perfused by a modification of the technique of Dodds which has been shown previously to sustain protein synthesis (10). The kidneys were removed and the blood washed out via a cannulated renal artery. Next the animal was exsanguinated; its red blood cells were used in the perfusion fluid. The portal vein was cannulated *in situ*, the liver flushed and removed with cannulas placed in the portal vein and the postcava. The right lateral lobe and the entire caudate lobe were isolated and only these segments perfused. Upon removal, both organs were immediately set in perfusion with a flow rate of approximately 0.4–0.5 ml/g per min. A pump pressure of 100 mm Hg for the kidney and gravity pressure of 15–20 cm H_2O above the liver were maintained. The perfusion fluid consisted of 30% canine erythrocytes which had been washed six times to remove all plasma proteins, 3.3% polyvinylpyrolidinone of mol wt 40,000, and 0.38% trisodium citrate in Tyrode solution. The organs were perfused from 1–6 hr and an evaluation of the system revealed a maintenance of perfusate pH, oxygen consumption, and production of bicarbonate and carbon dioxide. The average osmolality of the perfusion fluid was 286 mOsm/liter with minimal hemolysis with time. All measured values were within the limits for maintaining proper organ function.

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[1] *Abbreviations used in this paper:* B-12, vitamin B_{12}; IF, intrinsic factor; TC I, transcobalamin I; TC II, transcobalamin II.

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The perfusate as described above was evaluated both for its ability to bind B-12 and to promote uptake of $^{57}CoB_{12}$. 100 pg of $^{57}CoB_{12}$ was added to 2 ml perfusate; 4 pg remained after dialysis. The total uptake of this 4 pg by liver and kidney was about 2 pg. The relationship between hemoglobin found in the perfusate and its B-12 binding capacity was also determined. In no case was there enough hemolysate to bind a measurable amount of B-12. To further confirm that the perfusate did not contribute to the observations, a short experiment was run in which the Tyrode solution without red blood cells acted as the perfusion solution. The uptake of $^{57}CoB_{12}$ by the organs was similar to that when whole perfusate was used. Tyrode solution itself did not bind $^{57}CoB_{12}$.

$^{57}CoB_{12}$ obtained from Philips-Duphar, Amsterdam, Holland, with specific activity of 202 μCi/μg was used. The purity of the cyanocobalamin was verified by paper electrophoresis and assayed by *Euglena gracilis* for vitamin B-12. Gel filtration with Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) as described for fractionation of plasma proteins (11) was used to study the B-12 binding proteins in organ output and for isolation of TC II from canine serum.

In those studies where dog serum was the source of TC II, 100 pg of $^{57}CoB_{12}$ was added per 2 ml and of this 95–100% remained bound after dialysis. At no time was the binding capacity of serum exceeded (9), nor did any of the studies saturate the capacity of the organ for B-12. The erythrocyte binder used in three studies was prepared by repeated freeze-thawing of canine erythrocytes. It was, as identified by molecular size, an "R" binder.

The basic experimental procedure consisted of injecting solutions of $^{57}CoB_{12}$ or $^{57}CoB_{12}$ bound to proteins into the organ circulation and measuring uptake of $^{57}CoB_{12}$. The injection time was 45 sec and the material was injected directly into the cannula of the afferent vessel. Preliminary studies were done to determine organ uptake versus time. Of the labeled B-12 taken up by the organ, 95% or more was taken up in the first 2 min with only 1–2% or less taken up in subsequent samples collected over a period of 2 hr. Therefore 2 min was used as the basic collection period. B-12 uptake was usually calculated as the difference between input (injection) and 2 min output (collection). In a few studies the total organ was counted and its uptake of radioactivity calculated. Each day's perfusion was a study in itself and comparisons of uptake were made in the same organ under the same conditions.

Although we refer to uptake of TC II-B_{12}, only radioactivity was measured and the uptake of the intact complex was not proved. Finkler has shown that the HeLa cell takes up TC II-B_{12} intact (12) and we have assumed such is the case here.

RESULTS

$^{57}CoB_{12}$ and $^{57}CoB_{12}$ bound to canine serum, which is essentially all bound to TC II (9), were injected separately into the kidney and liver. Table I is the average of 12 comparable experiments which shows that uptake of serum (TC II)-$^{57}CoB_{12}$ by the liver was enhanced when compared to unbound $^{57}CoB_{12}$ while the uptake of unbound B-12 was greater in the kidney. The average uptake of free B-12 by the kidney was almost twice the uptake of serum (TC II)-$^{57}CoB_{12}$. The reverse was true in hepatic uptake, i.e., serum (TC II)-$^{57}CoB_{12}$ uptake was about twice that of free $^{57}CoB_{12}$. However, the amount of B-12 taken up per gram of renal tissue was always greater than that taken up per gram of liver perfused. In four experiments canine serum was labeled in vivo or in vitro and fractionated by gel filtration (11). The TC II thus isolated and bound to either 31.6 or 100 pg $^{57}CoB_{12}$/2 ml, was injected for uptake studies and gave results similar to serum bound $^{57}CoB_{12}$. In the kidney a mean of 84% of the free $^{57}CoB_{12}$ was taken up as compared to 43% of that bound to isolated TC II. The liver took up 37% of the free and 58% of the TC II-$^{57}CoB_{12}$. The picogram per gram uptake values had the same relationship as in Table I.

TABLE I

Uptake of 100 pg Injection by Canine Kidney and Liver of $^{57}CoB_{12}$ and Serum (TC II)-$^{57}CoB_{12}$

Uptake	Kidney: Free $^{57}CoB_{12}$	Kidney: Serum $^{57}CoB_{12}$	Liver: Free $^{57}CoB_{12}$	Liver: Serum $^{57}CoB_{12}$
Per cent total organ and SD	82±8	48±6	34±12	67±15
Avg pg/avg g perfused	3.6	1.9	0.4	0.7

In three experiments, free $^{57}CoB_{12}$, $^{57}CoB_{12}$ bound to B-12 binder in canine red blood cells, and serum (TC II)-bound $^{57}CoB_{12}$ were introduced into the liver and kidney. The erythrocyte binder, which bound 30 pg of the 100 pg added, seemed to have an inhibitory effect on renal uptake; it was half that of $^{57}CoB_{12}$ in serum. Uptake of the erythrocyte binder by the liver was equal to that of free $^{57}CoB_{12}$, but less than half that of $^{57}CoB_{12}$ in serum.

Preliminary analysis of the output of the kidney by gel filtration indicated that the $^{57}CoB_{12}$ existed bound to a substance with the elution volume of TC II. To evaluate this further, the liver was subjected to three sets of injections, Fig. 1. Serum (TC II)-$^{57}CoB_{12}$ had an uptake of 67% (60 pg) and free $^{57}CoB_{12}$ of only 27% (27 pg). The final hepatic injection was the renal output collected for 2 min after injection of $^{57}CoB_{12}$. It contained 20 pg $^{57}CoB_{12}$ and produced an uptake of 67% (14 pg) in the liver, in agreement with the per cent uptake after the initial serum (TC II)-B_{12} injection. On analysis of this renal output by gel filtration, the $^{57}CoB_{12}$ was bound exclusively to a protein of a molecular size corresponding to that of TC II (1, 11).

Additional studies were performed in which the perfusion fluid entering the liver flowed from the hepatic vein to the kidneys (Fig. 2). Of 100 pg unbound $^{57}CoB_{12}$ injected into the liver, this organ took up only 14 pg while the kidneys took up 10 pg and 9 pg from the liver output. When, in the same system, the 100 pg $^{57}CoB_{12}$ en-

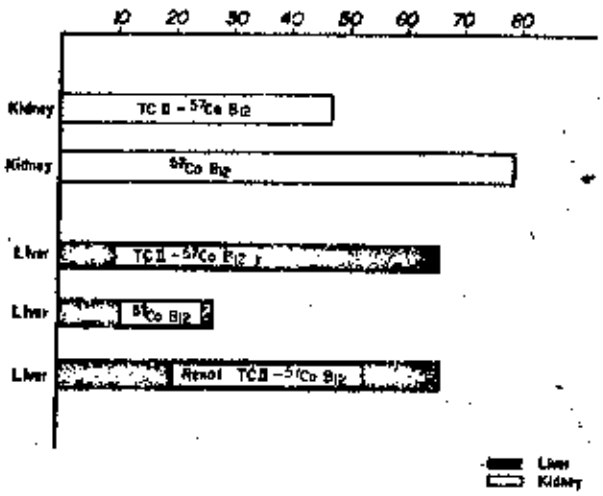

FIGURE 1 Per cent uptake of serum (TC II)-$^{57}CoB_{12}$ and $^{57}CoB_{12}$ by the kidney. Per cent uptake of serum (TC II)-$^{57}CoB_{12}$, $^{57}CoB_{12}$, and renal output (TC II)-$^{57}CoB_{12}$ by the liver.

tered the liver bound to TC II, hepatic uptake (42 pg) was definitely enhanced while the renal uptake, 8 pg and 10 pg of the hepatic perfusate, was not. These data reaffirmed the concept that TC II promoted uptake in the liver. Analysis of the liver output by Sephadex G-200 gel filtration showed $^{57}CoB_{12}$ bound predominately to a protein of a molecular size of ±121,000 and secondarily to a protein of ±38,000 molecular size. These correspond to the "R" binders of B-12 and TC II, respectively (1, 11).

DISCUSSION

Enhancement of B-12 uptake by TC II in isolated organs in a homologous system has been shown for the first time. The fact that the erythrocyte binder did not promote B-12 uptake by either organ showed that the simple presence of a binding protein was not enough. Possibly some

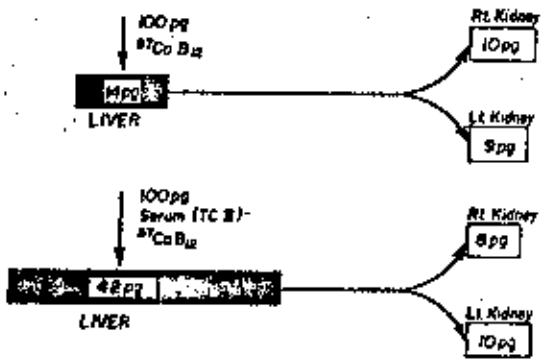

FIGURE 2 Uptake of $^{57}CoB_{12}$ and serum (TC II)-$^{57}CoB_{12}$ by the liver with flow of hepatic output to the kidneys. Renal uptake is given as picograms of B-12 extracted from perfusate coming from the liver.

binders other than TC II would be effective but only TC II and the erythrocyte "R" were tested. The increased uptake of TC II-B_{12} over that of free B-12 by liver is in agreement with uptake by cells in culture (4, 6), but the high uptake of free B-12 by the kidney was unexpected. The free B-12 thus taken up was not excreted in the urine in a single study. Neither was it recirculated as determined by long-term monitoring of the perfusate and of the organ itself.

The TC II-B_{12} uptake per gram was greater in the kidney although the difference could have been related more to conditions of flow, surface area exposed to TC II-B_{12}, etc. than to differences in reaction between TC II and its receptors. The possibility remains, however, that TC II exhibits tissue or organ selectivity in the distribution of B-12.

There was evidence that an organ receiving B-12 could modify its uptake by another organ. The binding of B-12 by TC II found in the kidney enhanced its uptake by the liver. The binding of B-12 to an inactive protein from the liver depressed B-12 uptake by the kidneys. This kind of interrelationship between organs could have strong influence on distribution of the vitamin and the principles delineated may apply to transport of other substances as well.

Before the detection and study of B-12 transport proteins in plasma there were many attempts to show that intrinsic factor (IF) or a related substance functioned in the circulation as well as in the gut. The usual models were slices or homogenates of rat liver. There were some studies of serum-bound B-12 in these systems (13). Subsequently, Gullberg (14) showed that the component of a porcine IF preparation active in the rat liver homogenate system was an "R" binder and not IF. Since in none of the earlier work were the B-12 binding components of IF or serum separated, it cannot be determined which were the active principles. More recently Toporek, Gizis, and Meyer (15) studied human B-12 binders by perfusion of whole rat liver. The native B-12 binding proteins of the rat plasma were not removed from the perfusate and their presence may have influenced the findings. Moreover, there was little uptake of free B-12, which must have become bound to the rat TC II, by the rat liver. The liver of the mouse takes up B-12 bound to murine TC II (7), and this contradiction must be explained before the perfusion system can be adopted. Taken as a group these heterologous systems, for reasons that have not been identified, appear to be nonspecific. Rat liver systems have taken up B-12 bound to human serum (13), porcine IF preparation (13), non-IF binders in porcine IF preparation (14), an incompletely identified component of human serum (16), leukocyte "R" binder (16), the "R" binder in human gastric juice (16), the third binder in human serum (15), and human TC II (15).

This lack of specificity is in sharp contrast to systems used to study intrinsic factor (14, 16) and the plasma B-12 binders in vitro (4, 6).

Production of TC II by the kidney has not been shown before. Tan (7) studied TC II production postnephrectomy in the mouse and considered liver to be the only source. A recent report of synthesis posthepatectomy (17), however, supports the present findings. Since TC II is the only plasma binder of its molecular size (1, 11), identification by gel filtration is reasonable. The ±120,000 mol wt binder coming from the liver requires further identification since several B-12 binders in the body are of that size (1).

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Comparison of Three Methods for Measuring Vitamin B_{12} in Serum: Radioisotopic, *Euglena gracilis* and *Lactobacillus leichmannii*

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Summary. The vitamin B_{12} content of 481 sera was estimated by a radioisotopic assay and 478 of the sera were also assayed by the *Euglena gracilis* method and 396 by the *Lactobacillus leichmannii* method. The serum vitamin B_{12} level in normal subjects ranged from 235 to 1470 pg/ml, mean 497 pg/ml (radioisotopic), 170–1144 pg/ml, mean 460 pg/ml (*E. gracilis*) and 155–1075 pg/ml, mean 471 pg/ml (*L. leichmannii*). In general, the radioisotopic assay gave higher results than the other two methods and the *E. gracilis* assay gave the lowest results. The *E. gracilis* assay gave the clearest distinction between normal subjects and patients with untreated pernicious anaemia. A large number of post-gastrectomy and folate deficient patients and occasional patients with other conditions gave subnormal results by one or both of the microbiological assays but normal values by the radioisotopic assay.

The serum vitamin B_{12} level measured by both the *E. gracilis* and radioisotopic assays rose with folic acid therapy in two folate deficient patients. In one of them, the values by the two assays were initially 100 pg/ml apart and remained this degree apart during the response, while in the other patient the two assays gave almost identical results both before and during the response to folic acid therapy.

Radioisotopic methods for measuring vitamin B_{12} in serum have been in use since 1961 and the correlation between these methods and the two most commonly used microbiological methods (the *Euglena gracilis* and *Lactobacillus leichmannii* assays) has usually been considered satisfactory. Both groups of methods have proved efficient in the diagnosis of vitamin B_{12} deficiency. In 1967, however, Lawrence & Klipstein reported that a radioisotopic method gave higher serum vitamin B_{12} values for some folate deficient sera than the *L. leichmannii* method, and in 1969 Raven *et al* reported that a radioisotopic method gave higher values for some sera (especially from post-gastrectomy patients and patients with folate or vitamin B_{12} deficiencies) than the *L. leichmannii* method. Similarly Robinson & Morgan (1969) noted that a radioisotopic method gave higher serum vitamin B_{12} values in some post-gastrectomy patients than the *E. gracilis* method.

Because of these discrepancies it was decided to assay a large number of sera from normal

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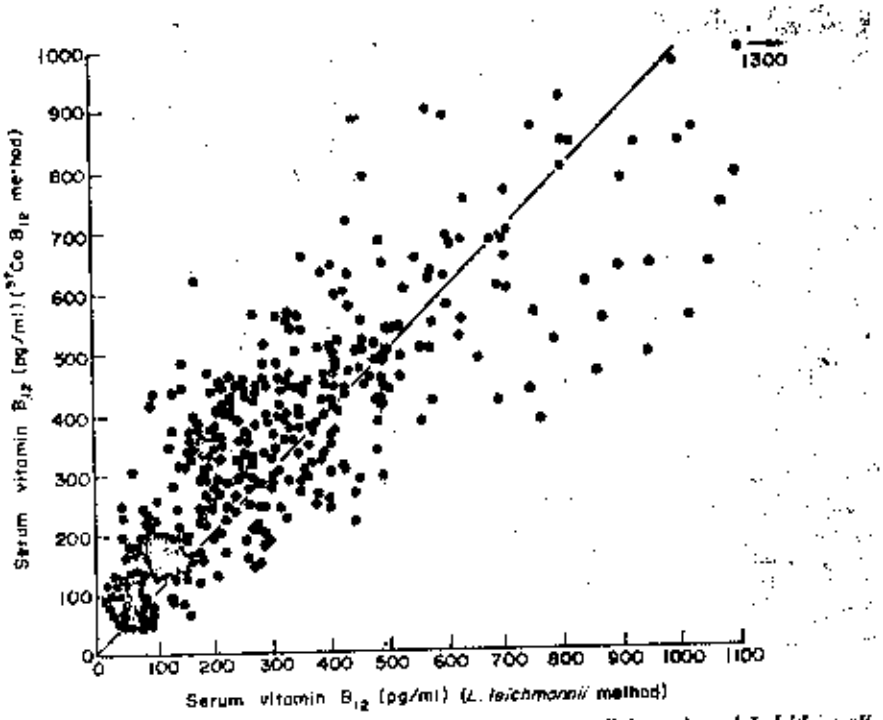

FIG 1. Comparison of the vitamin B_{12} values obtained for 395 sera by radioisotopic and *L. leichmannii* assays. In this and subsequent figures the line drawn through the ordinate represents the line of identity for the two results on the same serum.

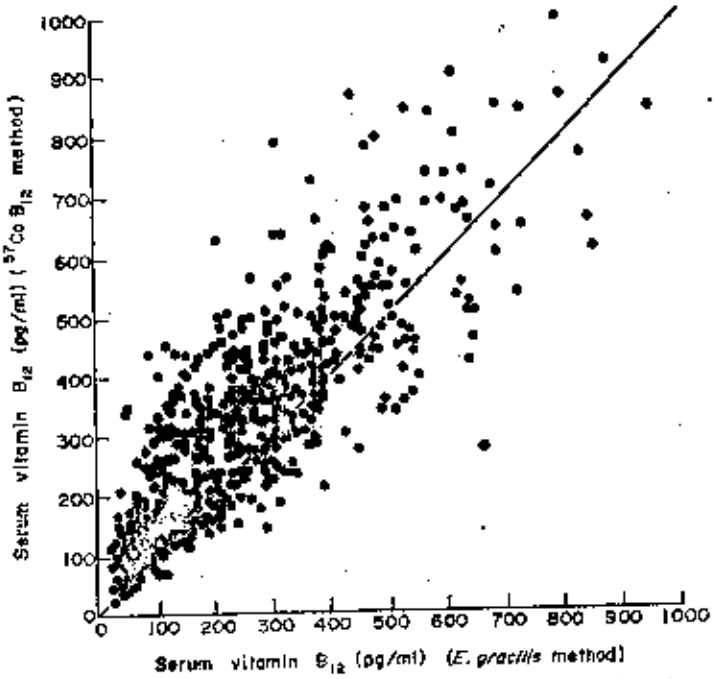

FIG 2. Comparison of the vitamin B_{12} values obtained for 478 sera by radioisotopic and *E. gracilis* assays.

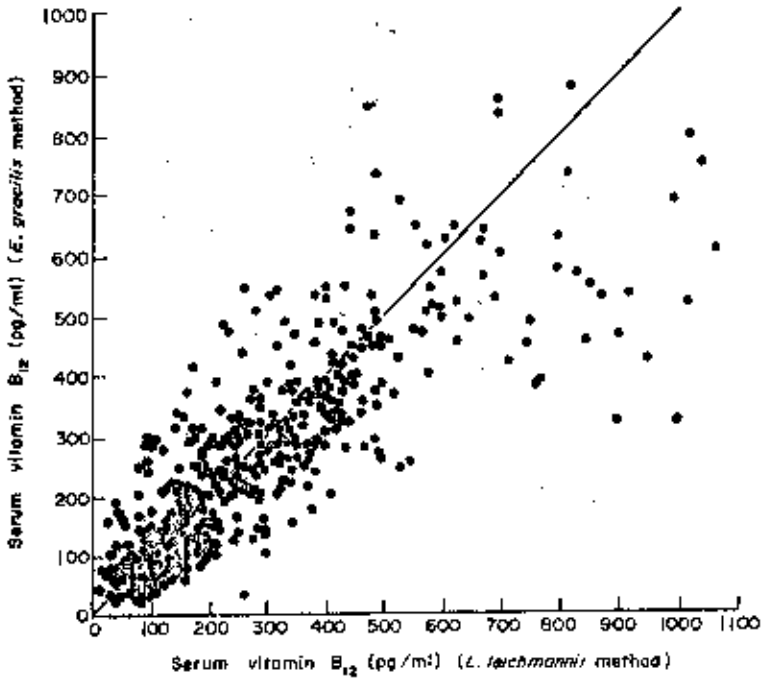

FIG 3. Comparison of the vitamin B_{12} values obtained for 395 sera by *E. gracilis* and *L. leichmann* assays.

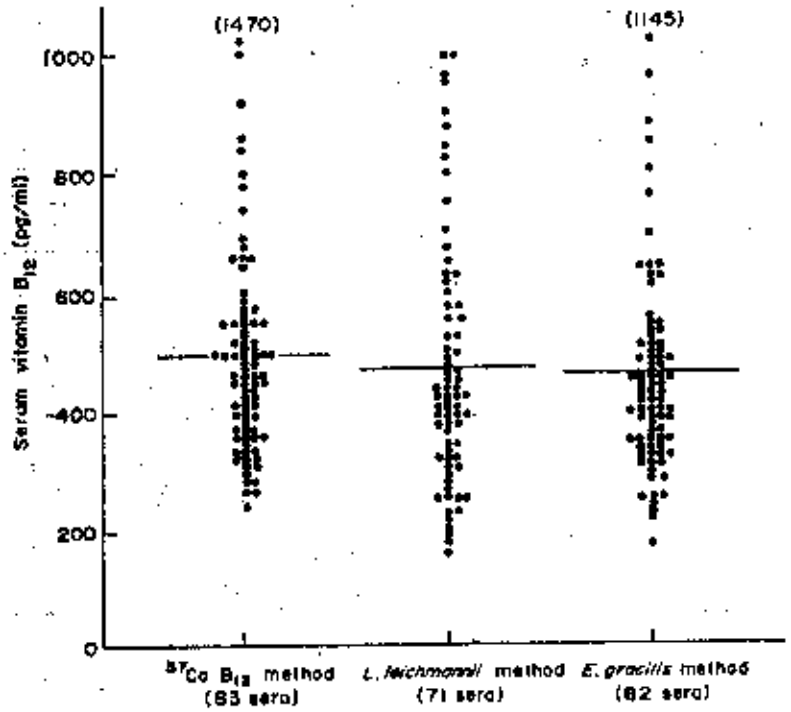

FIG 4. The vitamin B_{12} values obtained by the radioisotopic, *L. leichmannii* and *E. gracilis* assays for a group of sera from normal subjects. The unbroken lines represent the mean values.

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subjects and from patients with a wide variety of diseases by the *E. gracilis*, *L. leichmannii* and radioisotopic methods and this paper gives details of the results obtained.

MATERIALS AND METHODS

The radioisotopic method was that of Lau *et al* (1965) modified by Raven *et al* (1968, 1969). Individual serum backgrounds were used for each serum sample. The normal range with this method is 200–1000 pg/ml.

TABLE 1. Details of 26 sera in which the *E. gracilis* assay gave lower serum vitamin B_{12} values than either the radioisotopic or the *L. leichmannii* assays

Diagnosis	Serum vitamin B_{12} (pg/ml)		
	Radioisotopic	*L. leichmannii*	*E. gracilis*
Myelomatosis	870	750	450
Folate deficiency	840	925	535
Post gastrectomy	840	800	576
Dermatitis	790	1100	320
Normal	780	900	464
Renal failure	630	900	320
Normal	530	500	390
Normal	495	525	248
Normal	455	370	216
Post gastrectomy	450	325	210
Crohn's disease	405	340	150
Post gastrectomy	400	205	147
Iron deficiency	360	250	124
Post gastrectomy	330	290	104
Folate deficiency	320	265	96
Folate deficiency	290	210	144
Myxoedema	265	380	170
Post gastrectomy	260	240	128
Post gastrectomy	260	200	120
Post gastrectomy	260	200	104
Folate deficiency	240	195	88
Post gastrectomy	225	195	128
Pernicious anaemia	155	180	80
Folate deficiency	150	260	35
Crohn's disease	150	135	64
Post gastrectomy	75	80	16

The *Euglena gracilis* method was that of Hutner *et al* (1956) using the 'z' strain, and Anderson's (1964) modification. The normal range with this method is 160–925 pg/ml.

The *Lactobacillus leichmannii* method was that of Rosenthal & Sarett (1952) modified by Spray (1955) and Matthews (1962). The normal range with this method is 150–850 pg/ml though some sera from patients with untreated pernicious anaemia may have vitamin B_{12} values of between 150 and 180 pg/ml (Ardeman *et al*, 1966).

The radioisotopic and *L. leichmannii* assays were performed at Guy's Hospital and the *E.*

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gracilis assay at Hammersmith Hospital. A total of 481 sera from the following groups of subjects was assayed: normal (83), untreated pernicious anaemia (63), partial gastrectomy (154), megaloblastic anaemia due to folate deficiency (malnutrition, malabsorption, anticonvulsant therapy, 70), and a miscellaneous group (Crohn's disease, atrophic gastritis, tropical sprue, intestinal stagnant-loop syndrome, vagotomy, ulcerative colitis, renal failure, iron deficiency, myxoedema, myeloma, aplastic anaemia, disseminated sclerosis, alcoholism, liver damage, 111). The vitamin B_{12} levels of all 481 sera were measured by the radioisotopic method and of these 478 were assayed by the *E. gracilis* method and 395 by the *L. leichmannii* method.

TABLE II. Details of 26 sera in which the *L. leichmannii* assay gave lower serum vitamin B_{12} values than either the radioisotopic or the *E. gracilis* assays

Diagnosis	*Serum vitamin B_{12} (pg/ml)*		
	Radioisotopic	*L. leichmannii*	*E. gracilis*
Normal	515	285	505
Normal	460	225	480
Blind loop syndrome	450	210	390
Normal	395	170	420
Post gastrectomy	370	190	272
Folate deficiency	370	175	312
Normal	365	210	360
Vagotomy	365	180	300
Ulcerative colitis	360	190	312
Normal	355	155	385
Disseminated sclerosis	350	190	312
Post-gastrectomy	325	160	375
Malabsorption	255	100	290
Gastric atrophy	255	40	190
Folate deficiency	245	175	248
Folate deficiency: pregnancy	240	75	168
Idiopathic steatorrhoea	230	40	170
Malabsorption	200	100	240
Aplastic anaemia	195	150	208
Crohn's disease	195	75	200
Folate deficiency	190	80	140
Crohn's disease	185	125	200
Myelomatosis	180	115	280
Post gastrectomy	180	55	115
Sprue	130	25	100
Anticonvulsant megaloblastic anaemia	115	25	160

RESULTS

General Comparison of Radioisotopic, E. gracilis *and* L. leichmannii *Results*

The comparisons between the radioisotopic and *L. leichmannii* assays, the radioisotopic and *E. gracilis* assays and the *E. gracilis* and *L. leichmannii* assays are shown in Figs 1, 2 and 3. In general, the radioisotopic assay gave higher results than the other two assays. This was particularly marked for the comparison between the radioisotopic assay and the *E. gracilis* assay.

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There was also a better general correlation between the results of the two microbiological assays than between either microbiological assay and the radioisotopic assay though much higher values were occasionally found with one rather than the other microbiological assay.

A number of sera in which either the *E. gracilis* or the *L. leichmannii* assay gave much lower (usually by 100% or more) results than the radioisotopic assay and the other microbiological assay are listed in Tables I and II. Table III lists those sera in which both microbiological assays gave lower results than the radioisotopic assay. As many as 22 of these 28 sera came from postgastrectomy or folate deficient patients.

TABLE III. Details of 28 sera in which both the *L. leichmannii* and the *E. gracilis* assays gave lower serum vitamin B_{12} values than the radioisotopic assay

Diagnosis	*Serum vitamin B_{12} (pg/ml)*		
	Radioisotopic	*L. leichmannii*	*E. gracilis*
Alcoholism	900	575	620
Folate deficiency	790	460	460
Post gastrectomy	660	350	385
Post gastrectomy	625	150	210
Post gastrectomy	575	270	208
Hypochromic anaemia	445	150	240
Post gastrectomy	430	270	240
Post gastrectomy	425	250	260
Post gastrectomy	420	100	170
Post gastrectomy	415	230	255
Post gastrectomy	375	200	168
Adult coeliac disease (treated)	375	165	128
Folate deficiency	370	200	235
Post gastrectomy	340	200	200
Folate deficiency	340	185	184
Folate deficiency	340	170	192
Post gastrectomy	340	155	56
Folate deficiency	335	165	200
Folate deficiency	310	190	200
Folate deficiency	310	150	176
Post gastrectomy	310	145	120
Liver damage	305	185	235
Alcoholism	300	185	176
Post gastrectomy	295	180	192
Anticonvulsant therapy	290	185	152
Post gastrectomy	280	130	152
Folate deficiency	245	135	88
Folate deficiency (anticonvulsant drugs)	235	90	120

Normal, Vitamin B_{12} Deficient, Folate Deficient and Post-Gastrectomy Sera

The vitamin B_{12} values for a group of normal sera assayed by the three methods are shown in Fig 4. The results obtained by the radioisotopic assay are a little higher (range 235–1470, mean 497 pg/ml) than those obtained by the *L. leichmannii* assay (range 155–1075, mean 471 pg/ml) and the *E. gracilis* assay (range 170–1144, mean 460 pg/ml).

The vitamin B_{12} values for a group of sera from patients with untreated pernicious anaemia

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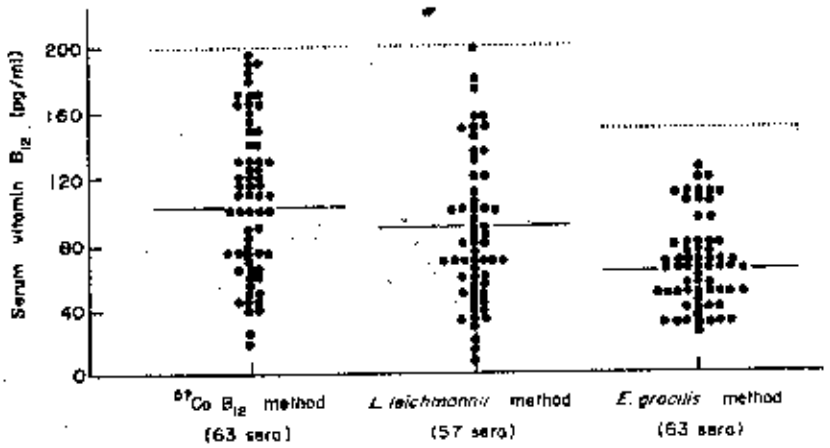

FIG 5. The vitamin B_{12} values obtained by the radioisotopic, *L. leichmannii* and *E. gracilis* assays for a group of sera from patients with untreated pernicious anaemia. In this and subsequent figures the unbroken lines represent the mean values and the dotted lines the lower limit of the normal range with each of the methods.

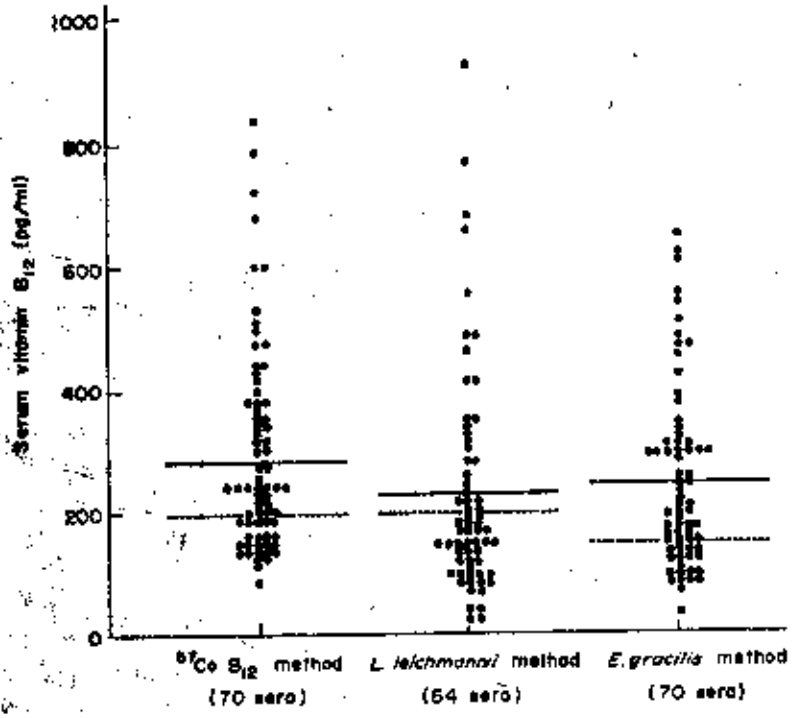

FIG 6. The vitamin B_{12} values obtained by the radioisotopic, *L. leichmannii* and *E. gracilis* assays for a group of sera from patients with megaloblastic anaemia due to folate deficiency.

are shown in Fig 5. The radioisotopic assay gave higher values (range 25–195, mean 106 pg/ml) than either the *L. leichmannii* (range 10–200, mean 88 pg/ml) or the *E. gracilis* assay (range 24–125, mean 66 pg/ml). Whereas sera from these patients had serum vitamin B_{12} values up to 200 pg/ml by either radioisotopic or *L. leichmannii* assays, only one serum had a vitamin B_{12} value (125 pg/ml) of more than 120 pg/ml by the *E. gracilis* assay.

The vitamin B_{12} values for a group of sera from patients with megaloblastic anaemia due to folate deficiency are shown in Fig 6. The folate deficiency was due to either inadequate diet, intestinal malabsorption or anticonvulsant drug therapy. Patients with folate deficiency

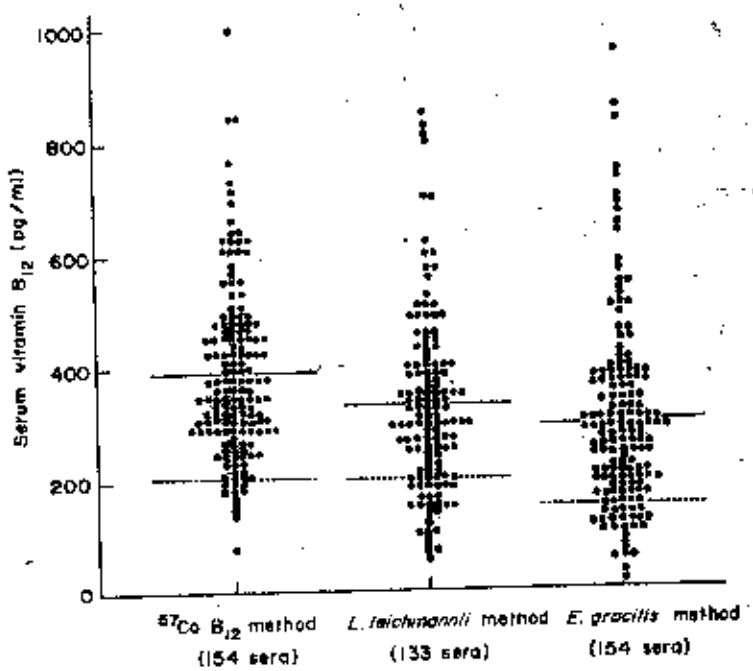

FIG 7. The vitamin B_{12} values obtained by the radioisotopic, *L. leichmannii* and *E. gracilis* assays for a group of sera from post-gastrectomy patients.

following gastric surgery were excluded from this group. The values obtained by radioisotopic assay (range 85–840, mean 287 pg/ml) are higher than those obtained by *L. leichmannii* (range 25–925, mean 231 pg/ml) or *E. gracilis* assays (range 35–650, mean 247 pg/ml). The *L. leichmannii* assay gave disproportionately more subnormal values (59%) than either the radioisotopic (33%) or the *E. gracilis* (29%) assays.

The serum vitamin B_{12} values for a group of post-gastrectomy patients are shown in Fig 7. As far as could be determined, none of the patients had received vitamin B_{12} therapy. The values obtained by the radioisotopic assay (range 75–1000, mean 386 pg/ml) are higher than those obtained by the *L. leichmannii* assay (range 55–810, mean 331 pg/ml) which in turn are higher than those obtained by the *E. gracilis* assay (range 16–960, mean 298 pg/ml). More sera gave subnormal values by the *E. gracilis* assay (14%) and the *L. leichmannii* assay (18%) than by the radioisotopic assay (4%).

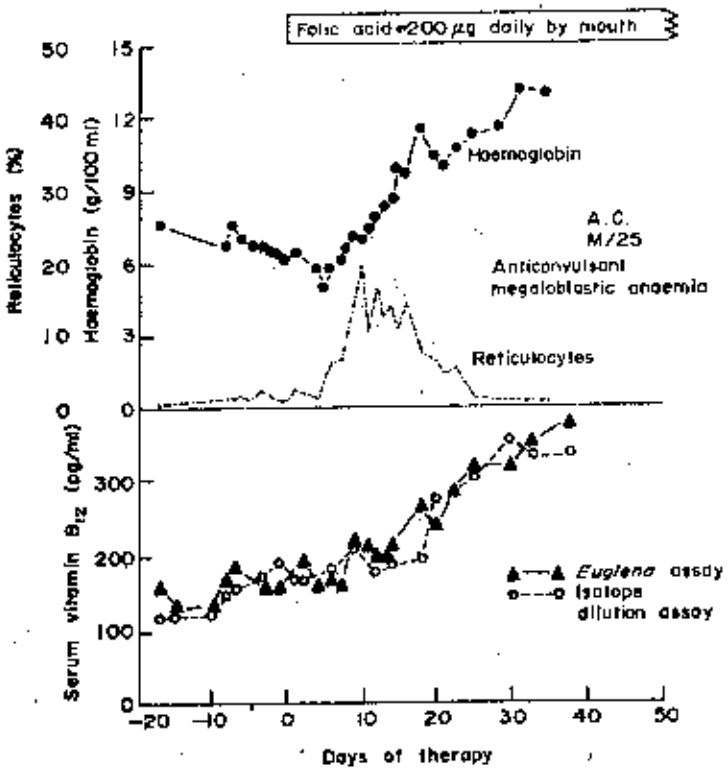

FIG 8. The changes in haemoglobin level, reticulocyte count and serum vitamin B_{12} level (determined by both radioisotopic and *E. gracilis* assay) observed in a patient with anticonvulsant drug-induced megaloblastic anaemia treated with folic acid 200 μg by mouth daily.

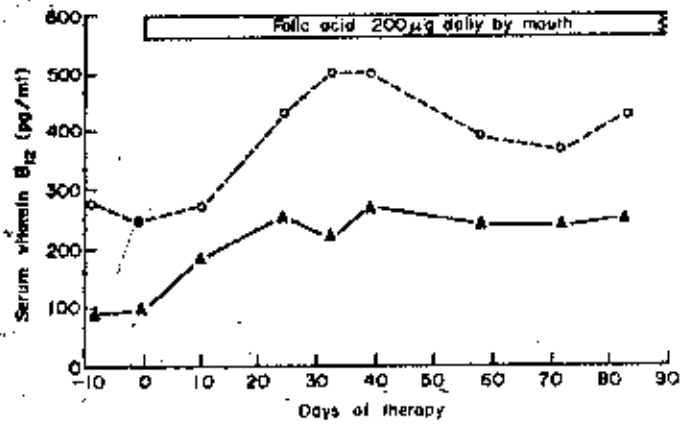

FIG 9. The changes in serum vitamin B_{12} level (determined by both radioisotopic (○) and *E. gracilis* (▲) assay) observed in a patient (A.B., F/57) with nutritional folate deficiency treated with folic acid 200 μg by mouth daily.

Effect of Folic Acid Therapy on the Serum Vitamin B_{12} Values in Two Patients with Folate Deficiency

Two patients with folate deficiency, one due to malnutrition, the other associated with anticonvulsant drug therapy, were treated with 200 μg of folic acid by mouth daily. Their serum vitamin B_{12} levels were measured before therapy and at frequent intervals during the haematological response by both the radioisotopic and *E. gracilis* methods. The values obtained are shown in Figs 8 and 9. In the case of the patient with anticonvulsant megaloblastic anaemia (Fig 8) both methods gave similar values throughout and the serum vitamin B_{12} level rose from approximately 120 pg/ml to over 300 pg/ml in 40 days by both assays. In the case of the patient with nutritional folate deficiency (Fig 9), the radioisotopic assay gave consistently higher values (between 100 and 200 pg/ml) than the *E. gracilis* assay, though with each method, the serum vitamin B_{12} values rose by a similar amount (150 pg/ml) during folic acid therapy.

DISCUSSION

The results of this study show that all three assays are satisfactory for diagnosing vitamin B_{12} deficiency in patients with untreated pernicious anaemia. In this respect the *E. gracilis* assay gave a particularly clear distinction from normal results while the *L. leichmannii* assay proved least satisfactory since the lowest normal value was 155 pg/ml whereas a proportion of pernicious anaemia patients gave results with this assay of up to 200 pg/ml. Ardeman *et al* (1966) also found that some sera from patients with untreated pernicious anaemia gave levels between 150 and 180 pg/ml with the *L. leichmannii* assay.

In general, the radioisotopic assay gave higher results than either of the other assays. This occurred at all levels of serum vitamin B_{12} values and in normal subjects as well as patients. The discrepancy between assays was most important when the serum vitamin B_{12} level was subnormal by one or both microbiological assays and normal by the radioisotopic assay. This situation arose most commonly in folate deficient and post-gastrectomy patients. For some of these post-gastrectomy patients whose sera gave widely discrepant results, repeat sera were taken at intervals of a month or more and also tested. Each assay gave a consistent result, but these continued to be 'set' 100 pg/ml or more apart.

The results in the two folate deficient patients also illustrate the point that it is the sera of particular patients which determine the degree of agreement or disagreement of the assays. In both patients, the serum vitamin B_{12} level rose, measured by both the *E. gracilis* and radioisotopic assay when folic acid was administered. In one patient, however, the results by the two assays on twenty separate serum samples were remarkably close to each other, whereas in the other patient the results with the radioisotopic assay on nine separate serum samples were consistently 100–200 pg/ml higher than with the *E. gracilis* assay.

It was not the purpose of the present study to say which assay gives the 'right' answer. Nor was an attempt made to explain why certain sera gave discrepant results with the different methods while other sera gave similar results by all three methods. The results show that the level of serum vitamin B_{12} measured with any of the three methods in individual patients must be interpreted carefully in the light of other clinical, haematological and biochemical findings.

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Vitamin B_{12}, A Cobalt Complex

Vitamin B_{12} was described by the undersigned (*Science*, April 16, p. 396) as a crystalline red compound which is highly active for producing hematological responses in patients with pernicious and other anemias (R. West. *Science*, April 16, p. 398; T. D. Spies, *et al.* *S. med. J.*, 1948, 41, 522, 523) and for the growth of *Lactobacillus lactis* (E. L. Rickes, *et al.* and M. S. Shorb. *Science*, April 16, pp. 396, 397), chicks (W. H. Ott, E. L. Rickes, and T. R. Wood. *J. biol. Chem.*, 1948, 174, 1047), and rats (G. A. Emerson, M. E. Zanetti, and T. R. Wood. To be published.) Independent isolation of an anti-pernicious anemia factor, presumably identical with B_{12}, has also been reported (E. L. Smith. *Nature, Lond.*, 1948, 161, 638).

Information on the chemical nature of B_{12} is of primary interest. The following observations on B_{12} have been made which appear useful for other chemical and biological studies.

Emission spectrographic analysis[1] of B_{12} has shown the presence of cobalt.[2] Vitamin B_{12} appears to be a cobalt coordination complex which, having 6 groups about the cobalt atom, could involve one or more organic moieties. The red color of B_{12} appears to be at least in part associated with its cobalt-complex character.

The presence of cobalt in vitamin B_{12} reflects significantly upon many biological studies which have shown that cobalt is an essential trace element in nutrition, and perhaps upon suggestions concerning cobalt as a trace contaminant in iron therapy of anemias (E. J. Underwood. *Proc. Soc. exp. Biol. Med.*, 1937, 36, 296). The nutritional significance of cobalt must be re-evaluated as the biological function of B_{12} is developed.

Cobaltous ion (1 μg/ml) was without activity for *L. lactis* as contrasted with the high potency of B_{12} (0.000013 μg/ml, half-maximal growth).

Randolph West has tested cobalt ion in two cases of pernicious anemia with negative results.[3] The average adult daily dietary intake of cobalt has been estimated at 100 μg (B. Ahmad and E. V. McCollum. *Amer. J. Hyg.*, 1939, 29A, 24).

Spectrographic examination of B_{12} also showed the presence of phosphorus. Although nitrogen was found to be present, tests for sulfur were negative.

Microbiological assay of an aqueous solution of B_{12} (74 μg/0.5 ml) showed that autoclaving for 15 min at 121° C did not change the activity within the experimental error of $11.4 \times 10^6 \pm 0.6 \times 10^6$ u/mg.

Vitamin B_{12} in 0.015 *N* sodium hydroxide solution (0.2 μg/ml) was inactivated (microbiological assay) at room temperature as follows: 20% (0.67 hr), 45% (6 hrs), 90% (23 hrs), 95% (95 hrs); it was inactivated in 0.01 *N* hydrochloric acid solution (10 μg/ml) as follows: 18% (3 hrs), 75% (23 hrs), 89% (95 hrs).

The cobalt-complex nature of vitamin B_{12} is an outstanding property.

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[1] We wish to thank Dr. Charles Rosenblum and Luise Anderson for this determination.

[2] Private communication from Dr. H. M. Walker, of Glaxo Laboratories, Ltd., discloses that Glaxo's factor also contains cobalt. E. L. Smith and L. F. J. Parker. *Proc. Biochem. Soc.*, in press; E. L. Smith. *Nature, Lond.*, in press.

[3] Personal communication; levels of 500 and 150 μg of cobaltous ion administered subcutaneously as acetate and chloride, respectively.

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Crystalline Vitamin B_{12}

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A crystalline compound which in microgram quantities has produced positive hematological responses in initial tests in patients with addisonian pernicious anemia has been isolated from liver.

The effectiveness of liver as a specific agent in the dietary treatment of pernicious anemia was described by Minot and Murphy in 1926 (*3*). After this discovery, many investigations were carried out upon the purification of the principle (or principles) in liver responsible for its therapeutic effectiveness. One of the difficulties in the development of purification methods was the lack of a sufficient number of available pernicious anemia patients for the testing of fractions resulting from purification attempts. Although many efforts have been made to develop a biological assay, no method has been described which has been definitely successful in guiding the fractionation work.

TABLE 1

APPROXIMATE VITAMIN B_{12} CONTENTS OF COMMERCIAL LIVER EXTRACTS FOR PARENTERAL USE
(15 U.S.P. injectable units/ml)

Source	Vitamin B_{12} content (microbiological assay)			
	LLD units/ml	μg/ml	μg/U.S.P. unit	Per cent of dry weight
Company A	72,000	6.5	0.4	0.003
Company B				
(Sample 1)	13,000	1.2	0.1	0.00055
(" 2)	19,040	1.7	0.1	.....
Company C				
(Sample 1)	154,000	14.0	0.9	0.014
(" 2)	80,000	8.0	0.5	0.0065
Company D				
(Sample 1)	29,000	2.6	0.2	0.001
(" 2)	38,000	3.5	0.2	0.0014

Commercial liver preparations, which are crude concentrates of the active principle, have been available for medical use for some time. The activity of these preparations is standardized by clinical tests.

Research in these laboratories in 1942, together with collaborative clinical tests conducted by Randolph West,[1] showed that further purification of the "anti-pernicious anemia" principle in commercial liver concentrates could be effected. Subsequently, these chemical and clinical studies were extended, and more recently Mary S. Shorb and George M. Briggs[2] collaboratively tested certain clinically highly active fractions for growth activity for *Lactobacillus lactis* Dorner and found them to be microbiologically active. This microorganism was found by Dr. Shorb to require two unidentified growth factors (*3*); one of them (LLD factor) appeared to be related to the activity of commercial liver preparations used in the treatment of pernicious anemia. For convenience in the testing of the fractions, use was made of an arbitrarily selected standard liver concentrate which was assigned a potency of 1,000 LLD units/mg.

Further purification of clinically active liver fractions[3] has led to the isolation, in minute amounts, of a crystalline compound which is highly active for the growth of *L. lactis*. This compound is being called vitamin B_{12}.[4] Its potency is about 11,000,000 LLD units/mg, and 0.000013 μg/ml of culture medium is capable of supporting half-maximal growth under the conditions used. This potency value was found by Dr. Shorb (*4*), using a 23-hr growth period. The compound crystallizes in the form of small red needles which, after drying, showed refractive indices of α, 1.616; β, 1.652; and γ, 1.664.[5] On the micro-stage, the crystals darken to black at about 210–220°, but do not liquefy below 300°.

Randolph West (*5*) has tested this crystalline compound for activity in the clinical treatment of pernicious anemia in relapse. In one patient a single intramuscular dose of 150 μg gave a very strong hematopoietic response; in two other patients doses of 3 and 6 μg, respectively, produced a prompt increase in the circulating reticulocytes, red cells, and hemoglobin. These results are supported by early tests conducted by Dr. West, in which three separate concentrates, containing by microbiological assay 2–5 μg of vitamin B_{12}, gave strongly positive responses in four patients.

The biological activity of the new vitamin is extremely high in terms of its activity in these tests on pernicious anemia. For example, using pteroylglutamic acid, hematopoietic responses have been obtained with doses of the order of 20,000–50,000 μg during the first 10 days of treatment (*1*).

It was of interest to determine the relative contents of vitamin B_{12} in several commercial liver extracts for parenteral use; the results of the assays are summarized in Table 1.

[1] Columbia University, College of Physicians and Surgeons.

[2] Department of Poultry Husbandry, Agricultural Experiment Station, University of Maryland. Dr. Briggs is now at the University of Minnesota.

[3] We are indebted to Dr. Shorb for many of the initial assays. We are also indebted to Miss Muriel Caswell and her colleagues of the Merck laboratories for microbiological assays.

[4] The reasons for this designation are as follows: The long expression "anti-pernicious anemia principle" is not advisable, largely because the biological role of this new compound in the treatment of pernicious anemia or other disease is yet to be learned. The term "LLD factor" implies a biological limitation and was used originally to name an activity. The biological activities of crude materials are frequently found later to be due to several chemically related entities, as exemplified in the vitamin and antibiotic fields. A trivial name based upon source or activity is undesirable. The name vitamin B_{12} has not been used in the B series and connotes only nutritional significance. We may in the future wish to designate a name based upon chemical structure.

[5] We wish to thank Charles Rosenblum and his associates for the determination of the optical properties.

One U.S.P. unit is defined (6) as that amount of liver extract required daily to produce satisfactory clinical and hematological responses in addisonian pernicious anemia. If it is assumed that this new crystalline compound is the only substance present in these preparations which is therapeutically active,[6] it is evident that clinical response should be obtained from the parenteral administration of approximately 1 μg of the new vitamin/day. The clinical responses obtained with single 3- and 6-μg doses of crystalline vitamin B_{12} are not inconsistent with the approximate equivalence of 1 μg of the vitamin and 1 U.S.P. injectable unit. It should be pointed out, however, that it is customary to administer 20–60 U.S.P. units of liver extract during the first two or three days to start remission of pernicious anemia in relapse. This dose range is equivalent to not more than about 20–60 μg of vitamin B_{12}.

Further research is in progress on the composition, structure, and biological activity of vitamin B_{12}.

During this research, we have had the pleasure and benefit of the interest and advice of Henry D. Dakin.

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CLEARANCE OF INTRAVENOUSLY INJECTED RADIOACTIVE COBALT-LABELED VITAMIN B_{12} IN CHRONIC MYELOID LEUKEMIA AND OTHER CONDITIONS

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THE INCREASED binding power of the serum of patients with chronic myeloid leukemia for vitamin B_{12} has been amply confirmed by a number of investigators and has been shown to be about 6 times that of the serum of normal subjects.[1, 10, 11] This binding protein has recently been shown to reside in the seromucoid fraction.[8] The average serum vitamin B_{12} concentration in such individuals has been estimated to be approximately 12 to 15 times that in normal subjects.[1, 7]

Following the intravenous injection of 1.5 μg. of radioactive cobalt (Co^{58})-labeled B_{12} with specific activity 0.57 μc. per μg., Mollin et al.[6] showed that the plasma clearance of radioactivity was slowest in patients with chronic myelocytic leukemia and in patients with pernicious anemia in severe relapse. Two subjects with chronic myelocytic leukemia were studied. One was given 1,000 μg. of nonradioactive vitamin B_{12} intramuscularly on the eleventh, twelfth, and thirteenth days after the intravenous injection. The intravenous injection of Co^{58}-labeled B_{12} was then repeated. It was observed that the rate of clearance was faster than it had been earlier but slower than in normal subjects. Miller et al.,[6] using an intravenous test dose of 4 μg. of Co^{60}-labeled B_{12} containing 3 μc. of activity, noted an even more marked delay in the clearance of radioactivity from the plasma of chronic myeloid leukemic patients. Cronkite and coworkers[3] similarly demonstrated the delayed clearance of Co^{60}-labeled B_{12} from the serum after injections of 1 to 3 μg. of the radioactive vitamin in patients with chronic myelocytic leukemia.

TABLE 1

CONDITIONS AND NUMBER OF SUBJECTS STUDIED

Condition	No. subj.
Chronic myeloid leukemia	7
Acute leukemia	3
Chronic lymphocytic leukemia	1
Multiple myeloma	2
Hematologically normal	3
Pernicious anemia (remission)	1
Polycythemia (remission)	1
Aplastic anemia	1
Hodgkin's disease	3
Carcinoma metastatic to liver	1
Chorioepithelioma	1
Cirrhosis of liver	1
TOTAL	27

It was the purpose of this investigation to study the serum clearance of intravenously injected radioactive B_{12} in leukemic subjects and other disease states as modified by loading doses of nonradioactive vitamin B_{12}.

MATERIALS AND METHODS

Subjects Studied. The subjects with leukemia in this investigation included both hospitalized patients and outpatients whose diagnosis was established by the usual abnormalities in the peripheral blood and bone marrow. Hematologically normal individuals served as controls. Patients with a variety of malignant tumors were also studied. All of the persons with chronic myeloid leukemia except 1 (I.B.) had received therapy before the tests were performed (Table 1).

Materials and Equipment. The Co^{58}-labeled B_{12} was supplied as a sterile solution with activity varying from 1.71 μc. per μg. to 3.78 μc. per μg. A dose of 1 μc. was calculated from the decay curve of Co^{58} and was injected intravenously. A standard was prepared at the same time by dilution of a suitable amount of the original material to 1,000 ml. Ten milliliters of this diluted solution served as a "plasma standard"; 100 ml. was used as a

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The radioactive cobalt (Co^{58})-labeled vitamin B_{12} was kindly supplied by Dr. N. S. Ritter, Merck & Co., Inc., Chemical Division, Rahway, N.J.

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TABLE 2

SERUM AND URINE LEVELS OF Co^{58}-LABELED B_{12} IN NORMAL SUBJECTS AND PATIENTS WITH MISCELLANEOUS CONDITIONS

Pt. init.	Pt. age, yr.	Pt. sex	Diagnosis	WBC, no./cu. mm.	Co^{58}-labeled B_{12} μc.	Co^{58}-labeled B_{12} μg.	% radioactiv. in serum, hr. 3	% radioactiv. in serum, hr. 24	% urinary excretion of I.V. dose, hr. 0-24	% urinary excretion of I.V. dose, hr. 24-48	% urinary excretion of I.V. dose, hr. 48-72
A.L.*	79	F	Arterioscler. heart dis.	7,800	1.0	0.950	6.5	2.1	...	...	...
				...	1.0	0.986	7.9	1.7	41.9†	1.8†	0.9†
R.A.*	18	M	Rheum. heart dis.	26,000	1.7	0.672	7.3	5.8	1.0	0.0†	...
				...	1.6	0.632	13.1	...	1.1	0.1	0.01
M.S.	61	M	Pern. anemia in remiss.	9,000	1.4	1.24	8.0	...	1.1	17.9†	6.5†
J.G.	47	M	Arterioscler. heart dis.	10,200	1.0	0.786	6.4	2.3	2.2	15.4†	8.7†
D.B.	7	F	Aplast. anemia	2,100	1.1	0.786	11.9	...	0.9	...	...
L.L.	72	F	Polycythemia	7,100	1.1	0.825	3.9	1.2	1.5	17.4†	4.2†
C.M.	71	F	Polycythemia	6,900	1.0	0.825	5.6	2.8	1.9	8.9†	5.1†
J.B.	45	M	Polycythemia	12,500	1.0	0.943	5.4	...	1.2	0.2	...
M.L.*	78	F	Ca. metast. to liver	7,800	1.0	0.328	8.5	1.5	...	...	...
				...	1.1	0.376	7.5	3.7	...	...	...
M.G.	40	F	Hodgkin's dis.	7,500	1.0	1.10	6.4	...	2.0	0.2	...
S.A.	19	F	Hodgkin's dis.	3,100	0.99	0.366	11.1	4.9	2.0	0.2	0.2
A.S.	68	M	Hodgkin's dis.	13,100	0.69	0.424	10.7	4.9	1.9	0.2	...
E.K.	28	F	Chorioepithel.	3,550	1.0	0.366	10.6	3.3	2.0	0.2	...
L.W.	52	F	Cirrhosis liver	10,600	0.88	0.482	3.8	2.3	2.0	0.2	...

*The second determination was made after B_{12} loading.
†One mg. of B_{12} within a 24-hour period.

"urine standard." Samples of plasma, serum, and urine were counted on the same day and compared with the standards.

The well counter was a modification of the type described by Hine and Miller,[8] consisting of a plastic scintillator well with a capacity of 80 ml. mounted on a photomultiplier tube surrounded by a 4-in. lead shield. With a background of 550 counts per minute (c.p.m.), 1 μc. of Co^{58} yields about 650,000 c.p.m. in this set-up. Counts were performed in duplicate for 5, 10, or 15 minutes. The counting error was less than 5%.

Procedures. Following the intravenous injection of approximately 1 μc. of Co^{58}-labeled B_{12} into the subject, a blood sample was taken

TABLE 3

SERUM AND URINE LEVELS OF Co^{58}-LABELED B_{12} IN PATIENTS WITH MYELOID LEUKEMIA

Pt. init.	Pt. age, yr.	Pt. sex	WBC, no./cu. mm.	Co^{58}-labeled B_{12} μc.	Co^{58}-labeled B_{12} μg.	% radioactiv. in serum, hr. 3	% radioactiv. in serum, hr. 24	% radioactiv. in serum, hr. 48	% urinary excretion of I.V. dose, hr. 0-24	% urinary excretion of I.V. dose, hr. 24-48
J.F.*	59	M	27,000	1.0	0.708	41.3	26.7	...	24.1†	5.3†
			...	0.98	0.708	13.9	4.8	...	1.9	...
B.D.*	64	F	79,500	1.4	1.08	34.2	21.4	18.1	16.7†	4.8†
			...	1.4	1.13	5.3	...	...	36.6†	7.1†
D.W.*	62	F	25,500	1.4	1.08	31.5	21.5	17.5	15.6†	6.8†
			...	2.0	1.13	3.7	...	...	79.7†	5.3†
H.M.*	45	F	79,400	1.5	1.13	19.0	...	10.9	28.1†	4.9†
			...	1.6	1.30	4.2‡	...	...	2.2	...
R.H.*	49	F	27,000	1.0	0.456	14.8	...	4.3	31.1†	8.1†
			...	1.0	0.472	7.6	...	...	39.4†	...
L.W.*	30	F	88,000	1.0	1.14	47.5	...	...	1.0	0.1
			...	1.1	1.34	6.9	...	...	4.9	0.2
I.B.¶	44	M	285,000	1.0	1.19	50.1	...	40.2	0.4	1.1
			...	1.0	1.34	67.8	...	...	1.4	0.2
			...	1.0	1.42	15.4	...	...	0.3	0.2
A.K.*	63	M	200,000	1.0	1.12	16.7	...	...	0.3	0.1
			133,000	1.0	1.20	2.6	...	...	3.2	0.4

*The second determination was made after B_{12} loading.
†One mg. of B_{12} within a 24-hour period.
‡Two-hour value.
¶The third determination was made after B_{12} loading.

TABLE 4

SERUM AND URINE LEVELS OF Co^{58}-LABELED B_{12} IN PATIENTS WITH VARIOUS LEUKEMIAS

Pt. init.	Pt. age, yr.	Pt. sex	Leukemia	WBC, no./cu. mm.	I.V. Co^{58}-labeled B_{12}		% radioactiv. in serum, hr.		% urinary excretion of I.V. dose, hr.	
					μc.	μg.	3	24	0-24	24-48
T.Z.	8	M	Acute stem cell	7,100	1.0	0.786	4.4	...	1.4	0.2
J.E.*	18	M	Acute lymphoblastic	4,700	0.2	0.058	7.8	3.1	2.6	0.01
B.G.	64	M	Chr. lymphocytic	124,000	1.3	0.386	8.4	5.3	1.0	1.3
					0.72	0.289	6.3	1.6	0.8	0.3
S.G.	65	F	Mult. myeloma	8,900	1.0	0.501	21.9	13.7	0.3	0.4
D.R.	23	M	Mult. myeloma	3,900	1.0	0.385	6.8	2.1	0.5	0.1

*Two determinations were made.

from the opposite arm in 1 minute. Subsequently, samples were withdrawn from either arm after 15 minutes and at 1 and 3 hours. In some cases, specimens were also taken at 24 and at 48 hours. Generally 20 ml. of blood was collected if possible. The blood was allowed to clot and the serum was separated. In instances in which this amount of serum could not be obtained, the volume was measured and made up to 10 ml. before counting. The percentage of the administered dose that remained in the serum at any given time was determined by the formula:[6]

$$\frac{\text{c.p.m./ml. serum} \times \text{plasma vol. (ml.)} \times 100}{\text{total c.p.m. injected}}$$

Plasma volume was estimated from the body weight using the formula:

$$\text{wt. (kg.)} \times 38 \text{ cc./kg.}$$

Urine collections were made at 24-hour intervals after the intravenous injection of Co^{58}.

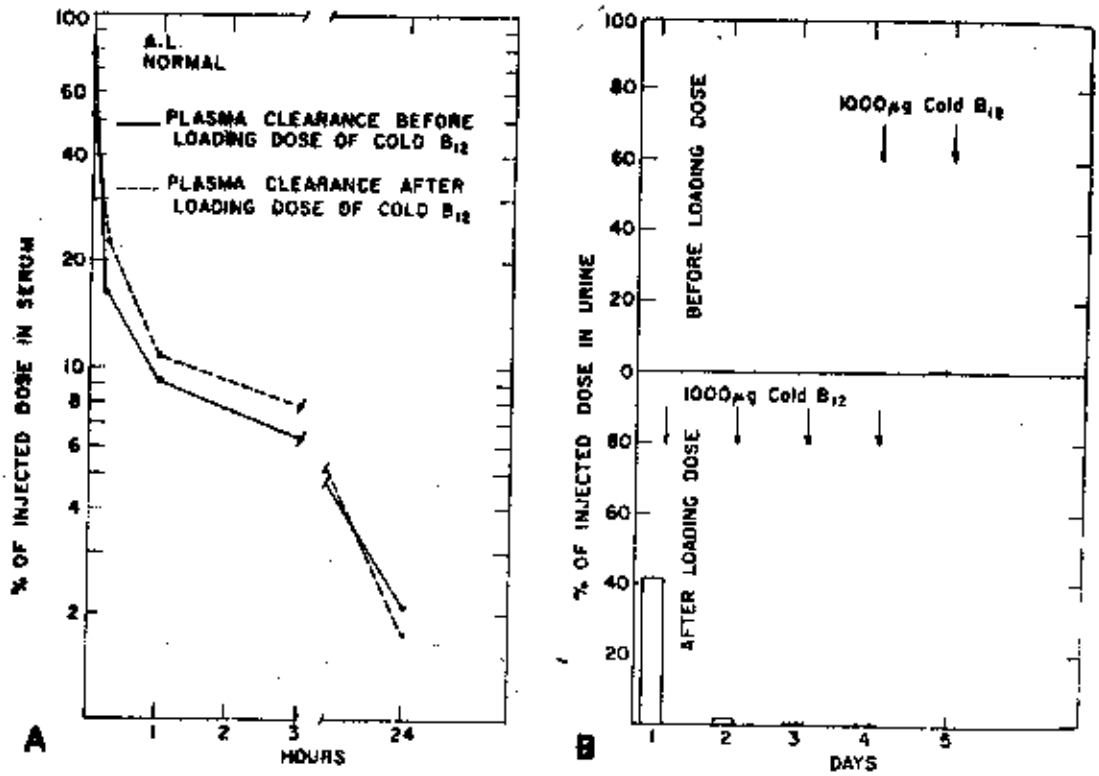

FIG. 1. A, Disappearance of an intravenously injected dose of Co^{58}-labeled B_{12} from the serum of a normal subject before and after an intramuscular loading dose of 1,000 μg. of "cold" B_{12}. B, The urinary excretion of radioactive B_{12} is high when the loading dose is given within the same 24-hour period.

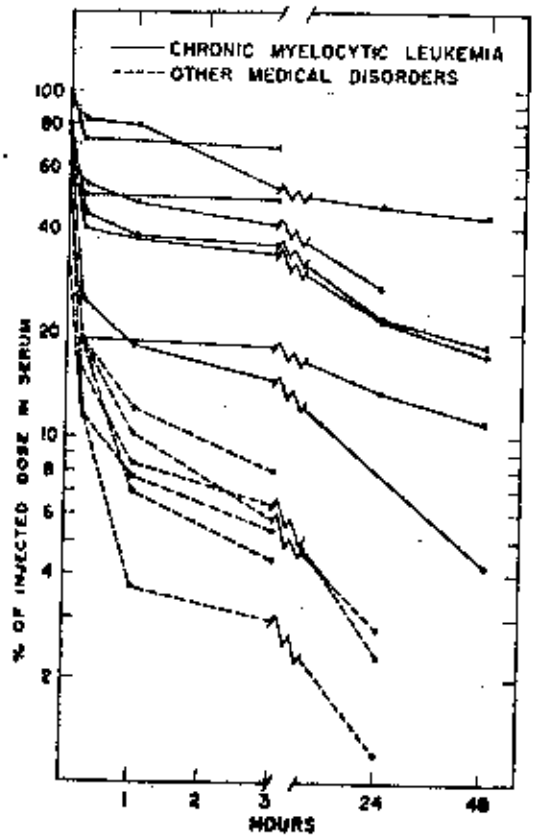

FIG. 2. Serum Co^{58}-labeled B_{12} clearance curves in chronic myelocytic leukemia contrasted with those for normal subjects and for those with other hematological abnormalities.

labeled B_{12}. The total urinary volume was measured. A 100-ml. aliquot was counted and compared against the 100-ml. urine standard the same day. The percentage excretion of the injected dose was calculated.

Loading doses of 1,000 μg. of nonradioactive vitamin B_{12} were injected intramuscularly at various intervals before or after administration of the radioactive B_{12}. This was frequently given after the 5-hour blood sample had been drawn and again 24 hours later. However the timing of the loading dose varied in different experiments. Twenty-four hour urinary collections were continued for at least 2 days after each injection.

RESULTS

Disappearance of Co^{58}-Labeled B_{12} Activity from the Serum. In normal subjects and in those persons with conditions other than myeloid leukemia, the serum radioactivity declined rapidly (Table 2). The mean value for retention of radioactivity in 7 patients with chronic myeloid leukemia and 1 with acute myeloid leukemia, A.K. (Table 3), was 36.2% (range 14.8 to 67.8%). In individuals with chronic myeloid leukemia, significant levels of radioactivity persisted after 48 hours. With 1 exception, S.G. (Table 4), an individual with multiple myeloma and a blood urea nitrogen of 100 mg. per 100 cc., less than 12% of the radioactivity remained in the serum after 5 hours in patients with other leukemias. The mean for 19 cases, prior to any vitamin B_{12} loading was 9.0% (range 3.8 to 21.9%). Excluding the aforementioned case, the highest level observed was 13.1%.

There was no significant change in the serum clearance of Co^{58}-labeled B_{12} in the normal and nonmyeloid groups after loading doses of "cold" B_{12} (Fig. 1). However, after 1,000 μg. of "cold" B_{12} the 8 subjects with myeloid leukemia showed significantly faster disappearance of radioactivity, resembling the normal curves. After 5 hours, radioactivity in the serum fell to a mean value of 7.5% (range 2.6 to 15.4%). These changes are summarized in Figs. 2 and 3.

Urinary Excretion Studies. In the group

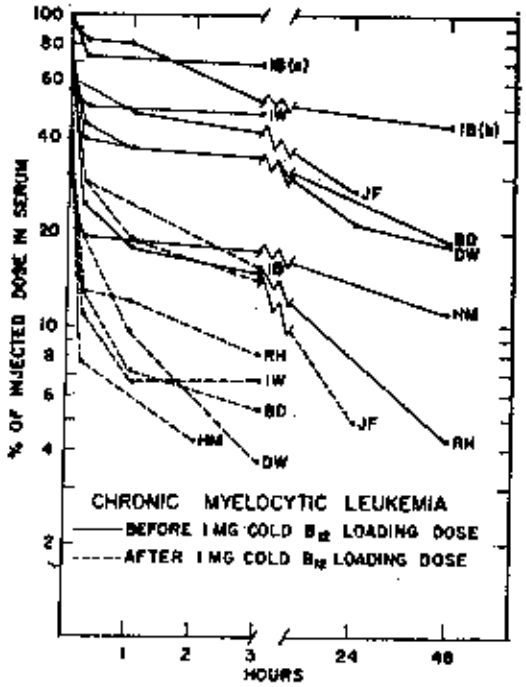

FIG. 3. Serum Co^{58}-labeled B_{12} clearance curves in chronic myelocytic leukemia before and after loading doses of "cold" B_{12}.

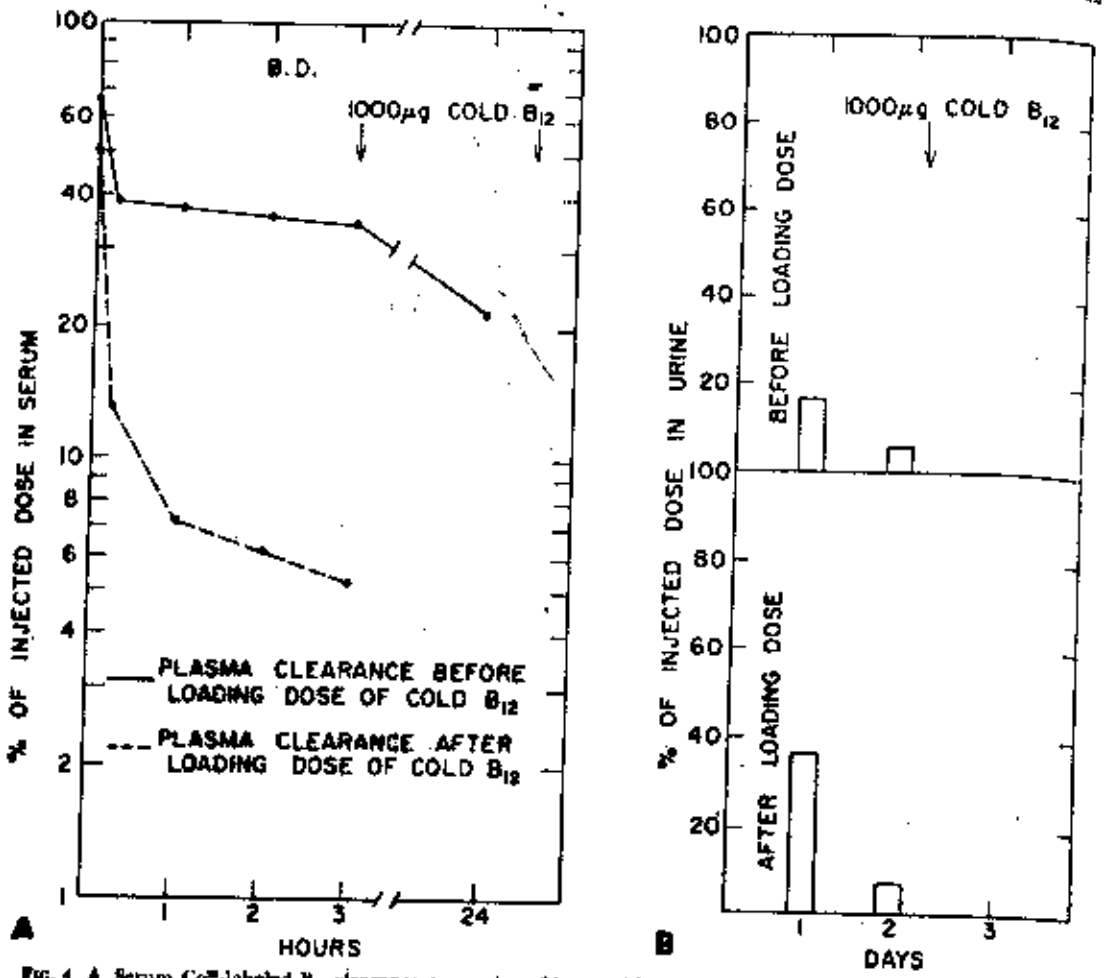

FIG. 4. A, Serum Co^{60}-labeled B_{12} clearance curves in a 64-year-old patient with chronic myeloid leukemia before and after a 1,000-μg. loading dose of "cold" B_{12}. B, There is an interval of 2 days between the 2 curves.

studies, 17 individuals without myeloid leukemia excreted an average of 1.5% (range 0.3 to 2.2%) of the intravenous dose of radioactive vitamin B_{12} in the first 24-hour urinary collection. The excretion in the second 24 hours was negligible (Tables 2 and 4). Three subjects with myeloid leukemia (I.W., I.B., and A.K.) who did not receive loading doses of "cold" B_{12} in the first 24 hours showed an average urinary excretion of 0.8% (Table 3).

When a loading dose of 1 mg. of nonradioactive B_{12} was administered intramuscularly in the same 24-hour period as the intravenous Co^{60}-labeled B_{12} injection, the urinary excretion of radioactivity was greatly enhanced. Figure 1A indicates a normal individual (A.L.) who excreted 41.9% of the administered radioactivity after a "flushing" dose of "cold" B_{12}.

Patients with chronic myeloid leukemia also showed a high urinary excretion of radioactive B_{12} after a loading dose of the "cold" vitamin (Fig. 4). These values ranged from 36 to 79% of the administered dose. In order to determine whether the rapid drop in serum radioactivity induced by vitamin B_{12} saturation was due to increased urinary excretion or increased tissue binding, the 3-hour loading dose was omitted after the second intravenous clearance test. It is evident that in patients J.F. and H.M. this rapid decline occurred although there was little or no urinary excretion of radioactive B_{12}. In patients A.K. and I.W., loading doses were separated from intravenous radioactive B_{12} administration by 3 or 4 days. Similarly, there was a negligible urinary excretion but a sharp fall in serum activity (Fig. 5). Subject I.B. had 2 consecutive intravenous injections of Co^{60}-labeled B_{12} 1 week apart without "cold" B_{12} loading (Fig. 6). The level of activity in the serum remained

high, indicating no observable effect from the second intravenous dose of vitamin B_{12}. Four days were allowed to elapse and the patient was then given 1,000 μg. of "cold" B_{12} intramuscularly. Two days later, a third clearance test was performed. Again there was a rapid fall in serum radioactivity with a negligible urinary excretion.

Discussion

The vitamin B_{12} binding substance in normal subjects and patients with chronic myeloid leukemia has been shown to be an acidic protein in the seromucoid fraction related to orosomucoid.[11] It has been suggested that the increased binding power of myeloid leukemic serum was due to an increased amount of this substance rather than to an abnormal protein. Myeloid leukemic leukocytes, normal leukocytes, and erythrocytes do not bind vitamin B_{12}.[8, 10, 18] More recent studies[5] have shown a high Co^{60}-labeled B_{12} binding capacity of intact normal leukocytes that is increased after sonic disintegration of these cells. There has been some difference of opinion as to the vitamin B_{12} binding capacity of normal and leukemic serum. Miller and Sullivan[7] estimated the unsaturated vitamin B_{12} capacity of normal subjects to be 0.64 mμg. per ml. and the total binding capacity to be 2.23 mμg. per ml. The corresponding values for chronic myeloid leukemic subjects were 8.87 mμg. per ml. and 16.86 mμg. per ml. Meyer, Bertcher, and Cronkite,[4] using a slightly different technique, found values of Co^{60} binding capacity of serum in chronic myeloid leukemia to be as high as 28 mμg. per ml.

All investigators are agreed that the total vitamin B_{12} content of the serum, as well as the binding capacity for vitamin B_{12}, is increased in chronic myeloid leukemia. It is therefore not surprising that an intravenously administered dose of radioactive B_{12} is retained longer and at higher levels in the serum of a leukemic individual than in a normal subject. Mollin et al.[9] used a test dose of 1.5 μg. of vitamin B_{12} containing 0.86 μc. of activity or less. Miller et al.[6] used a 4-μg. intravenous dose containing 3.0 μc., asserting that this amount exceeded the total plasma vitamin B_{12} by about threefold but was within

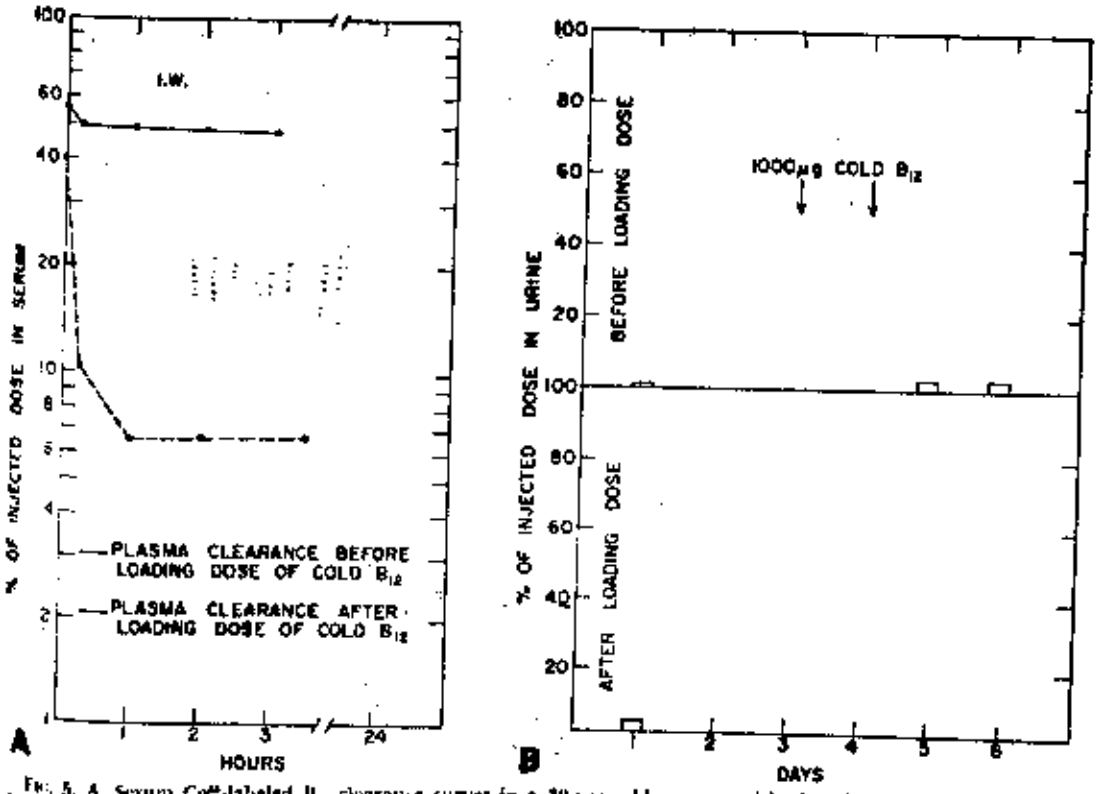

FIG. 5. A. Serum Co^{58}-labeled B_{12} clearance curves in a 30-year-old woman with chronic myeloid leukemia before and after loading doses of "cold" B_{12}. B. There is an interval of 6 days between the 2 curves.

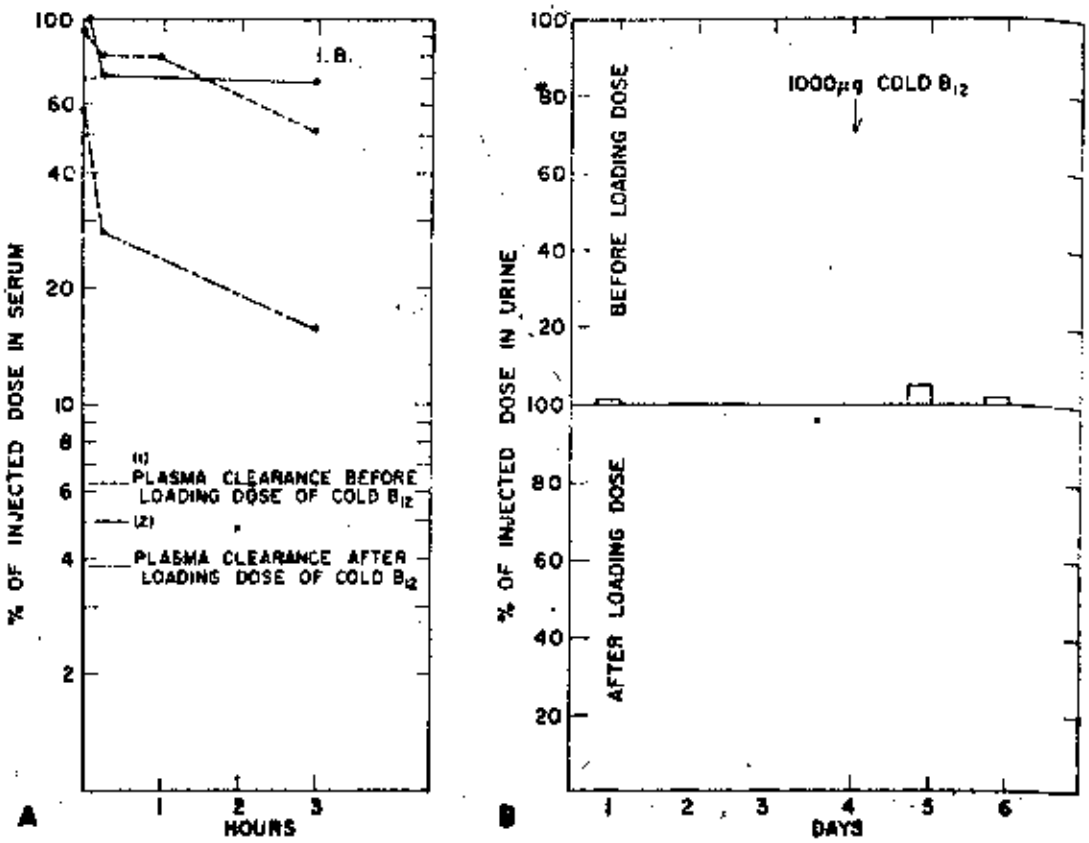

FIG. 6. A, Serum Co^{58}-labeled B_{12} clearance curves in a 44-year-old man with chronic myeloid leukemia. Two consecutive intravenous injections of radioactive B_{12} were first given 1 week apart (curves 1 and 2). B, Four days after the second injection a single loading dose of 1,000 μg. of "cold" B_{12} was given. Two days later the third clearance test was performed.

the total binding capacity of chronic myelogenous leukemic serum. In our studies, for the most part, a dose of 1.0 to 1.5 μc. of Co^{58}-labeled B_{12} was employed, which was found to give convenient counting levels. The amount of vitamin B_{12} varied from 0.06 μg. to 1.42 μg., since it did not appear that the dosage in this range was critical. Moreover, smaller quantities of vitamin B_{12} more nearly approximated tracer amounts. In patient J.E., an 18-year-old man with acute lymphoblastic leukemia, 2 determinations, using first 0.06 and then 0.89 μg. intravenously gave approximately the same disappearance curve (Table 4).

Mollin and his group[9] and Miller et al.[6] have shown that the clearance of intravenously injected radioactive B_{12} from the blood of chronic myeloid leukemic patients is delayed. We have confirmed these findings and have found a normal clearance in a variety of other conditions, including lymphocytic leukemia, Hodgkin's disease, and polycythemia in remission. Normal values were also obtained in subjects with pernicious anemia in remission, aplastic anemia, choriopithelioma, and 1 individual with metastatic carcinoma of the liver. In pernicious anemia in relapse, the delayed clearance is probably due to defective tissue uptake.

It is of interest that after a loading intramuscular dose of 1 mg. of nonradioactive B_{12} to subjects with granulocytic leukemia, the serum clearance curve of Co^{58}-labeled B_{12} can be modified to a normal pattern. The urinary measurements revealed that this more rapid disappearance of activity from the serum is not dependent on increased urinary excretion. External counting over the liver and spleen was not done in our studies. However, others[4] have shown that there is no increased uptake over these organs during the first 5 hours in either normal subjects or leukemic patients. Rachmilewitz et al.[12] found that the activity measurable over the liver after an intravenous

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injection increased slowly over the next 6 to 8 days. This slow accumulation would not account for the initial rapid fall in serum levels. We must therefore assume, as suggested by Miller et al.,[6] that diffuse general tissue binding of vitamin B_{12} occurs and that the material is then gradually transferred to the liver, where it is stored. When the plasma receptors are saturated, tissue binding is enhanced.

SUMMARY

1. The clearance of intravenously injected radioactive cobalt (Co^{58})–labeled vitamin B_{12} from the serum has been studied in 27 persons, including 8 with myeloid leukemia.

2. The disappearance of activity was delayed in myeloid leukemic subjects regardless of the clinical and hematological status, but rapid in those with the other conditions studied.

3. Loading doses of nonradioactive vitamin B_{12} administered to these leukemic patients converted the delayed clearance to one in the normal range.

4. The mechanism of this conversion was not due to increased excretion of radioactivity. It is thought that saturation of plasma receptors by the loading doses thereby enhanced tissue binding of vitamin B_{12}.

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Thiamine-responsive megaloblastic anemia

An 11-year-old Caucasian girl is presented who had a megaloblastic anemia responsive only to thiamine. Other abnormalities included diabetes mellitus, aminoaciduria, and sensorineural deafness. Initially the anemia, refractory to vitamin B_{12} and folic acid therapy, responded to administration of a multiple vitamin preparation. Vitamin supplementation was withdrawn followed by a recurrence of anemia 3½ months later. The implicated vitamins were then administered sequentially. A reticulocytosis followed administration of thiamine. Anemia again recurred 4 months after cessation of vitamin supplementation. On this occasion the anemia was corrected by the oral administration of 20 mg. thiamine daily. Thiamine blood levels and activities of 3 thiamine-dependent enzymes of the patient's blood cells were normal, excluding a generalized defect of thiamine metabolism. The patient therefore appeared to have a thiamine-dependent megaloblastic anemia. This represents the first demonstration of a role for this vitamin in DNA metabolism.

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DURHAM, N. C.

MEGALOBLASTIC ANEMIAS of childhood are rare and are most often the result of vitamin B_{12} or folic acid deficiency. Rarer causes are hereditary orotic aciduria,[1] sideroblastic anemias,[2] and malignancies of the di Guglielmo variety.[3] This report presents a patient whose megaloblastic anemia was unresponsive to folic acid and vitamin B_{12} but responded to the administration of thiamine. Anemia recurred twice during periods of normal dietary thiamine intake and was corrected by supplementation with the vitamin. This unique response of a megaloblastic anemia to thiamine led to additional studies of the patient, including the activities of known thiamine-dependent enzymes, blood thiamine levels, and the incorporation of nucleosides into lymphocyte nucleic acids.

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CASE REPORT AND CLINICAL STUDIES

The patient, an 11-year-old Caucasian girl, was born November 11, 1954, following an apparently normal gestation. The birth weight was 3.6 kilograms; growth and development during infancy were normal. The family history revealed no relatives with deafness, diabetes, or

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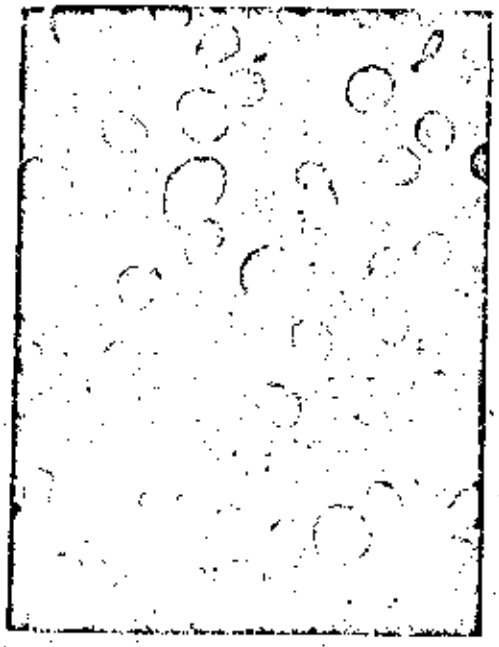

Fig. 1. Peripheral blood smear showing macrocytosis, anisocytosis, and poikilocytosis.

Fig. 2. Bone marrow showing megaloblasts.

unexplained anemia. At 3 years of age the patient developed diabetes mellitus, and at 4½ years of age hearing loss was first noticed. Bilateral sensorineural deafness was complete by age 7½ years. Hemoglobin determinations done at age 1, 7, and 10 7/12 years were 10.5, 11.5, and 10 Gm. per cent, respectively. In February, 1966, a severe anemia was first detected; the hemoglobin was 6.0 Gm. per cent. Oral iron was administered for 2½ months without response, and a packed erythrocyte transfusion was then given. On April 5, 1966, a smear of a bone marrow aspirate revealed megaloblastic changes. A serum vitamin B_{12} level measured 756 $\mu\mu$g per milliliter (Bioscience Laboratories). Oral folic acid (5 mg. 3 times daily for 2 weeks) and parenteral vitamin B_{12} (100 μg daily for 1 week) were administered without hematologic response. The patient was then referred for evaluation.

Upon admission to Duke University Medical Center Hospital on June 28, 1968, the patient appeared pale and was obviously deaf. The weight was 32 kilograms, the height 134 cm. The tympanic membranes were normal in appearance. A soft precordial hemic murmur was audible; otherwise, the heart was normal. The liver, spleen, and lymph nodes were not palpably enlarged. There were no edema, peripheral neuropathies, or other signs of beriberi.

The results of the admitting laboratory work were as follows: hemoglobin 5.8 Gm. per cent, hematocrit 19 per cent, leukocytes 3,500 per cubic millimeter, platelets 145,000 per cubic millimeter, reticulocytes 4 per cent. The differential leukocyte count was: 4 bands, 49 polymorphonuclear forms, 32 lymphocytes, 11 monocytes, 4 eosinophils. The peripheral blood smear (Fig. 1) revealed moderate poikilocytosis and anisocytosis with numerous macrocytes, giant platelets, and a slight degree of nuclear hypersegmentation of the granulocytes. A smear of a bone marrow aspirate showed erythroid hyperplasia with numerous megaloblasts (Fig. 2). Little stainable iron and no ring sideroblasts were present. Hemoglobin electrophoresis revealed A hemoglobin. The sickle cell preparation was negative. Erythrocyte autohemolysis was 0.17 per cent at 24 hours, 0.84 per cent at 48 hours. Incubated erythrocyte osmotic fragility was normal. The serum iron concentration was 115 μg per cent; the total iron binding capacity was 300 μg per cent.

Beginning on July 3, 1966, Berocca-C,* 2 ml., was administered parenterally once daily for 5 consecutive days. A reticulocyte response of 10 per cent was first apparent on the fourth day of treatment and reached a peak of 19 per cent on

*Berocca-C (Roche) contains per 2 ml.: thiamine HCl 10 mg., riboflavin 10 mg., pyridoxine 20 mg., nicotinamide 80 mg., panthenol 20 mg., biotin 0.2 mg., ascorbic acid 100 mg.

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the eighth day of treatment. The reticulocytosis was followed by a rise in hemoglobin to a maximum of 9.8 Gm. per cent on July 19, 1966 (Fig. 3). At the same time the leukopenia and thrombocytopenia were corrected. A posttreatment bone marrow obtained on July 13, 1966, showed improvement but not complete resolution of the megaloblastic changes. Deca-Vi-Sol,† 1 chewable tablet daily, was administered from July 6 through July 17, 1966.

On July 6, 1966 (after 3 days treatment with Berocca-C) the patient experienced transient hypoglycemia. The diabetes had been well controlled with an 1,800 caloric diet, NPH insulin 40 units, and regular insulin 20 units per day. During the following 10 days, however, hypoglycemic attacks necessitated a decrease of insulin dosage to approximately one half the pretreatment level. Supplemental vitamins were not administered after discharge from the hospital, and a balanced 1,800 calorie diabetic diet was continued unchanged.

Follow-up laboratory tests on August 2, 1966, and August 29, 1966, revealed no change in the hematologic status. Blood smears continued to show moderate anisocytosis and poikilocytosis. By late August the insulin requirements had increased to 50 units N.P.H. insulin daily.

Signs of hematologic relapse were present on Oct. 24, 1966, approximately 3½ months after the first remission. The hemoglobin was 7.8 Gm. per cent, hematocrit 24 per cent, leukocyte count 5,400 per cubic millimeter, reticulocytes 1.8 per cent, platelets 107,500 per cubic millimeter, erythrocytes 2.34 × 10^6 per cubic millimeter, MCV 102 cubic microns, MCH 33 $\mu\mu g$, MCHC 33 per cent. The morphologic abnormalities of the peripheral blood smear were again present. The bone marrow was frankly megaloblastic. Stainable iron was present, but there were no ring sideroblasts.

During the second admission to the hospital the vitamins present in Berocca-C were given in a sequential manner to ascertain which vitamin, if any, had produced the original response. The vitamins given, the reticulocytosis, and the hemoglobin response are shown in Fig. 4. A reticulocytosis followed the administration of thiamine. The reticulocyte count peaked at 22 per cent, 9 days after the initiation of thiamine, and was followed by a rise of hemoglobin to 13.6 Gm. per cent. The borderline thrombocytopenia and leukopenia were also corrected, but insulin requirements did not decrease as previously. In an attempt to again induce improvement of the diabetes, biotin, pantothenic acid, and finally Berocca-C were administered. The insulin requirements did not change.

Following the last injection of Berocca-C on Jan. 5, 1967, all supplemental vitamins were discontinued. No medication except insulin was taken. Hematologic remission continued for 4 months until May, 1967. Upon readmission in relapse, the hemoglobin was 8.5 Gm. per cent, erythrocytes 2.46 × 10^6 per cubic millimeter, leukocytes 6,000 per cubic millimeter, hematocrit 26 per cent, reticulocytes 6.3 per cent, platelets 180,000 per cubic millimeter, MCV 106 cubic microns, MCH 35 $\mu\mu g$, MCHC 33 per cent. The bone marrow and peripheral blood smears showed the previously described abnormalities.

Administration of oral uridine 1.0 Gm. per day for 5 days (May 11 to 15, 1967) and yeast DNA 1.5 Gm. per day for 5 days (May 16 to 20, 1967) failed to produce a hematologic response. Following these trials, thiamine, 20 mg. per day orally, was begun on May 22, 1967. Reticulocytosis began on the fourth day of treatment and peaked at 14.5 per cent on the eleventh day. The hemoglobin rose to 13.7 Gm. per cent by the twenty-eighth day of thiamine administration (Fig. 5). The leukocyte and platelet counts

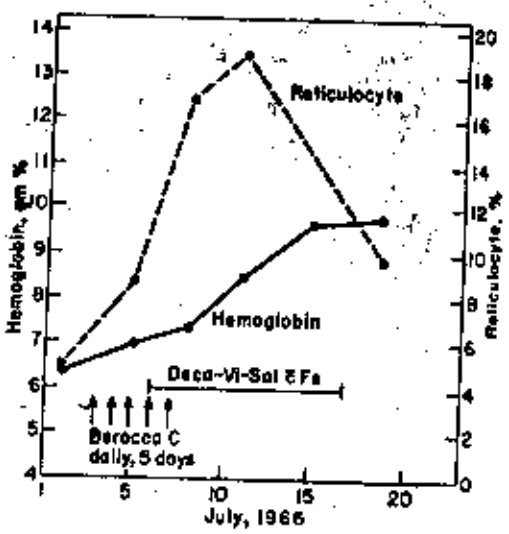

Fig. 3. Reticulocytosis and rise of hemoglobin following administration of a multiple vitamin preparation, Berocca-C.

†Deca-Vi-Sol (Mead Johnson) contains per tablet: vitamin A 4,000 units, vitamin D 400 units, ascorbic acid 75 mg., thiamine 1.2 mg., riboflavin 1.5 mg., niacinamide 15 mg., pyridoxine 1.2 mg., pantothenic acid 5 mg., vitamin B_{12} 3 μg, biotin 40 μg, ferrous fumarate 40 mg.

were within the normal range during the second relapse and did not change with treatment.

Following each of the hematologic responses, the abnormalities of the peripheral blood smear were not completely corrected; anisocytosis and poikilocytosis of erythrocytes persisted.

In addition to the anemia, diabetes, and deafness, other abnormalities were present. An electroencephalogram obtained during hematologic relapse showed the following features: spike wave abnormalities of the temporal region of a 55½ per second frequency and not exceeding 100 microvolts amplitude. In the parietal region, 6 per second high voltage discharges with amplitudes in the 200 microvolt range were seen. There was no alteration of this bizarre tracing with therapy. The patient had never had any seizure activity. Although her motor development was normal, her intellectual development was somewhat impaired. The deafness and unwillingness to cooperate with simple instructions had made an exact evaluation of intelligence impossible, but her achievement at school was on a kindergarten level.

Other laboratory findings included the following: serum sodium 136 mEq. per liter, potassium 5 mEq. per liter, carbon dioxide 28 mEq. per liter, calcium 9.8 mg. per cent, phosphorus 4.1 mg. per cent; blood urea nitrogen 9 mg. per cent; total serum protein 7.8 Gm. per cent, albumin 4.6 Gm. per cent, globulin 3.2 Gm. per cent, IgG 1,150 mg. per cent, IgA 100 mg. per cent, and IgM 59 mg. per cent, PBI 6.9 μg per cent, serum glutamic-oxalacetic transaminase 15.4 units, serum glutamic-pyruvic transaminase 40 units, zinc turbidity 16 units, alkaline phosphatase 7.0 Bodanski units, and prothrombin time 83 per cent of normal. Routine urinalysis revealed: specific gravity 1.005, pH 6.5; the reactions for protein, sugar, and acetone were

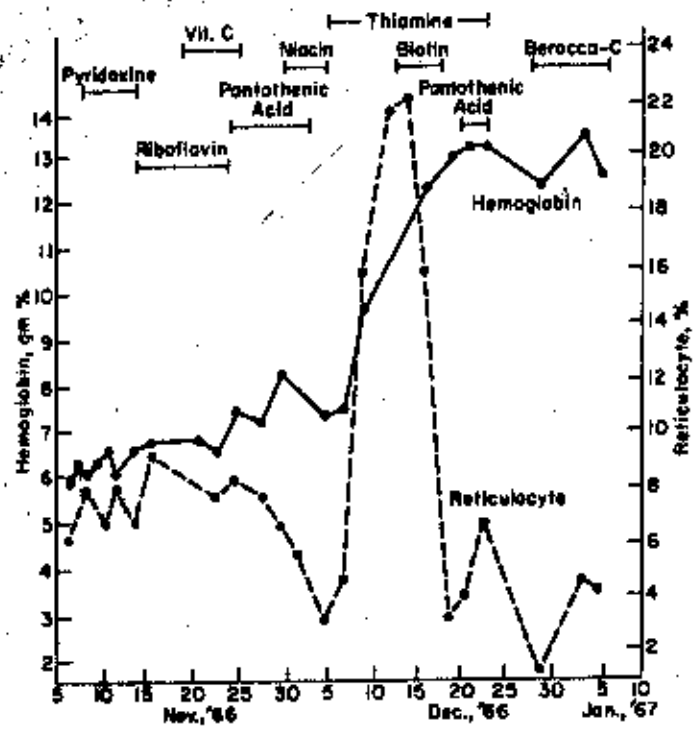

Fig. 4. Reticulocytosis and rise of hemoglobin following administration of thiamine. Lack of response to pyridoxine, riboflavin, vitamin C, niacin, and pantothenic acid is shown. The daily dosages and dates of administration of the vitamins are as follows: pyridoxine 25 mg., Nov. 8-13, 1966; riboflavin 30 mg., Nov. 14-22; ascorbic acid 50 mg., Nov. 19-23; pantothenic acid 250 mg., Nov. 24-Dec. 2; niacin 50 mg., Nov. 30-Dec. 4; thiamine 50 mg., Dec. 5-16; thiamine 25 mg., Dec. 17-22; biotin 10 mg., Dec. 12-16; pantothenic acid 63 mg., Dec. 20-22; Berocca-C 2 ml., Dec. 28-31; Berocca-C 1 ml., Jan. 1-5, 1967. All vitamins were given intramuscularly except biotin and riboflavin, which were taken orally.

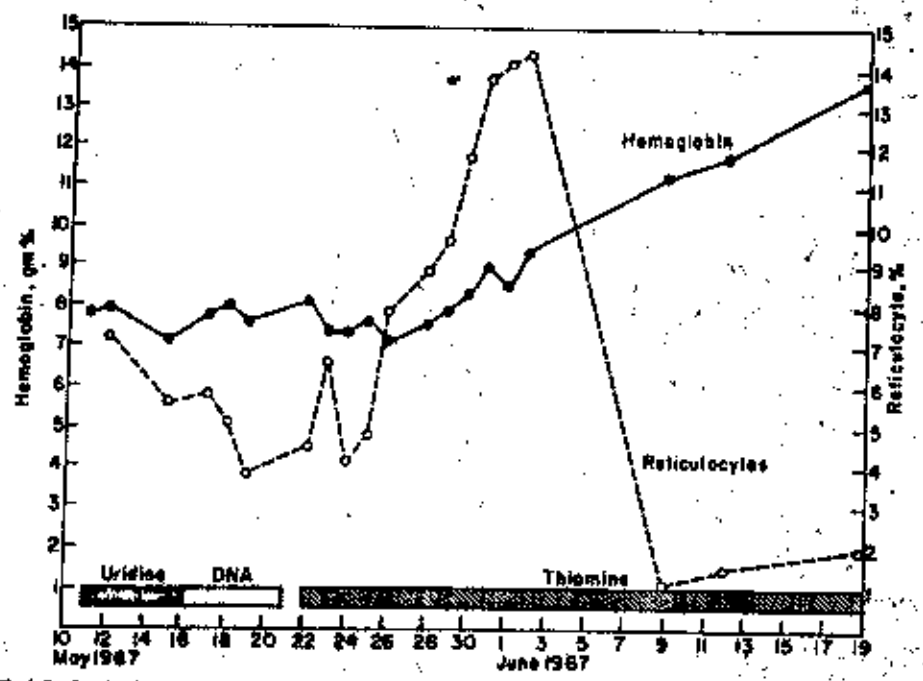

Fig. 5. Reticulocytosis and rise of hemoglobin following administration of oral thiamine 20 mg. per day. Lack of response to uridine and DNA is shown.

negative; the microscopic examination revealed an occasional epithelial cell. Urinary $FeCl_3$, DNPH, and CTAB tests were negative.

Additional studies of special interest included the following: Activities of the erythrocyte enzymes, orotidylic pyrophosphorylase, and orotidylic decarboxylase[4] were determined and were not decreased. Urinary screening test for hereditary orotic aciduria[5] was also negative. Urinary formiminoglutamic acid excretion was not excessive after histidine load.[6] Urinary excretion of methylmalonic acid and xanthurenic acid during relapse was also normal. The diabetes was unresponsive to administration of tolbutamide. An electrocardiogram during relapse was normal.

Following the last remission the patient took 20 mg. thiamine orally per day and maintained a normal hemoglobin for more than one year.

SPECIAL STUDIES

The response of the megaloblastic anemia to thiamine suggested at least two possibilities regarding the underlying defect in this patient. The disorder might involve primarily the metabolism of thiamine and result secondarily in the malfunction of numerous thiamine-dependent enzymatic processes, or the defect may lie in a specific thiamine-dependent enzyme so that it alone operates suboptimally. To investigate these possibilities, the activities of 3 thiamine-dependent enzymes were assayed and blood thiamine levels were measured. The megaloblastic features of the anemia led to studies of the incorporation of uridine and thymidine by lymphocytes and of lymphocyte transformation following stimulation by phytohemagglutinin. The finding of elevated urinary amino acids led to their quantitation in urine and plasma.

METHODS

Erythrocyte transketolase. The method used was that reported by Wolfe and associates.[7] Erythrocyte transketolase is assayed by cycling 2-C^{14}-glucose through the pentose shunt of the glycolytic pathway, a process requiring the activity of this thiamine-de-

pendent enzyme. Loss of enzymatic activity is reflected by a deficient production of carbon dioxide-C^{14} during the second transit of the sugar molecule through the pentose pathway. An erythrocyte suspension of known concentration was prepared from heparinized blood by centrifugation and by removal of plasma and buffy coat, followed by resuspension in an equal volume of buffer of pH 7.4 constituted as follows: 20 mM. sodium phosphate, 5 mM. magnesium chloride, 15 mM. sodium chloride, and 4 mM. potassium chloride. This buffer was also used in the other enzyme assays to be described. The reaction mixture consisted of a 0.4 ml. volume of the erythrocyte suspension and 0.2 ml. of aqueous 0.025 per cent methylene blue with 0.06 μc (5.06 μmoles) 2-C^{14}-glucose made up to a 1 ml. volume with buffer. Duplicate samples were run with and without thiamine hydrochloride in a final concentration of 10^{-4} M. A blank was prepared by omitting the erythrocyte suspension. The samples were incubated at 37° C. in a shaking water bath for 3 hours. The $C^{14}O_2$ was collected in hydroxide of Hyamine which was counted in a toluene-phosphor mixture in a Packard Tri-Carb Liquid Scintillation Counter. The results are expressed as mμmoles of CO_2 per 10^8 erythrocytes per hour. This and the following assays described were performed with blood taken from the patient during relapse. No supplemental thiamine was being administered. The enzymatic activities of control blood samples were determined simultaneously.

Leukocyte pyruvic dehydrogenase. Pyruvate is decarboxylated by leukocyte pyruvic dehydrogenase to form acetyl-CoA and carbon dioxide. The activity of this thiamine-dependent enzyme was assayed by the recovery of carbon dioxide-C^{14} following the incubation of leukocytes with 1-C^{14}-pyruvate. A modification of the method published by Dreyfus and Hauser[8] was used. Cell suspensions were prepared by removal of plasma from heparinized whole blood following centrifugation. Since erythrocytes lack this enzyme, no attempt was made to remove them. Sedimented cells were resuspended in a volume of the buffer described above to provide a concentration of leukocytes from 5,000 to 10,000 per cubic millimeter (Coulter Counter). A volume of 0.7 ml. of this suspension was combined with 0.4 ml. of the buffer containing 0.017 μc (72 μmoles) 1-C^{14}-sodium pyruvate. From this point the assay was performed with and without thiamine, as described for the erythrocyte transketolase assay. Results are expressed as mμmoles CO_2 per 10^6 leukocytes per hour.

Leukocyte α-ketoglutaric dehydrogenase. The carboxyl carbon of α-ketoglutaric acid is released as CO_2 upon the conversion of this acid to succinyl-CoA in the presence of α-ketoglutaric dehydrogenase. The activity of this thiamine-dependent enzyme was assayed by the collection of carbon dioxide-C^{14} when labeled α-ketoglutaric acid was used as the substrate. Leukocytes were prepared and counted as previously described. A 0.5 ml. volume of the suspension was added to 0.5 ml. of buffer containing 0.08 μc (6.35 μmoles) 1,2-C^{14}-α-ketoglutaric acid. From this point the assay was performed with and without thiamine, as described for the transketolase assay. Enzymatic activity of a normal control sample was determined simultaneously. The results are expressed as mμmoles CO_2 per 10^6 leukocytes per hour.

Blood thiamine assay: Blood thiamine was assayed according to the method of Haugen.[9] Thiamine pyrophosphate was hydrolyzed with phosphatase to form the free vitamin which was oxidized by potassium ferricyanide to the fluorescent thiochrome. The fluorescence was measured after extraction of the thiochrome into isobutanol. Powdered Taka-diastase (Parke-Davis) was used as a source of phosphatase.[10] All samples were measured in duplicate.

Leukocyte transformation and incorporation studies. The basic medium was Eagle's MEM[11] with Earle's balanced salt solution. The pH was adjusted to 7.4 with 5 per cent sodium bicarbonate. For the studies designed to demonstrate thiamine dependency, the medium was identical except that thiamine

was omitted in preparation. Phytohemagglutinin P was obtained from Difco Laboratories. Plasmagel was obtained from the Laboratorie Roger Bellon. H^3-thymidine and H^3-uridine were obtained from New England Nuclear Corp.

Heparinized blood was obtained from the patient when in relapse and mixed with ¼ volume of Plasmagel for sedimentation of the red cells. One milliliter of cells suspended in the plasma-Plasmagel mixture was transferred to a stoppered test tube containing 4 ml. of culture medium containing penicillin and streptomycin as the antibiotics. Phytohemagglutinin P was added in a volume of 0.1 ml. Cultures were incubated at 37° C. for 72 hours. Velban (Grand Island Biological Co.) was added two hours before harvesting to effect metaphase arrest. The cells were harvested and prepared after the method of Moorhead and associates.[18]

In the studies with H^3-thymidine and H^3-uridine incorporation, 4 μc of the tritiated nucleotide was added to the media 6 hours before harvesting. After the slides were fixed and stained, they were dipped in Kodak nuclear tract emulsion (NBT2) and kept in the Cambridge box for 6 weeks before being developed.

Aminoacid studies. Aminoacid analyses were performed on aliquot parts of 24 hour urine collections. Plasma aminoacids were determined after precipitation of the protein with picric acid which was removed by passage through a column of Dowex 2 x 10. The aminoacids were separated and quantitated with a Beckman Spinco amino acid analyzer using the conditions described by Spackman and associates.[19]

Loading tests were performed by oral administration of 150 mg. per kilogram of the amino acids lysine and valine; the tests were carried out before and during therapy with thiamine. Because of technical limitations only a 3 hour sample was obtained after loading, this being a time when the values would be expected to have returned to near control levels in the normal individual, but could be expected to be elevated if there were delayed clearance.[14]

RESULTS

Erythrocyte transketolase. Averaged values of duplicate determinations are expressed as mμmoles CO_2 per 10^8 erythrocytes per hour. Activities for the patient's erythrocyte transketolase were 12.0 with thiamine added to the reaction mix, and 11.9 without added thiamine. Corresponding activities for the control subject were 6.3 and 4.9, respectively.

Leukocyte pyruvic dehydrogenase. Results are expressed as mμmoles CO_2 per 10^9 leukocytes per hour. Averaged values for activity of the pyruvic dehydrogenase for the patient were 1.6 with and 1.5 without added thiamine. For the control the respective values were 2.0 and 2.3.

Leukocyte α-ketoglutaric dehydrogenase. With enzyme activities expressed as mμmoles CO_2 per 10^6 leukocytes per hour, averaged values for duplicate samples for the patient were 0.45 with added thiamine and 0.29 without added thiamine. For the control the values were 0.51 with added thiamine and 0.38 without added thiamine.

Blood thiamine assay. Values for duplicate samples of total blood thiamine for the patient during relapse averaged 4.8 μg per cent. Normal values by this method range from 2.48 to 10.77 μg per cent with an average of 5.28 μg per cent.[9]

Leukocyte transformation and incorporation studies. The studies of lymphocyte transformation consequent to phytohemagglutinin stimulation were indistinguishable in the media with and without added thiamine in terms of the percentage of large lymphocytes and mitotic figures. No difference was demonstrated in tritiated thymidine or uridine incorporation with or without added thiamine in the media, as judged from the number of grains per cell. Normal control cells run simultaneously could not be distinguished from the patient's cells by the response given.

Amino acid studies. The urinary content of amino acids in a 24 hour specimen were: valine 61 mg. per 24 hours before therapy and 47 mg. per 24 hours during therapy with thiamine, compared with a normal range of 4 to 6 mg. per 24 hours; lysine 74 mg. per 24 hours before and 203 mg. per 24 hours

Table I. Plasma amino acids (milligrams per 100 ml.). The quantitative results of plasma aminoacids in the control period and 3 hours after valine and lysine loading (150 mg. per kilogram) are presented. The loading tests were run before therapy was instituted and several days after high doses of thiamine had been administered. Average adult control values are listed for comparison[32]

Amino acid	Fasting	Valine load		Lysine load		Average adult control[32]
		Before thiamine therapy	After thiamine therapy	Before thiamine therapy	After thiamine therapy	
Aspartic acid	Trace	0.09	Trace	0.40	Trace	0.5
Threonine	0.76	1.99	2.29	3.35	1.49	2.0
Serine	0.78	1.89	1.69	3.41	1.72	1.4
Proline	0.99	4.62	0	2.70	0	2.6
Glutamic acid	0.81	2.43	1.10	4.16	1.04	1.1
Glycine	1.20	3.48	1.67	4.24	1.40	2.8
Alanine	1.35	8.44	4.9	7.97	4.29	3.8
Half cystine	Trace	Trace	Trace	0	Trace	1.0
Methionine	Trace	Trace	0.37	1.06	0.55	0.35
Isoleucine	Trace	0.22	1.46	2.98	1.76	1.6
Leucine	0.81	1.69	1.99	7.37	1.98	2.0
Tyrosine	Trace	0	1.07	0	1.2	1.4
Phenylalanine	Trace	0	0.69	0	0.83	1.6
Histidine	1.35	1.01	1.65	3.52	1.30	1.7
Arginine	Trace	Trace	0.91	2.56	Trace	2.1
Lysine	1.26	3.98	1.68	20	4.36	2.8
Valine	1.46	38.16	27.81	9.75	3.2	3.0

during therapy, compared with normal range of 7 to 48 mg. per 24 hours.[25] The other amino acids were present in normal quantities. The finding of elevated valine and lysine levels in the urine prompted the investigation of the plasma levels and loading response to these 2 amino acids. The plasma sample was obtained before and 3 hours after the load to discriminate abnormal clearance of the loaded dose from the normal.[24] The values obtained with the valine loading were abnormal and were the same before and after thiamine therapy. Lysine clearance was definitely abnormal before, but normal after, therapy was instituted. There was a small but significant rise in proline with both loading tests before therapy, and a decrease to undetectable levels in response to these loading tests after therapy was commenced (Table I).

DISCUSSION

All of the usual causes of megaloblastic anemia may be excluded in this patient. Classical pernicious anemia is excluded by the normal serum vitamin B_{12} level, the failure of response to parenteral vitamin B_{12}, and the normal urinary excretion of methylmalonic acid. Folic acid deficiency is similarly excluded by the lack of response to folate therapy and by the normal urinary excretion of formiminoglutamic acid. di Guglielmo's erythremic myelosis is a progressive, malignant process unresponsive to known hematinics and usually terminates fatally within months.[8] Hereditary orotic aciduria is excluded by assays of the activities of the enzymes, orotidylic pyrophosphorylase and orotidylic decarboxylase, and by the urinary screening test for this disorder.[4, 5] Megaloblastic changes are also found in the bone marrow of some patients with sideroblastic anemia. Signs of folate deficiency and a partial response to this vitamin or to pyridoxine are sometimes observed. Characteristic findings are increased numbers of ringed sideroblasts in the marrow and an excessive excretion of urinary xanthurenic acid. The patient's anemia, on the other hand, was unresponsive to both pyridoxine and folic acid,

the bone marrow was not iron loaded, and the urinary excretion of xanthurenic acid and formiminoglutamic acid was not elevated. Isolated cases of megaloblastic anemia totally unresponsive to therapy or responding to unusual therapeutic regimens have been reported.[14-19] These disorders, however, do not present the same features as the patient reported here.

The response of this patient's anemia to the administration of thiamine is unique and suggests one of several possible derangements of thiamine metabolism: deficient intake, defective absorption, excessive excretion, inadequate utilization, or an increased requirement. The first three possibilities may be excluded for the following reasons: (1) Clinical signs of beriberi were not present, (2) blood thiamine levels were normal, (3) erythrocyte transketolase activity was not reduced. The transketolase assay is an especially sensitive indicator of thiamine deficiency, and low blood levels of this vitamin for any reason (decreased intake, inadequate absorption, or excessive excretion) would be expected to result in decreased activity.[20] Furthermore, anemia, if present at all, is a late manifestation of thiamine deprivation. The presence of a severe anemia without any other signs of beriberi is not readily explained on the basis of a generalized thiamine deficiency.

Thiamine pyrophosphate is a cofactor for several essential enzymatic reactions such as the transketolation reaction and the decarboxylation of α-ketoacids. In an attempt to exclude a generalized failure of thiamine utilization in this patient, 3 thiamine-dependent enzymes were assayed: erythrocyte transketolase, leukocyte pyruvic dehydrogenase, and leukocyte α-ketoglutaric dehydrogenase. The activities of these particular enzymes were found to be normal, and their activities were not significantly altered by the addition of thiamine hydrochloride in vitro. The finding of 3 normally functioning thiamine-dependent enzymes in this patient's blood cells demonstrates that the defect does not affect all enzymes utilizing this vitamin cofactor.

The most reasonable explanation for the response of this child's anemia to thiamine is an increased requirement for this vitamin, a thiamine-dependent state. Whereas the ingestion of normal dietary amounts of thiamine was insufficient to prevent the development of anemia, it was corrected by the administration of amounts significantly greater than the accepted minimum daily requirement. This finding suggests that normal hematopoiesis is made possible in this child by the elevation of tissue thiamine concentrations. High thiamine intake may load tissues and expand stores 2 to 3 times normal,[21] but once supplementation is withdrawn, a prompt decrease of stores to pretreatment level is to be expected. In the studies of Brin,[20] for example, human volunteers were given 5 mg. thiamine daily for 5 days, an amount adequate to replete all stores, and thereafter thiamine-poor diets were ingested. Biochemical evidence of thiamine deficiency was apparent 8 days later, and symptoms of depletion appeared within 21 days. Apparently so long as this patient's tissue level of thiamine could be maintained by supplementation at superphysiologic levels, normal hematopoiesis was possible. With physiologic levels of tissue thiamine, however, hematopoiesis was retarded and within weeks resulted in anemia. The response of the disorder to large but not to physiologic amounts of the vitamin has a parallel in pyridoxine-responsive anemia.[2]

The megaloblastic features of the anemia suggest that the postulated defect involves the synthesis of nuclear material (DNA). Until now there has been no evidence to implicate thiamine in DNA synthesis. To study this possibility further, DNA and uridine were administered to the patient. In addition, lymphocytes obtained during hematologic relapse were incubated with labeled thymidine and uridine and examined autoradiographically. The administration of DNA and uridine failed to produce a hematologic response, and the patient's lymphocytes incorporated thymidine and uridine normally. In addition, lymphocytes stimulated with

phytohemagglutinin underwent characteristic blast transformation. From these studies the mechanism of impairment of nucleic acid metabolism accounting for the megaloblastic changes could not be demonstrated. The failure to demonstrate an abnormality in lymphocyte nucleotide incorporation during transformation suggests that the thiamine deficiency is limited to the myelocytic and erythroid series.

Whether this patient's diabetes and deafness should be considered an integral part of the thiamine dependency is difficult to assess. Defective DNA synthesis, implied by the megaloblastic anemia, could also result in functional derangements of the pancreatic islet cells and of the cochlear nerve cells. The failure of the deafness and diabetes to respond to therapy does not necessarily indicate a lack of association with thiamine dependency. Therapy was not administered until years after the onset of symptoms, and the disorders in these tissues may be irreversible.

A role for thiamine in lysine and valine metabolism has not been described. Thiamine administration greatly altered postloading plasma lysine levels, implying a functional role for this vitamin in lysine metabolism. The abnormal urinary and plasma levels of valine after loading, however, was not changed by therapy. If the normal metabolism of this amino acid is in fact dependent upon thiamine, an irreversible lesion in this case must be postulated.

One could postulate that this patient's disease involves a single defective enzyme requiring excessive thiamine concentrations for normal activity. In the presence of usual physiologic concentrations of the vitamin, this enzyme alone functions suboptimally and results in megaloblastic anemia. In contrast, a generalized thiamine deficiency alters the function of multiple thiamine-dependent enzymes and produces an entirely different clinical spectrum characterized as beriberi. Megaloblastic anemia is absent in beriberi, but this absence does not necessarily indicate that thiamine plays no role in blood formation. Rather, it suggests that lack of thiamine affects other metabolic processes more profoundly. Thus, a failing heart or a neurologic deficit may result in death before thiamine tissue concentrations fall to levels limiting hematopoiesis. Only when metabolic pathways most sensitive to thiamine deficiency are spared would the role of thiamine in blood formation be demonstrable. If a single thiamine-dependent enzyme is defective so that it alone requires increased concentrations of the vitamin, disordered hematopoiesis could occur in the absence of either thiamine deficiency or signs of beriberi. This postulated single-enzyme defect would, therefore, allow the demonstration of the heretofore unsuspected participation of thiamine in hematopoiesis.

SUMMARY

An 11-year-old Caucasian girl is presented with a megaloblastic anemia, refractory to folate and vitamin B_{12}, but responsive to treatment with thiamine. The initial response followed the administration of a multiple vitamin preparation. Withdrawal of supplemental vitamins resulted in a relapse within 3½ months. When the implicated vitamins were given sequentially, a reticulocytosis followed the administration of thiamine. A second relapse of the anemia occurred 4 months after cessation of vitamin supplementation. On this occasion the anemia was corrected by 20 mg. oral thiamine daily. The response of anemia to superphysiologic quantities of this vitamin supports a thiamine-dependent state and demonstrates a previously unsuspected participation of thiamine in hematopoiesis. Additional abnormalities include diabetes mellitus, aminoaciduria, and sensorineural deafness.

The assay of urinary methylmalonic acid was performed by Frank A Oski, M.D., Hospital of the University of Pennsylvania, Philadelphia, Pa. The assay of urinary xanthurenic acid was performed by Dr. Ernest E. McCoy, University of Virginia School of Medicine, Charlottesville, Va. The amino acid analysis was performed courtesy of Dr. Paul Webster, Veterans Administration Hospital, Durham, N. C.

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The Site of Absorption of Co^{58}-Labeled Vitamin B_{12} in Man

An Investigation Made by Intestinal Intubation with Polyethylene Glycol as a Marker Substance

By Vagn Rønnov-Jessen and Jacob Hansen

UNTIL RECENT YEARS it was believed that the absorption of vitamin B_{12} in man took place in the upper and middle portions of the small intestine.[4,7,8] However, studies in animals, especially dogs, have revealed that considerable absorption takes place in the ileum, whereas it is negligible in the proximal small intestine[2,10] unless very large doses are given.[11,15]

Clinical observations in patients, after extensive resections of the ileum or intestinal short circuitings with exclusion of considerable portions of the ileum, seem to indicate that in man the absorption of B_{12} also takes place almost exclusively in the distal portions of the small intestine.[1,5,14] The methods of investigation did not show, however, whether the activity was localized to the visceral contents or the intestinal wall itself.

In this paper we report our study of the absorption of vitamin B_{12} in the various divisions of the human small intestine.

Materials and Methods

The series consisted of 8 men and 2 women between 22 and 71 years of age. None of them showed signs of organic disorders in the alimentary tract, but a few of them suffered from minor dyspeptic complaints. The diagnoses are listed in table 1.

After the small intestine had been intubated, the patients received an aqueous test meal, to which was added Co^{58}-labeled vitamin B_{12} and a marker substance. After this, samples of intestinal juice were simultaneously collected from different levels of the small intestine.

A tri-bore polyethylene tubing was used (Portex). Each channel had a sectional area of about 3 sq. mm. The tubing of all three channels was perforated with four or five holes over a length of about 10 cm: in channel 1, 30 to 50 cm. from the distal end of the tubing; in channel 2, 80 to 100 cm. proximally to this; and in channel 3 a further 80 or 100 cm. above the most distal hole in channel 2. The lumen of every channel was occluded with a metal stopper below the most distal hole.

The tubing was introduced through the nose and drawn out at the mouth. A small rubber pouch containing about 10 Gm. of mercury was sewn to the distal end of the tubing after which the patient swallowed it. Its progress in the small intestine was controlled by x-rays with a view to placing the rubber pouch in the caecum. The bag with mercury thus showed the position of the distal end of the tubing and the metal stoppers the location of the perforations inside the small intestine. During fluoroscopy radio-opaque solution was injected into the channel leading to the distal holes. This outlined loops of the tubing and by delineating the contours of the surrounding gut, the contrast fluid sometimes helped to decide the distal position of the tubing. When this was in its place, or if after 48–60 hours it would no longer pass anally, the test meal was administered.

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The test meal consisted of 500 ml. of water with an addition of 0.5 μg. of Co^{58}-labeled vitamin B_{12} with a specific activity of 1 μc. per μg. vitamin B_{12}. In order to secure satisfactory absorption, 100 mg. of hog intrinsic factor* were added. In addition, ½ per cent of polyethylene glycol (PEG) with a molecular weight of about 4000, was added as a marker substance. Twenty to thirty minutes after the administration of the test meal each channel was connected to a water suction pump with a receptacle. Suction was intermittent from the upper and middle channels so the amount of the test meal which reached the distal end of the tubing would not be reduced too much. Suction was continued for 4–5 hours with a view to recovering at least 3–4 samples of 10–15 ml. from each level.

A radiographic survey of the abdomen at the end of the experiments showed the position of the tubing had remained unaltered in all cases.

The PEG concentration of the samples was determined by the colorimetric method described by Hydén[12] and the radioactivity was measured in a well type scintillation detector. Samples and background counting rates were counted up to 10,000 counts. The test meal was used as a standard.

The percentage activity of the collected samples as compared with that of the standard sample was determined by the following formula:

$$1. \quad \frac{\text{Activity of sample}}{\text{Activity of standard}} \times 100 \text{ per cent}$$

The concentration of PEG in the samples was also calculated in percentages of that of the standard solution:

$$2. \quad \frac{\text{Extinction of sample}}{\text{Extinction of standard}} \times 100 \text{ per cent}$$

The vitamin B_{12} percentage which remained in the samples was calculated from the ratio of 1 to 2:

$$3. \quad \frac{\text{Activity of sample}}{\text{Activity of standard}} \times \frac{\text{Extinction of standard}}{\text{Extinction of sample}} \times 100 \text{ per cent}$$

Samples with a PEG concentration below 15 per cent of the standard were rejected, as PEG in such concentrations could not be determined with sufficient accuracy. The measurement of the activity also became increasingly unreliable in these low concentrations, the background counting rates sometimes amounting to 50 per cent or more of the entire counting rates.

Results

One intubation of the small intestine was carried out in each patient. In 4 cases (numbers 2, 4, 7 and 8) the pouch with mercury was successfully placed in the caecum. When the distance from the nostrils to the pylorus was estimated at 60 cm., the distances from the pylorus to the caecum were 320, 240, [illegible] and 330 cm., respectively, in these cases. In 1 case (number 9) the tubing passed down to the middle of the transverse colon and when it was withdrawn a "creasing" or "folding" of the gut took place so it only measured 210 cm. from the pylorus to the caecum. In these 5 cases the position of the perforations inside the intestine could be easily determined since their places on the tubing were known.

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*Intrinsic factor was prepared from fresh hog pyloric mucosa which was desiccated in vacuum and defatted. One hundred mg. of the preparation corresponds with 600 mg. fresh pyloric mucosa. The preparation was standardized by its effect on the Schilling test in patients with pernicious anemia.

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In the last 5 cases in which the tubing did not reach the caecum, only the distance of the perforations from the pylorus was known. According to Blankenhorn et al.[8] and our observations in the first four experiments, the length of the small intestine was estimated at 300 cm. Holes at a distance of less than 100 cm. from the pylorus were considered to be situated in the proximal third of the gut, holes at distances between 100 cm. and 200 cm. (from the pylorus) in the middle third. When the distance exceeded 200 cm., they were considered to be in the distal third of the gut. This occurred in experiments number 6 and 10.

As a rule, the sampling periods varied between 5 and 20 minutes although they were sometimes of longer duration because the suction was initially unsuccessful. Very often the suction started suddenly and then the "effective" period was in reality only of few minutes duration.

In 9 subjects a total of 42 samples were collected from the proximal third of the small intestine, 36 samples from the middle third in 8 cases and 26 samples from the anal third of the small intestine in 7 cases. The results are given in table 1. The activities actually found in the samples are indicated in per cent of the activities expected from the concentration of PEG. Figure 1 gives a graphical representation of the average values found in the various experiments.

In 6 of the experiments (1, 2, 5, 6, 8 and 10) samples from the oral third of the small intestine showed values around 100 per cent, whereas in the 3 other experiments (3, 4 and 7) they grouped themselves just under 90 per cent. The difference is significant whether it is calculated on the basis of single samples (12.1 per cent, S.E. ± 2.23 per cent ($p < 0.001$)) or from the average values (12.9 per cent ± 1.69 per cent ($p < 0.001$)).

In 7 experiments samples were collected from both the upper and middle third of the small intestine. The difference between the values is significant, whether it is calculated on the basis of the single samples (6.2 per cent, S.E. ± 2.41 per cent ($p < 0.02$)) or by comparison of the average values of each experiment (5.9 per cent ± 2.14 per cent ($p < 0.05$)).

In 6 experiments there were samples from both the middle and anal third. The difference between the results of the two groups is highly significant, both when calculated on the basis of the single sample (24.3 per cent, S.E. ± 4.31 per cent ($p < 0.001$)) and when the average values are compared (27.4 per cent ± 5.85 per cent ($p < 0.01$)).

Discussion

As far as we have been able to ascertain, previous investigations of the absorption of vitamin B_{12} in the human small intestine have not been done along the lines described above. The properties of PEG as a marker substance —water solubility, no absorption in the small intestine or to the mucous membrane, protein or the intestinal juice, no destruction in the alimentary tract, and an exact method for quantitive determination—have been prerequisites for obtaining reliable results.[6,12,13,16,17]

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Table 1.—*Absorption of Vitamin B_{12} in Various Parts of the Human Intestine*

	Sex, Age Diagnosis	Oral third of Intestine	Middle third of Intestine	Anal third of Intestine
1.	♂ 54 Psychoneurosis Coronary sclerosis ?	92–101–109	97–98	– – – –
2.	♂ 48 Psychoneurosis	106–105–106	95–97–102–95 82–69	49
3.	♂ 64 Remote myocardial infarction Gastroduodenitis ?	91–93–92–93–97–75	79–89–88–86–78–77–84–99	– – – –
4.	♂ 35 Psychoneurosis	90–81–89–97	– – – –	106–73–40–61–81
5.	♂ 49 Cerebral arteriosclerotic disease	112–100–102–91–101–99–97–98	– – – –	– – – –
6.	♂ 22 Psychoneurosis Constipation	91–92–106–118–112	115–101–100–108–105	86–82–70–76–75–79–110
7.	♂ 71 Aerophagia	94–85–96–94	88–85	85–79
8.	♂ 63 Psychoneurosis	102–102–97–102–102–94	100–95–91–85–89–96–88	55–65–50–44
9.	♀ 51 Psychoneurosis Aerophagia	– – – –	90–88–89	68–63–69–70
10.	♀ 50 Dyspepsia Hypercholesterolemia	102–104–101	104–96–82	82–78–69

Ten persons were given a test meal containing Co^{58}-B_{12} (500 ml. specific activity 0.5 μg. per 0.5 μc. of Co^{58}). PEG was used as marker substance. The activities actually found in samples from upper, middle and lower third of the small bowel are indicated in per cent of the values expected from the concentration of PEG.

As previously mentioned, it is generally accepted that the absorption of vitamin B_{12} in the human alimentary tract takes place almost exclusively in the ileum, whereas it is negligible in the duodenum and jejunum. The results of this study corroborate this in that we have found a considerable absorption of vitamin B_{12} taking place during the passage of the test meal from the middle to the anal third of the small intestine, whereas only a modest, though significant, absorption takes place from the oral to the middle third. The results of our investigations of the absorption in the oral portion of the small intestine can only be explained by the assumption that in certain cases an absorption also takes place in the proximal portion of the jejunum. As mentioned above, in 3 cases out of 9, the content of B_{12} was reduced in samples from the oral third of the small intestine, collected 95, 50 and 50 cm. distally to the pylorus. If this reduction is due to erroneous determinations, there is every probability, in view of the grouping of the samples just under 90 per cent, that the error had been made in the measurements of the standard samples. This would imply that the contents of PEG had been

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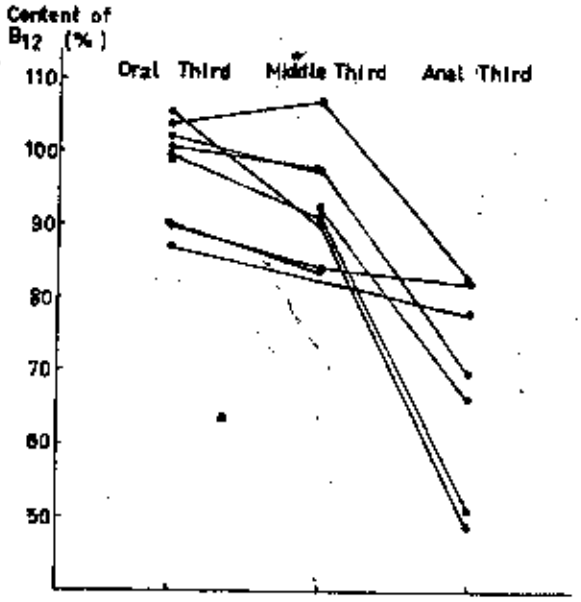

Fig. 1.—Content of Co^{58}-vitamin B_{12} in various parts of the human intestine after a test meal containing Co^{58}-B_{12} and PEG as marker substance. The content is indicated in per cent of the values expected from the concentration of PEG.

underestimated or the radioactivity overestimated, or both. If so, these experiments (cf. formula 3) would also be expected to reveal a low content of B_{12} in the middle and anal third of the small intestine. As shown in figure 1, this was not the case.

The findings must, therefore, be presumed to indicate that removal of vitamin B_{12} has taken place from the test meal. This removal is likely indicative of an absorption, but the possibility cannot be excluded that it has only been reversibly adsorbed to the mucous membranes of the stomach and the proximal portion of the small intestine.

Clinical symptoms distinguishing these 3 patients (3, 4, 7) from the other 6 could not be demonstrated. The absorption of B_{12} in the proximal portions of the small intestine might be due to concentration of the test meal during its passage down the gut. This was not the case, however, since it was found that the maximum concentration of PEG in proportion to the test meal in these 3 cases was 60, 120 and 110 per cent, whereas in the others it ranges from 40 to 100 per cent.

Our observations are supported in part by the results obtained by Doscherholmen et al.[9] who administered Co^{60}-labeled vitamin B_{12} to eleven very sick patients. After death, 2 hours to 113 days later, it was found that in 7 cases the radioactivity of the small intestine was localized to the ileum, and in 2, evenly distributed in the whole gut. In 2 cases it was almost exclusively localized to the jejunum which, according to these authors, might suggest an absorption of B_{12} in the jejunum in certain cases.

The limited number of patients in our study does not permit an evaluation

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of how frequently the absorption of B_{12} begins in the jejunum. Comparing our results and those of Doscherholmen et al.[9] it can be concluded that absorption in the jejunum takes place not infrequently.

Summary and Conclusion

Intestinal intubation was performed with a tri-bore tubing in ten subjects without signs of gastrointestinal disease. After administration of a test meal containing Co^{58}-labeled vitamin B_{12} and with polyethylene glycol as a marker substance, samples of intestinal juice were collected from the upper, middle and anal thirds of the small intestine.

During the passage of the test dose from the upper to the middle third of the gut a modest, but significant, absorption of about 6 per cent was discovered. During the passage from the middle to the anal third of the intestine a considerably larger absorption took place, on an average of about 25 per cent of the oral dose.

These results agree entirely with those obtained from experiments performed in animals and with clinical observations in man after operations on the small intestine. The most outstanding feature was that, contrary to the generally accepted view, our study seems to indicate that in certain cases the absorption of B_{12} begins far up in the jejunum. This seemed to be the case in 3 out of 9 experiments. The study throws no light on the cause of this.

Summario in Interlingua

Intubation intestinal con tubos tri-luminate esseva executate in dece subjectos sin signos de morbo gastrointestinal. Post le administration de un repasto experimental, continente vitamina B_{12} marcate con Co^{58}, con glycol polyethylenic como substantia de marcage, specimens de succo intestinal esseva colligite ab le tertio superior, le tertio intermedie, e le tertio anal del intestino tenue.

Durante le passage del dose experimental ab le tertio superior al tertio intermedie del intestino, un modeste (sed significative) absorption de circa 6 pro cento esseva discoperite. Durante le passage ab tertio intermedie ad le tertio anal del intestino, un considerabilemente plus marcate absorption occurreva, amontante al media a circa 25 pro cento del dose oral.

Iste resultatos se trova completemente de accordo con illos obtenite in experimentos animal e con le observationes in patientes human post operationes in le intestino tenue. Le plus notabile characteristica essova que—per contrasto con le generalmente acceptate notion—nostre studio pare indicar que in certe casos le absorption de vitamina B_{12} comencia multo in alto in le jejuno. Isto pareva esser le caso in 3 de 9 experimentos. Le studio non provide datos que poterea explicar iste constatation.

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Tissue Distribution and Storage Forms of Vitamin B_{12} Injected and Orally Administered to the Dog.* (28011)

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Previous attempts by the present authors (1,2,3) to compare the behavior of orally administered Vit. B_{12} with that of injected material have been inconclusive. Thus, 30 days after administration to normal humans, 0.19% of a tracer amount (0.5 μg) of injected radioactive Vit. B_{12} is excreted (1) per day; and in an independent study (2), the eventual daily output was found to be 0.23% of a 3 μg dose. By contrast the total excretion rate of normal Vit. B_{12} from body stores (3) appears to be only ≈ 0.03% per day. Such a divergence may result from incomplete mixing of radioactive Vit. B_{12} and body stores of the vitamin. Alternatively it may actually reflect the functioning of different compartments attending the several modes of administration involved. The possibility of degradation or transformation of vitamin within tissues and organs must also be considered. Because of the apparent discrepancy, experiments were initiated in humans

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and in dogs in which Vit. B_{12} was administered orally and by injection to the same test subject. A double labeling procedure was employed to permit simultaneous observations of both modes of administration, thus ruling out subject variability. By this means it should be possible to ascertain the role of mode of administration. This paper reports measurements of comparative retention by various tissues of the dog and the nature of the retention form of the vitamin in the liver.

Materials and methods. Vit. B_{12} preparations labeled with cobalt-57 and with cobalt-60 were administered to 2 dogs (mongrels; weights ≈ 10 kg) for a period of about 2 weeks. During this period each animal received 22 oral doses of Vit. B_{12}-Co^{57}, each containing 0.11 μg (and 0.6 μc) of vitamin administered in frozen gelatin capsules, and 7 intravenous injections of ≈ 2.1 μg (≈ 2.0 μc) of Vit. B_{12}-Co^{60}. Total Vit. B_{12} fed was 2.4 μg (13.2 μc) orally and 14 μg (13 μc) intravenously.

The dogs were sacrificed 4 weeks after receiving the last dose. Weighed samples from all organs and tissue were digested in 2 ml of 6N NaOH, and measured in a well type scintillation detector by means of a single channel gamma ray spectrometer.

Storage forms of Vit. B_{12} were studied by the reverse isotope dilution method(4,5). Portions (10-15 g) of liver were homogenized with water, and the radioactive components liberated either by acid nitrite or by enzymatic digestion, in the presence of crystalline cyanocobalamin (Vit. B_{12}) and/or hydroxocobalamin added previously as carriers (≈ 1 mg of each cobalamin). Cyanocobalamin and/or hydroxocobalamin fractions were then isolated, and purified for measurement of recovered vitamin (spectrophotometrically) and of associated Co^{57} and Co^{60} (gamma ray scintillation spectrometry). The percentage of initial radioactivity recoverable as the 2 cobalamins may be computed from the fractional recovery of radioactivity and vitamin in the purified fractions. Details of isolation and purification procedures, as well as calculations involved in such dilution procedures, have been published elsewhere(4,5,6,7). Papain(8) and trypsin(9) were employed for enzymatic digestion.

Results. Tissue radioactivities in cpm per gram due to Co^{57} and Co^{60} observed in 47 different tissue samples from each of 2 dogs are reported graphically in Fig. 1, which shows log-log plots of the tissue specific Co^{60} activities and the corresponding Co^{57} values.† The slopes of these plots indicate that, with relatively few exceptions the average ratio of Co^{57}/Co^{60} is approximately constant in all tissue specimens examined. This ratio has an average value of 8 for both animals. Taking into account the value of 19.3 for the ratio of specific radioactivities, in cpm per μg of Vit. B_{12}-Co^{57} to that of Vit. B_{12}-Co^{60} under respective conditions of spectral measurement, one computes that the average relative amounts of injected Vit. B_{12} (-Co^{60}) retained by tissue are 2.4 times the corresponding values for orally administered Vit. B_{12} (-Co^{57}). This is in accord with normal expectation for these modes of administration.

Results of reverse isotope dilution assays of both dog livers are recorded in Table I. To insure equilibration of labeled vitamin in tissue with carrier vitamin, samples were boiled for 15 minutes or 1-2 hours after enzymatic digestion prior to isolation of cobalamins instead of the customary 5 minutes (for trypsin) or 15 minutes (for papain) to destroy excess enzyme. Treatment of both specimens with nitrous acid in the presence of cyano carrier indicates that all of the radioactivity can be isolated as the cyanocobalamin. This is in agreement with a previous report(4). However, use of carrier hydroxocobalamin indicated that most of the radioactivity could also be recovered as the hydroxocobalamin. Obviously, use of nitrite in treating tissue samples leads to an equilibration of radioactivity with either of the carrier cobalamins. Accordingly, nitrite will not maintain the integrity of the several cobalamins in tissue, but serves only to indicate a total cobalamin radioactivity. A similar effect was noted in a blank experiment with sodium metabisulfite(10) added to a

† A preliminary report of this observation was made in Ref. 3.

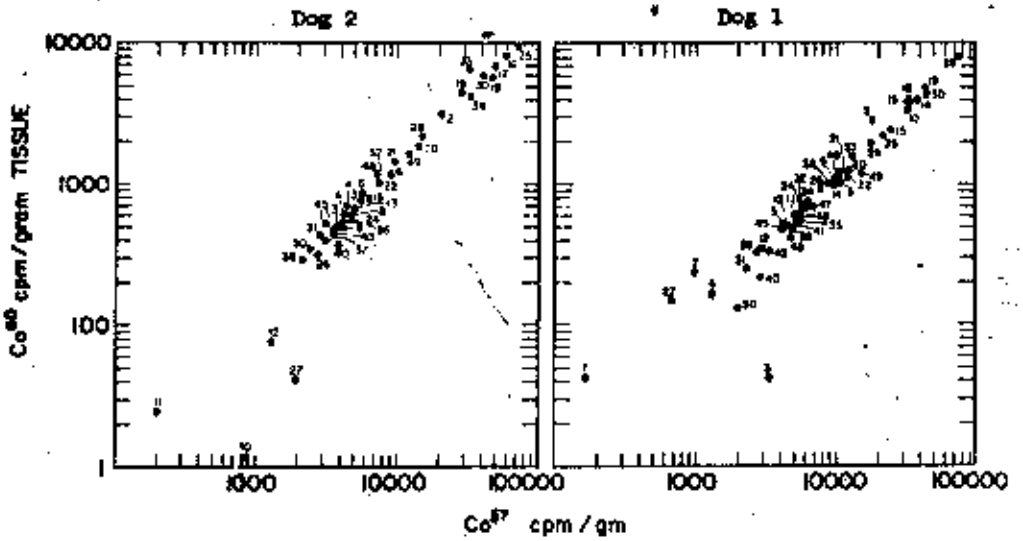

FIG. 1. Radioactivities in tissues of 2 experimental animals receiving oral vitamin B_{12}-Co^{57} and intravenous vitamin B_{12}-Co^{60}. Numbers indicate the following tissues:

1. Aorta
2. Heart
3. Skin
4. Skeletal muscle
5. Diaphragm muscle
6. Thyroid
7. Parathyroid
8. Lung
9. Thymus
10. Cartilage (sternum)
11. Bone
12. " marrow
13. Urinary bladder
14. Gall "
15. Spleen
16. Kidney
17. Adrenals
18. Pancreas
19. Liver
20. Submaxillary gland
21. Axillary lymph node
22. Mesenteric lymph node
23. Cerebrum
24. Thoracic duct
25. Pituitary
26. Femoral nerve
27. Cornea
29. Testis
30. Prostate
31. Esophagus
32. Stomach-cardia-wall
33. Stomach-fundus-mucosa
34. Stomach-fundus-wall
35. Stomach-pylorus-wall
36. Duodenum-mucosa
37. Duodenum-wall
38. Jejunum-mucosa
40. Ileum-mucosa
41. Ileum-wall
42. Cecum-mucosa
43. Cecum-wall
45. Colon-wall
46. Rectum-wall
47. Tonsil
48. Popliteal lymph node
49. Parotid
50. Omentum fat

mixture of inactive livers, papain, radioactive hydroxocobalamin-Co^{57} and cyanocobalamin-Co^{60} and both cobalamins added as carriers.

Differentiation between the cobalamins is possible, however, by digesting tissue with papain to liberate radioactive components. Papain treatment of the liver of Dog 1 in the presence of both carriers yields a cyanocobalamin fraction accounting for 7-21% of total tissue radioactivity§ and a hydroxocobalamin fraction containing 66-100% of tissue radioactivity. Furthermore the percentages of administered cobalt-57 and cobalt-60 recovered in the cobalamin fractions appeared to be the same. Subsequent examination of these fractions by chromatography on paper indicated high purity; and reprocessing of eluates from paper by solvent extraction and ion exchange resins yielded identical recovery results. Similar enzymatic treatment of liver from Dog 2 in presence of cyanocobalamin carrier yielded a cyanocobalamin fraction with 10-15% of total tissue radioactivity, and with hydroxocobalamin carrier, a hydroxo-fraction with ≈ 100% of initial activity. In the presence of both carriers, values were 7-26% in the cyanocobalamin fraction, and 62-100% in the hydroxocobalamin isolate. In all instances, cobalt-57 and cobalt-60 were comparable in behavior.

A limited number of trypsin digestions also indicated transfer of cyanocobalamin radioactivity to hydroxocobalamin. However, cobalamin extraction after trypsin treatment

§ A preliminary report of this observation was made in 2nd European Symposium on Vitamin B_{12} and Intrinsic Factor, Hamburg, Aug. 2-5, 1961. Enke Verlag, Stuttgart, 1962, p306.

TABLE I. Reverse Isotope Dilution Assay of Dog Livers.

Dog	Tissue treatment	Carriers	Exp series	Boiled (min)	% recovered as Cyanocobalamin Co^{57}	Cyanocobalamin Co^{60}	Hydroxocobalamin Co^{57}	Hydroxocobalamin Co^{60}
1	HNO_2	Cyano	A	60	99.6	97.3	—	—
		Hydroxo‡	M	60	—	—	95.0	95.0
	Papain	Cyano	H*	15	6.7	7.4	101	108
		Hydroxo	H*	60	19.7	21.4	66.6	66.4
2	HNO_2	Cyano	A	60	97.6	97.8	—	—
		Hydroxo‡	J	60	—	—	92.7	92.7
	Papain	Cyano	C	15	9.6	10.8	—	—
		"	C	15	16.5	13.8	—	—
		Cyano + hydroxo	D	15	6.6	6.2	100	100
			F*	60	25.2	26.1	64.5	60.6
			E	60	17.7	17.1	70.4	69.2
			E	120	22.8	21.7	62.1	61.5
		Hydroxo‡	J	60	—	—	100	105
	Trypsin	Cyano	B	15	45.2	47.2	—	—
		Cyano + hydroxo	G†	60	39.0 (20.0)	38.2 (25.9)	45.0	46.3
		Hydroxo	L	60	—	—	118	112

* These isolates were purified further by chromatography on paper, elution, solvent extraction, and ion exchange resins. Results obtained were essentially the same as in the standard procedure.

† Further purification of the cyanocobalamin fraction yielded the lower results shown in parentheses.

‡ Plus trace of cyanocobalamin = 1% of hydroxocobalamin carrier weight.

was much more difficult, and required prolonged boiling, indicating incomplete liberation of cobalamins (from binding proteins?). This is borne out by the lower results obtained in Exp. G (Table I) after further purification of isolates by chromatography on paper. This equivocal behavior was not observed with papain.

Discussion. In an earlier investigation(4) of the stability of injected Vit. B_{12}, it was concluded that "Vitamin B_{12}-Co^{60} is retained by dog livers as an intact cobalamin, probably as cyanocobalamin." It now appears that this result reflects the method of treating tissue rather than the stability of cyanocobalamin, since the use of nitrite, and probably other reducing agents, does not assure the inviolability of the cobalamin structures. Nitrous acid may be used in the isotope dilution assay for total cobalamins; it will not serve to differentiate between cyano- and noncyanocobalamins in tissue.

Differentiation was possible by subjecting tissue to enzymatic digestion. By this means it was demonstrated that the bulk of the administered cyanocobalamin is converted during storage in liver to a noncyano form of cobalamin which is isolable as hydroxocobalamin. Whether this conversion is limited to the hydroxo-stage, or whether conversion proceeds to the cobamide coenzyme(11,12), is not discernible from these experiments since isolations were not performed in the dark; and the cobamide coenzyme could easily have degraded to hydroxocobalamin(12). That conversion of cyanocobalamin to its coenzyme does occur in the rabbit was reported by Fenrych *et al.*(13).

The similarity in behavior of oral and injected Vit. B_{12} is worthy of note. Cobalt-57 and cobalt-60 are similarly distributed between the 2 cobalamin fractions isolated from the liver, indicating identical conversion of oral and parenteral Vit. B_{12} retained in this organ. This is in accord with the essential constancy of the Co^{57}/Co^{60} in all tissues. This suggests that Vit. B_{12} and conversion products retained by the animal are incorporated in a common pool regardless of mode of administration. Cooperman *et al.*(14) have already reported that oral Vit. B_{12}-Co^{60} injected subcutaneously into dogs equili-

brates in similar fashion with endogenous tissue vitamin by 14 days after administration. Comparable results were obtained by Gräsbeck *et al.*(15) with rats. One must conclude that the different fractional excretion rates of injected and endogenous Vit. B_{12} are not determined by differences in mode of administration. A possible explanation lies in the rate of conversion of administered cyanocobalamin to noncyanocobalamins, cobamide coenzyme and/or bound forms of either. Thus Doscherholmen and Hagen(16) have reported 5 components in urinary excretion by the human. Chemical changes such as those discussed, and not necessarily different physical compartments, could account in part for the complex nature of the excretion pattern observed with Vit. B_{12}.

Summary. 1. The distribution of oral and intravenous Vit. B_{12} was determined simultaneously in dogs using vitamin preparations labeled respectively with cobalt-57 and cobalt-60. 2. Thirty-one days after administration, the ratio of Co^{57}/Co^{60} was constant in all tissues, indicating the existence of a common pool for cobalamin and related compounds regardless of mode of administration. 3. Conversion of administered labeled Vit. B_{12} to a noncyano form was demonstrated by reverse isotope dilution analysis of livers. This is true regardless of mode of administration. 4. It is suggested that chemical changes in the vitamin account in part for the complex excretion pattern of labeled Vit. B_{12}.

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Comparative Absorption of Vitamin B_{12} Analogues by Normal Humans. II. Chloro-, Sulfato-, Nitro- and Thiocyanato- *vs* Cyanocobalamin. (22264)

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The first(1) paper of this series reported results of comparative oral absorption studies of chlorocobalamin and cyanocobalamin employing the urinary excretion method of Schilling(2). These tests were interpreted as indicating the superiority of cyanocobalamin over chlorocobalamin for alimentary absorption by humans. Results of oral tolerance tests with humans, and of fecal excretion measurements with rats, confirming the low chlorocobalamin absorption, are reported herein. In addition, similarly inferior absorptions of sulfato-, nitro- and thiocyanatocobalamins are reported, based upon urinary and fecal excretion studies in humans, and upon fecal excretion by rats.

Methods, Materials. The preparation and properties of the radioactive and normal chlorocobalamins and cyanocobalamins employed in these experiments have been described(1). Sulfatocobalamins were obtained photochemically as for chlorocobalamin except that irradiation was performed in 0.01 M H_2SO_4 solution. Samples of the nitroco-

TABLE I. Urinary Excretion of Radioactivity by Humans.

Cobalamin Oral		Cobalamin Inj., 1 mg	Avg % of dose ± stand. dev.
Cyano	2 μg	Cyano; chloro	≈12*
Sulfato	"	Sulfato	3.5 ± 1.5
		Chloro	2.6 ± .8
		Cyano	3.7 ± 1.8
Nitro	"	Nitro	2.0 ± .8
		Chloro	3.1 ± .5
		Cyano	1.9 ± .2
Thiocyanato	"	"	2.0 ± .9
Nitro	.5	"	5.8 ± 3.6
Cyano	"	"	17.6 ± 9.0

* Avg of accumulated standard test data.

balamins and thiocyanatocobalamins were prepared from sulfatocobalamins by treatment with acid nitrite and KSCN respectively. Paper chromatograms,* distribution coefficients and absorption spectra attested to the identity of the radioactive and normal forms. Specific activities of the radioactive cobalamins were: cyano-≈200 μc/mg; sulfato-≈130 μc/mg; nitro-≈130 μc/mg and thiocyanato-≈171 μc/mg. The urinary excretion(2,3) and the Heinle-Welch(4) fecal excretion (5-6 days) methods employed to compare the several labeled cobalamins have been adequately described elsewhere. In all cases the subjects (groups of 4-6) were young, healthy adult males under continual supervision to insure complete collection of excreta. Urine and feces radioactivity was determined by scintillation counting. Two series of oral tolerance tests were performed with chloro- and cyanocobalamin as described by Chow(5). Subjects were fed 3 or 5 mg of test substance in aqueous solution after first removing 15 ml of fasting blood for microbiological assay of serum for vitamin B_{12}. Two hours after ingestion, another 15 ml sample of blood (still fasting) was removed for vit. B_{12} assay. Aged subjects, *i.e.* over 65 years, were employed in order to reveal more readily resultant changes in serum vit. B_{12}. Only differences in excess of 40 μμg/ml are considered experimentally significant(5).

* The authors are grateful to R. D. Babson of the Research Laboratories of Merck & Co., for these chromatographic tests.

Normal adult male rats of the Johns Hopkins colony, weighing an average of 300 g per animal, were employed (in groups of 6) in fecal excretion experiments with cyano-, chloro- and nitrocobalamin-Co^{60}. A 50 mμg dose of labeled analogue was fed to each rat by stomach tube and feces collected for 5 days. All collections were measured for cobalt 60 content by scintillation counting of feces homogenates. Actually, excretion of radioactivity was negligible after the first 24-48 hours.

Results. Human urinary excretion results are compiled in Table I. Table II contains the results of 2 fecal excretion studies with 0.5 μg of cyano- and nitrocobalamin-Co^{60} administered to human subjects. Although daily measurements were performed, only cumulative excretions for the entire collection periods are of interest. Group average cumulative excretions measured, and average absorption values computed therefrom by difference, are reported.

Results of the 2 oral tolerance tests are compiled in Table III. Initial concentrations and maximum difference, observed after 2 hours, are reported in terms of μμg/ml of serum.

TABLE II. Fecal Excretion of Radioactivity by Humans.

Study	Cobalamin (.5 μg oral)	Avg % exc. ± stand. dev.	Avg % absorbed
1st* (5 days)	Cyano	18.0 ± 7.1	82.0
	Nitro	41.8 ± 13.8	58.2
2nd (6 days)	Cyano	13.7 ± 4.4	86.3
	Nitro	46.5 ± 6.9	53.5

* 1 mg vit. B_{12} injected 2 hr after oral dose of tracer.

TABLE III. Serum Levels (μμg/ml) in Humans.

Study	Cyano Initial	Cyano Difference (2 hr)	Chloro Initial	Chloro Difference (2 hr)
1st	280	682	222	0
3 mg cyano;	146	854	216	0
3 mg chloro	251	70	157	0
	169	286	642	0
	227	94	570	303
2nd	99	204	169	76
3 mg cyano;	122	158	140	41
5 " chloro	157	141	251	46
	227	94	490	0
	198	64	146	0

TABLE IV. Fecal Excretion of Radioactivity by Rats.

Cobalamin (.050 μg oral)	Avg % exer. ± stand. dev.	Avg % absorbed
Cyano	65.4 ± 7.2	34.6
Chloro	90.6 ± 9.5	9.4
Nitro	85.2 ± 16.3	14.8

Table IV is a summary of the findings from the fecal excretion study with cyano-, chloro- and nitro-cobalamin-Co^{60} administered to rats. Here too, group average cumulative excretions (after 5 days) and computed absorptions, are reported.

Discussion. Application of the urinary excretion method at 2 μg to sulfato-, thiocyanato- and nitrocobalamin reveals lower responses than are obtained with cyanocobalamin, as can be seen from the summary Table I. For nitrocobalamin, this is true also at the 0.5 μg oral level. The low sulfato-excretions (~3% of the 2 μg dose) are practically identical with those reported previously for the chloro-analog, and are about one-fourth the average 12% figure typical of vit. B_{12} itself. Similar behavior characterizes nitrocobalamin and thiocyanato cobalamin *i.e.* low responses of 1.9-3.1% of the 2 μg dose independent of the choice of injected analogue. Even at 0.5 μg oral intake, the urinary nitrocobalamin output of 5.8% is much less than the 17.6% elimination of cyanocobalamin. Furthermore as in the case of labeled chloro- (1), the nature of the injected cobalamin is immaterial. Thus sulfato-, chloro-, nitro- and cyano-cobalamin are indistinguishable in their capacity to induce cobalamin excretion upon injection, which again points to the equivalence, perhaps through interconvertibility of cobalamins or conversion to another common structural analogue, of the several cobalamins once introduced into the blood stream.

The similarity in behavior of the chloro- and sulfato-forms is understandable in terms of their identical electrolyte and hydrophilic properties(6,7) and spectra. Less evident is the reason for the slight responses obtained with nitrocobalamin and thiocyanato-cobalamin. These compounds are characterized as electrically neutral cobalamins(7,8) since the substituent anions are relatively tightly bound(9) and because of their relative organophilic tendencies(9,10). In this respect they resemble cyanocobalamin rather than aquocobalamin salts, and might have been expected to exhibit a pronounced absorption closely approximating that of vit. B_{12} itself. Despite evidence for slight dissociability in certain cases, addition of cyanide ion converts all known cobalamins to the cyano form. The current results then point to the unique importance of the latter group in governing the metabolic activity of the cobalamins, rather than to the dissociability of the substituent anion. It is conceivable that even the low non-cyano responses are themselves due to partial interactions with traces of cyanide ion in gastric juice or upper intestinal contents rather than to an inherent activity of these cobalamins.

That the extent of urinary excretion after injection is an index of absorption has been verified by independent methods of demonstrating absorption.† In the case of chlorocobalamin, oral tolerance tests (Table III; first study) with humans failed to reveal any increase in serum cobalamin concentration in four out of five subjects fed 3 mg doses; and even feeding of 5 mg (Table III; second study) produces a barely significant rise in 3 out of 5 persons. By contrast, ten individuals receiving 3 mg of cyanocobalamin each exhibited serum levels 2 hours after ingestion which were on the average approximately 2-3 times normal. Further evidence for the superior absorption of cyanocobalamin is provided by the fecal excretion measurements with rats. It is obvious from Table IV that the absorption (9.4% of the 50 mμg oral dose) of chlorocobalamin is only about one-fourth the 34.6% absorption typifying cyanocobalamin.

Similar confirmation has been obtained in

†G. B. J. Glass, N. Y. Medical College has observed(11) by hepatic uptake method that the cpm of liver projections after oral administration of 0.5 μg of chlorocobalamin-Co^{60} is only one-half to one-third the radioactivity noted after administration of cyanocobalamin-Co^{60}. This is in accord with conclusions presented in our paper.

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studies comparing the fecal excretion of labeled nitrocobalamin with that of cyanocobalamin by rats and humans. In the rat studies (Table IV) nitrocobalamin absorption (14.8%) is less than half the 34.6% value obtained with the cyano- form. In the first of the human studies (Table II), fecal excretions of nitro- and cyano- forms were respectively 41.8% and 18.0% of the 0.5 μg oral dose which presumably correspond to absorptions of 58.2% and 82.0%. Respective absorption figures of 53.5% and 86.3% obtained in the second study are in excellent agreement. When one considers that "zero" absorption values, as are obtained with pernicious anemia patients(12), may range from 0 to 24% (average=12%), these figures would indicate a 2 to 1 superiority in absorption in favor of cyanocobalamin.

It is significant that all methods support the observations, based upon the urinary excretion method, that cyanocobalamin excels the other cobalamins as an oral absorption form. One may conclude further that, as regards the cobalamin family, the injection-excretion test can serve as an index by which to judge comparative absorption.

Summary. 1. Urinary excretion measurements indicate considerably lower oral absorption of sulfato-, nitro- and thiocyanatocobalamin than is exhibited by cyanocobalamin. In the blood stream, however, the several cobalamins are equivalent in inducing the excretion of ingested vit. B_{12}. 2. That cobalamin urinary excretion responses truly constitute an absorption index was verified in studies comparing chlorocobalamin with cyanocobalamin, by serum level assays in humans and by fecal excretion experiments with rats. 3. Inferior absorption of orally administered nitrocobalamin was confirmed by fecal excretion observations in humans and rats. 4. The superior absorption of cyanocobalamin (vit. B_{12}) over other cobalamins studied is attributed to either the chemical specificity of the substituent cyano group or the tightness of its binding in the cobalt coordination sphere.

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Tissue Distribution of Radio-B_{12} after Intravenous Injection of Gastric Juice, Saliva and Plasma-Bound Co^{57} B_{12}

II. In Rats

By William S. Rosenthal, Herbert Weisberg and George B. Jerzy Glass

WE HAVE PREVIOUSLY SHOWN in man that vitamin B_{12} (B_{12}) binders of normal human gastric juice (NHGJ)[1] and pernicious anemia gastric juice (PAGJ)[2] enhance hepatic uptake and delay blood clearance of an i.v. tracer dose of $Co^{57}B_{12}$. This extended the prior work of others[3-7] using in vivo and in vitro animal systems which demonstrated that gastrointestinal B_{12} binders with and without intrinsic factor (IF) activity enhance B_{12} uptake by liver tissue.

In all in vivo studies, however, precise quantitative comparisons of the effects of different B_{12} binders on hepatic B_{12} uptake could not be made because of interference by extrahepatic tissues. The role of the reticuloendothelial system in the removal of bound B_{12}, though questioned,[8] was also not evaluated. Therefore, in the present investigation, we studied the hepatic uptake of a variety of gastrointestinal B_{12} binders by means of an in situ rat liver perfusion technic without recirculation of blood which permitted rapid and precise quantitative comparisons. In addition, the effect of depression of reticuloendothelial system activity on hepatic removal of bound B_{12} was also studied.

The results indicate that, compared with unbound B_{12}, binding of B_{12} to PAGJ, human saliva, hog intrinsic factor concentrate (HIFC), and boiled HIFC increased B_{12} uptake by rat liver. On the other hand, B_{12} bound to NHGJ was removed by rat liver in a manner quantitatively similar to that observed with unbound B_{12}. This result is markedly different from our previous finding in man.[1] Depression of reticuloendothelial system activity induced by india ink did not affect the hepatic removal of HIFC-bound B_{12}.

Methods and Materials

Hepatic uptake was quantitated by means of a rat liver perfusion technic without recirculation.[9] Perfusions were performed in 48 nonfasting female Sprague-Dawley rats weighing 275-325 Gm. Whole rat blood was obtained by cardiac puncture in a heparinized

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syringe from rats of the same strain and size used for perfusion, filtered through the nylon mesh filter of a standard blood transfusion set to remove clots, and oxygenated with a mixture of 95 per cent O_2 and 5 per cent CO_2. The blood was warmed to 37 C. by passage through a water bath and the entire perfusion system maintained at that temperature by enclosure in a plexiglass constant-temperature box. After pentobarbital anesthesia of the test animal, cannulas were positioned in the inferior vena cava and portal vein, and ligatures were placed to isolate the liver from the rest of the circulation. The oxygenated blood was then perfused for 10 minutes via the portal vein through the liver at a constant rate of 4 ml. per minute maintained by a motor driven syringe. The effluent was collected by means of a cannula positioned opposite the hepatic venous outflow into the inferior vena cava. The cannula was connected to a manifold which permitted separation of the effluent into ten 1-minute samples. Throughout the perfusion, the livers appeared grossly normal and examination by light microscopy at the end of the perfusion demonstrated no abnormalities.

Extraction ratios $\frac{A-V}{A}$ have been calculated from the perfusate concentration (A) and the effluent concentration (V) during the fourth and tenth minutes. The fourth minute was chosen because intrahepatic mixing was complete at that time as determined in a separate investigation.[10] The tenth minute was the end of the perfusion.

Material for Perfusion

1. *Unbound B_{12} (Table 1)*

The B_{12} concentration of whole rat blood determined before the addition of radio-B_{12} in 6 pools, derived from 6 donor rats each, ranged from 300-800 pg./ml. using a radioassay.[11]* $Co^{57}B_{12}$ (or $Co^{60}B_{12}$ in those experiments utilizing higher B_{12} concentrations) was added to whole rat blood to produce final radio-B_{12} concentrations ranging from 12 pg./ml. to 13,633 pg./ml.† These values do not include endogenous serum B_{12} since the latter is not completely miscible in vitro with added crystalline B_{12}.[12]

In 21 rats the following final radio-B_{12} concentrations and perfusion rates were obtained:

Group	Radio-B_{12} Concentration (pg./ml.)	Radio-B_{12} Perfusion Rate (pg./100 Gm. rat/min.)
A (6 rats)	12 to 60	15 to 71
B (7 rats)	111 to 410	140 to 459
C (4 rats)	1379 to 4301	1872 to 6325
D (4 rats)	7902 to 13,633	9503 to 16,875

2. *Hog Intrinsic Factor Concentrate-Bound B_{12}*

a. Radio B_{12} was incubated for 30 minutes at room temperature with 0.1 mg. of purified HIFC (WES 727)‡ in physiologic saline solution. The HIFC-bound B_{12} was then separated by Sephadex G-25 gel filtration according to the method described in this laboratory.[13]

In 8 perfusions HIFC-bound B_{12} was added to the perfusate to produce radio-B_{12} concentrations of 174 to 8907 pg./ml. resulting in radio-B_{12} perfusion rates ranging from 224 to 11,419 pg./100 Gm. rat/minute.

*We are indebted to Dr. Sheldon Rothenberg for performing these determinations.

†$Co^{57}B_{12}$ (8-18 μc./μg.) and $Co^{60}B_{12}$ (1 μc./μg.) were generously supplied by Dr. Elmer Alpert and Dr. Charles Rosenblum of Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania.

‡Purified hog intrinsic factor concentrate was generously provided by Dr. Leon Ellenbogen, Lederle Laboratories, Pearl River, N.Y. Five mg. of this material gave optimum B_{12} absorption with the Schilling test.

b. Samples of HIFC-bound B_{12} were heated in a water bath at 100 C. at pH 6 for 30 minutes. Gel filtration of aliquots of this boiled material demonstrated that it was still bound to radio-B_{12}. Despite lack of change in binding capacity, this heated material showed markedly reduced ability to enhance B_{12} absorption in a pernicious anemia patient as measured by the Schilling test. This is in accord with previous work[14-16] showing the inactivation of IF by heating with little effect on B_{12} binding.

In 4 perfusions this heat treated HIFC bound to $Co^{60}B_{12}$ was added to the perfusate to produce final HIFC-bound $Co^{60}B_{12}$ concentrations varying from 12,000 to 15,364 pg./ml resulting in final perfusion rates of 12,606 to 18,412 pg./100 Gm. rat/minute.

c. In 4 additional perfusions, one hour prior to perfusion with nonheated HIFC-bound B_{12}, 16 mg. of india ink in 1 per cent gelatin solution per 100 Gm. rat was injected into the tail vein of the test rat according to the method of Halpern et al.[17] in order to depress the activity of the reticuloendothelial system in the liver. At the time of the perfusion, the liver was diffusely black and some carbon particles were still in circulation.

3. *Human Gastric Juice-Bound B_{12}*

a. NHGJ was prepared by a modification of the method described by Wullerstein et al.[18] NHGJ was collected on ice in separate batches following maximal histamine stimulation (0.4 mg./10 Kg.) from 2 duodenal ulcer patients. The juice was allowed to stand for 24 hours after collection and was then brought to pH 7, centrifuged, and sterilized by Berkefeld V filtration. IF activity in the juice was demonstrated by the guinea pig intestinal mucosal homogenate assay.[19]

This material was added in 4 perfusions to produce final radio-B_{12} concentrations of 86 to 3913 pg./ml., resulting in perfusion rates of 106 to 4797 pg./100 Gm. rat/minute.

b. Aliquots of PAGJ-bound B_{12} prepared for injection in human subjects[2] were added in 4 perfusions to produce final radio-B_{12} concentrations of 106 to 244 pg./ml., resulting in perfusion rates ranging from 172 to 309 pg./100 Gm. rat/minute. Lower concentrations of radio-B_{12} were used in these experiments, since PAGJ is a poor binder and greater amounts of radio-B_{12} would have required excessive dilution of the blood perfusate.

4. *Saliva-Bound B_{12}*

Four ml. of postprandial saliva obtained from a normal individual was incubated at room temperature for one-half hour with $Co^{57}B_{12}$ or $Co^{60}B_{12}$. Bound B_{12} was separated by Sephadex G-25 gel filtration, as described above. This material was added in 3 perfusions to produce final radio-B_{12} concentrations of 92 to 1247 pg./ml. resulting in final perfusion rates of 129 to 1568 pg./100 Gm. rat/minute.

Results

1. *Unbound B_{12}*

Results obtained in 21 perfusions with unbound B_{12} show (Table 1) that variations in B_{12} concentration of the perfusate did not affect hepatic extraction ratios except at the highest concentrations tested (Group D). This latter group is therefore omitted from all statistical comparisons with bound radio-B_{12}. There was no significant difference between the mean extraction ratios over the range of concentrations from 12 to 4301 pg./ml, even though the latter radio-B_{12} concentration is approximately 10 times the normal rat serum B_{12} level. In each group the mean extraction ratio declined significantly ($p<.05$) between 4 and 10 minutes. At the highest perfusate concentration (Group D), mean extraction ratios were significantly lower ($p<.05$) than each of the other groups both at 4 and 10 minutes, with little B_{12} being removed at 10 minutes. At the lowest perfusate concentration (Group A), radio-B_{12} was probably completely bound by serum binders.

Table 1.—Removal of Radio-B_{12} by Rat Liver

Test Material in Perfusate	Number of Perfusions	Radio-B_{12} Concentration (pg./ml.)	Radio-B_{12} Perfusion Rate (pg./100 Gm. rat/min.)	Extraction Ratio (mean ± SE) 4 min.	10 min.
1. Unbound Radio B_{12}					
Group A	6	12 to 60	15 to 71	0.279 ± .039	0.127 ± .040
Group B	7	111 to 410	140 to 459	0.308 ± .074	0.161 ± .077
Group C	4	1379 to 4301	1672 to 6325	0.215 ± .041	0.082 ± .018
Group D	4	7902 to 13,633	9503 to 16,875	0.092 ± .019	0.034 ± .010
2. HIFC-Bound Radio B_{12}					
a. Nonheated	8	174 to 8907	224 to 11,419	0.798 ± .055	0.784 ± .044
b. Boiled	4	12,000 to 15,364	12,866 to 18,412	0.663 ± .015	0.634 ± .005
c. Nonheated, 1 hr. after india ink	4	1002 to 7901	1270 to 10,803	0.837 ± .014	0.805 ± .017
3. Human Gastric Juice-Bound Radio B_{12}					
a. NHGJ	4	86 to 3913	100 to 4797	0.255 ± .093	0.168 ± .082
b. PAGJ	4	106 to 244	172 to 309	0.406 ± .067	0.373 ± .068
4. Saliva-Bound Radio B_{12}	3	92 to 1247	129 to 1588	0.511 ± .044	0.448 ± .043

2. *HIFC-Bound B_{12}*

Results obtained in 16 perfusions with HIFC-bound B_{12}, B_{12} bound to boiled HIFC, and HIFC-bound B_{12} after india ink injection are shown in Table 1 and Figures 1 and 2.

a. The mean extraction ratio in 8 rats perfused with nonheated HIFC-bound B_{12} was much higher than with unbound B_{12} ($p<.001$) at both 4 and 10 minutes. In addition the difference between the 4 and 10 minutes values was not significant.

b. The mean extraction ratios at 4 and 10 minutes in 4 rats perfused with boiled HIFC-bound B_{12} (Fig. 1) were also significantly greater ($p<.001$) than that observed with B_{12} alone but not significantly different from that observed with nonheated HIFC.

c. In 4 rats injected with india ink prior to perfusion with nonheated HIFC-bound B_{12} (Fig. 2), the mean extraction ratios at 4 and 10 minutes were again significantly greater ($p<.001$) than that observed with B_{12} alone and not significantly different from that observed with nonheated and boiled HIFC in rats without prior india ink injection.

Thus, hepatic removal of B_{12} in rats was significantly enhanced by prior binding to HIFC. This enhanced removal was not affected either by impairing intrinsic factor activity by boiling, or by depression of reticuloendothelial system activity. All perfusions with HIFC-bound B_{12} were characterized by a lack of significant change in the extraction ratio between the fourth and tenth minutes of the perfusion. This contrasts with the significant decline observed with unbound B_{12}.

3. *Human Gastric Juice-Bound B_{12}*

Results obtained in 4 perfusions with NHGJ-bound B_{12} and 4 others with

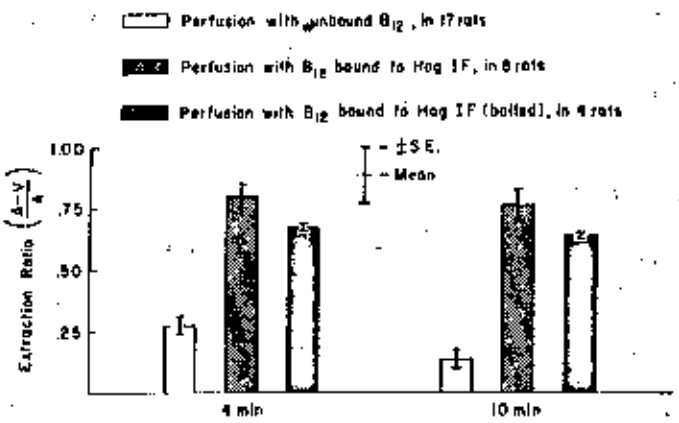

Fig. 1.—Results obtained in 17 rat liver perfusions with unbound radio-B_{12} (Groups A, B, and C) compared with results obtained in 8 perfusions with radio-B_{12} bound to nonheated HIFC and in 4 perfusions with boiled HIFC.

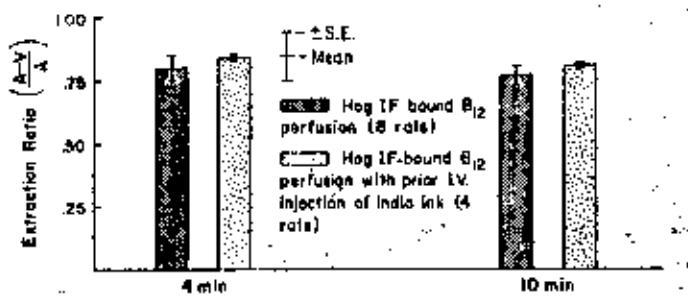

Fig. 2.—Results obtained in 8 rat liver perfusions with B_{12} bound to nonheated HIFC compared to those in 4 perfusions with nonheated HIFC-bound B_{12} during depression of reticuloendothelial system activity with india ink.

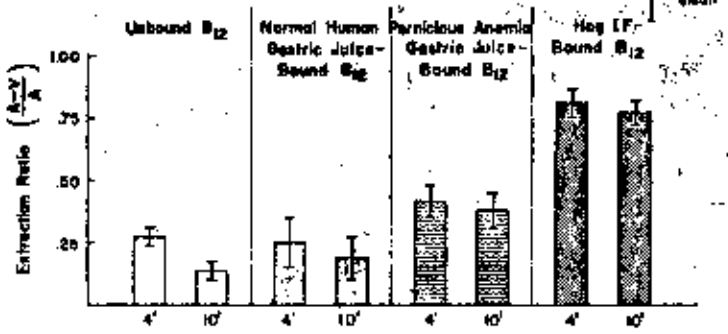

Fig. 3.—Comparison of results obtained in 17 perfusions with unbound B_{12} (Groups A, B, and C), 4 perfusions with NHGJ-bound B_{12}, 4 perfusions with PAGJ-bound B_{12}, and 8 perfusions with nonheated HIFC-bound B_{12}.

PAGJ-bound B_{12} are shown in Table 1 and Figure 3. For comparison, results obtained with B_{12} alone and HIFC-bound B_{12} are also shown.

a. Mean extraction ratios for NHGJ-bound B_{12} at 4 and 10 minutes were not significantly different from that observed with B_{12} alone, but significantly less ($p<.01$) than that observed with HIFC-bound B_{12}.

b. With PAGJ-bound B_{12} the 4-minute extraction ratio was not significantly greater than that of B_{12} alone or bound to NHGJ; however, the 10-minute extraction ratio of PAGJ-bound B_{12} was significantly greater ($p<.01$) than that of B_{12} alone or bound to NHGJ. The lack of change in the extraction ratio during the perfusion between 4 and 10 minutes resembled that seen with HIFC-bound B_{12}.

4. *Saliva-Bound B_{12}*

The mean extraction ratios obtained in 3 perfusions with saliva-bound B_{12} (Table 1) were significantly greater ($p<.01$) than that of B_{12} alone or bound to NHGJ, but not significantly different from that of PAGJ-bound B_{12}, both at 4 to 10 minutes. It was, however, significantly less ($p<.01$) than that observed with HIFC-bound B_{12}.

Discussion

The rat liver perfusion system used in this study amplified our investigations in man [1,2] and permitted quantitation of hepatic B_{12} uptake under a variety of conditions.

In comparison with previous studies of others on the influence of various binding materials[3-7] on hepatic B_{12} uptake, the rat liver perfusion system without recirculation used in this study had the following advantages:

a. The uptake phenomena in our rat liver system reflected activity at the cell membrane, while in liver homogenates[8] and slices[4,5] uptake may also reflect binding by intracellular binders.

b. The B_{12} clearance from the blood in our rat liver system was restricted to liver without the participation of nonhepatic tissue in this process, which is not the case in studies using the intact rat.[6]

c. Because of the constant perfusion rate and timed collection of the entire effluent with no recirculation, removal rates could be accurately determined. In perfusions with recirculation, quantitative determinations of B_{12} removal may be influenced by variations in perfusion rate, changes in concentration of materials, accumulation of metabolic end products in the perfusate, and difficulties in direct effluent sampling. Recirculation of the effluent furthermore introduces qualitative and quantitative changes of the B_{12} binders in the perfusate with repeated passage through the liver which modifies the B_{12} removal process.[7] These considerations are eliminated in our perfusion system since there is no recirculation of the perfusate.

We have shown in the present study that the extraction ratio at 4 and 10 minutes for perfused unbound B_{12} is constant over a wide range of perfusion rates up to 6325 pg./100 Gm. rat/min. Above this rate the extraction ratio decreases significantly, however. This wide range of radio-B_{12} concentrations was used in order to evaluate whether uptake was different at dosage levels

which were a fraction of the endogenous serum B_{12} level and up to 20 times that level. In the lowest range, the added radio-B_{12} was most probably completely bound to serum binders because of the low B_{12} concentration. Binding of B_{12} to serum therefore did not alter hepatic removal rates. This contrasted with the perfusion experiments using HIFC and boiled HIFC-bound B_{12} where extraction ratios were approximately 3 times greater and did not fall even at perfusion rates as high as 18,412 pg./100 Gm. rat/min. This high clearance of rate of approximately 80 per cent per minute for HIFC-bound B_{12} corroborates the rapid blood clearance of HIFC-bound B_{12} found by others in the intact rat.[8] This rapid clearance was not found, however, in our previous work with homologous IF in man[1] or by others using homologous IF in the rat,[8] indicating that HIFC functions in rat and man in a manner not comparable to homologous IF binders.

The uptake of NHGJ-bound B_{12}, in this study, was not significantly greater than that of unbound B_{12}, despite the preservation of IF activity of the former as shown in the guinea pig intestinal mucosa homogenate system. This failure of NHGJ to enhance rat liver uptake is in line with the findings of others in the intact rat and rat liver homogenates[3] and slices.[20] It differs, however, from the results obtained with a sequential incubation technic on rat liver slices using human gastric juice.[21]

Some species differences between the responsiveness of rat and human liver to B_{12} binders derived from human and hog stomach exist therefore. The reason for the similarity in clearance of unbound and NHGJ-bound B_{12} is not apparent, especially since uptake of PAGJ-bound B_{12} and B_{12} bound to human saliva by rat liver was significantly greater than that of unbound B_{12}. Although this might be due to cleavage of the NHGJ-B_{12} complex in the perfusate, this does not seem likely since similarly treated NHGJ-B_{12} was taken up more rapidly by liver and maintained higher blood levels over a 24-hour period than unbound B_{12} after i.v. injection in man. These results suggest, therefore, that species difference is a feature of IF binders in this test system and not of the non-IF binders of PAGJ and saliva, which are more likely related to gastric and salivary mucous substances. It is also possible that the divergent behavior of HIFC and homologous human and rat IF may be due to the non-IF binders present in crude HIFC preparations.

Finally, the possibility has been suggested[8] that the enhancement of hepatic B_{12} uptake when bound to saliva and gastric binders may represent the uptake of foreign substances by the reticuloendothelial system of the liver. The results obtained in this study after reticuloendothelial system depression would speak against this. Using an identical dose of india ink, Benaceraff et al.[22] have shown interference with reticuloendothelial system uptake of a second colloid. Such interference with the uptake of HIFC-bound B_{12} after india ink injection was not observed in the present study. Furthermore, Schwartz et al.[23] have demonstrated that binding of B_{12} to HIFC diminishes splenic B_{12} uptake after intraperitoneal injection in rats. This would not be the case if reticuloendothelial system uptake was responsible for the removal of injected HIFC-bound B_{12}. The significant difference observed between the uptake of NHGJ- and PAGJ-bound B_{12} also makes this unlikely.

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Summary

Hepatic uptake of unbound radio-B_{12} and radio-B_{12} bound to various B_{12} binders was studied in 48 rats using a method of in situ liver perfusion of homologous whole blood without recirculation. Compared with unbound radio-B_{12}, radio-B_{12} bound to pernicious anemia gastric juice, human saliva, hog intrinsic factor concentrate, and boiled hog intrinsic factor concentrate increased uptake of B_{12} by rat liver. B_{12} bound to normal human gastric juice, however, was removed in a manner quantitatively similar to that observed with unbound B_{12}.

Depression of reticuloendothelial system activity by india ink injection prior to perfusion of hog intrinsic factor concentrate-bound B_{12} did not significantly alter hepatic B_{12} removal rates. Comparison with results obtained in similar experiments, but without prior reticuloendothelial system depression, suggests that the removal process was accomplished by hepatic parenchymal cells rather than reticuloendothelial cells of the liver.

These results suggest that B_{12} binders of hog intrinsic factor concentrate, pernicious anemia gastric juice, and saliva may function in the membrane transport of B_{12} in the liver in a manner both quantitatively and qualitatively different from those of serum. This transport function is obviously not limited to intrinsic factor, since materials with and without intrinsic factor activity can produce this effect.

SUMMARIO IN INTERLINGUA

Le acceptation hepatic de vitamina B_{12} radioactive non ligate e ligate a varie ligatores de B_{12} esseva studiate in 48 rattos, utilisante un methodo de perfusion hepatic in sito de homologe sanguine total sin recirculation. In comparation con non-ligate radio-B_{12}, le radio-B_{12} ques es ligate a succo gastric ab anemia perniciose, a saliva human, a concentrato de porcin factor intrinsec, e a bullite-concentrato de porcin factor intrinsec augmentava le acceptation de B_{12} per hepate de ratto. Tamen, B_{12} ligate a normal succo gastric human esseva eliminate in un maniera quantitativemente simile a illo observate con non-ligate B_{12}.

Le depression del activitate reticuloendothelial per le injection de tinta de China ante le perfusion de B_{12} ligate a concentrato de porcin factor intrinsec non alterava significativemente le elimination hepatic de B_{12}. Le comparation con resultatos obtenite in simile experimentos, sed sin previe depression del systema reticuloendothelial suggestiona que le processo de elimination esseva complite per hepatic cellulas parenchymal plus toste que per hepatic cellulas reticuloendothelial.

Le resultatos del presente studio suggestiona que le ligatores de B_{12} in concentrato de porcin factor intrinsec, in succo gastric ab anemia perniciose, e in saliva function possibilemente in transporto membranal de B_{12} in le hepate in un maniera que es quantitativemente e qualitativemente differente ab illo de sero. Iste function de transporto es, per consequente, non restringite a factor intrinsec, proque materiales con e sin activitate de factor intrinsec pote producer le effecto.

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Tissue Distribution of Radio-B_{12} after Intravenous Injection of Gastric Juice, Saliva and Plasma-Bound Co^{57} B_{12}

I. In Man

By William S. Rosenthal, Herbert Weisberg and George B. Jerzy Gl

WHETHER INTRINSIC FACTOR (IF) functions outside the gastrointestinal tract is controversial. Normal human gastric juice (NHG has been shown to potentiate the maturation effect of vitamin B_{12} on megaloblastic bone marrow in tissue culture,[1,2] although this finding has been contested.[3,4] Hog intrinsic factor concentrate (HIFC) has also been shown to enhance uptake of B_{12} by rat liver slices,[5,6] homogenates,[7] in the intact animal when injected intravenously,[8] and in isolated perfusions.[9] Brody et al.[10,11] found that the i.v. injection of B_{12} mixed with HIFC increased the blood clearance of B_{12} in pernicious anemia patients to normal. On the other hand, the blood clearance of homologous IF-bound B_{12} in rats was found by Taylor and Medenica[12] to proceed at a significantly slower rate than B_{12} bound to HIFC. In previous work in man, we have shown that homologous IF in NHGJ enhances hepatic uptake of B_{12} after intravenous injection while producing a delay in its blood clearance.[13,14]

Whether these findings in man are specifically a function of intrinsic factor active binders is not known since B_{12} binders without IF activity have not been studied. In the present investigation, therefore, we have extended our previous work with NHGJ[13,14] to the study of the effect of non-IF B_{12} binders in pernicious anemia gastric juice (PAGJ) and autologous plasma in man. The results obtained indicate that a property of the B_{12} binders of PAGJ, but not of plasma binders, is responsible in man for the enhancement of B_{12} uptake by liver and delay in its blood clearance. B_{12} uptake by arm tissue was not significantly different for any of the B_{12} binders tested.

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Materials

Pernicious Anemia Gastric Juice-Bound B_{12}

PAGJ was collected on ice in separate batches during maximal histamine stimulation from 2 pernicious anemia patients in remission. The diagnosis of pernicious anemia was established by the presence of gastric atrophy on suction biopsy, histamine fast anacidity, failure to absorb radio-B_{12}* by the urinary excretion test[15] and hepatic uptake test,[16] but with improved absorption after administration of radio-B_{12} when given with IF. During collection of the juice, the patients were instructed not to swallow their saliva. Samples contaminated with blood or bile were discarded. The gastric juice obtained was then filtered through gauze, centrifuged to remove precipitated materials, and the supernatant passed through a Berkefeld V filter.

The sterile juice was then divided into 10 ml. aliquots and stored aseptically at −20 C. for periods up to 2 months. No IF activity was detected in these juices using the guinea pig intestinal mucosa homogenate[17] and charcoal assay[18,19] methods.†

Twenty-four hours before administration, a 10 ml. aliquot of the juice was thawed and incubated with 1 μg. sterile $Co^{57}B_{12}$ for one-half hour at 25 C. This mixture was concentrated to a volume of 2 ml. by dialysis against 30 per cent Carbowax 6000.[20] In order to separate bound from unbound B_{12}, the concentrated mixture was subjected to gel filtration on Sephadex G-25.[21] The B_{12} bound material appearing in the fractions of the excluded peak was pooled and resterilized by Millipore filtration. After this treatment, the material for injection contained .029-.034 μg. $Co^{57}B_{12}$ (≈.3 μc.) in a 10 ml. volume.

Plasma-Bound B_{12}

Whole blood (350 ml.) was obtained by phlebotomy from each of 2 normal young adult males, anticoagulated with acid citrate-dextrose solution, and the plasma separated under sterile conditions by centrifugation; 150 ml. of plasma derived from each phlebotomy was then incubated with 0.05 μg. $Co^{57}B_{12}$ (≈.7 μc.) for one-half hour at room temperature and injected into the same donors. The $Co^{57}B_{12}$ injected was shown to be over 95 per cent bound on Sephadex G-25 gel filtration. This was as expected since the amount of B_{12} added did not exceed the binding capacity of normal plasma.[22]

It will be noted that in the present study the dosage range of PAGJ bound $Co^{57}B_{12}$ injected was from .029 μg. to .034 μg., the dose of plasma-bound $Co^{57}B_{12}$ was 0.05 μg., and the dose of $Co^{57}B_{12}$ injected unbound was 0.1 μg. Over this dosage range, B_{12} behaves as a tracer, as has been shown in previous work from this laboratory[23] and elsewhere.[24,25]

Methods

Ten studies of hepatic and arm uptake and blood clearance of bound and unbound B_{12} were performed on 4 individuals free of hepatic or hematologic disease. In all studies, 0.1 μg. of unbound $Co^{57}B_{12}$ (≈1.5 μc.) was first injected i.v. and changes in hepatic and arm radioactivity measured every 5 minutes during the first hour, every 1 to 2 hours during the first day, and at intervals for the following week. The method and data used for measuring blood clearance and hepatic uptake of injected NHGJ bound and unbound radio-B_{12} have been described previously.[14]

Blood samples were drawn every 5 minutes for the first 20 minutes and every 10 minutes thereafter during the first hour. Subsequently, blood sampling was carried out at the same time as hepatic radioactivity measurements. The blood was hemolyzed and radioactivity measured in a 1 x 1 inch NaI crystal well type scintillation detector with a gamma spectrometer and scaler.

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*$Co^{57}B_{12}$ (8-18 μc./μg.) and $Co^{60}B_{12}$ (1 μc./μg.) were generously supplied by Dr. Elmer Alpert and Dr. Charles Rosenblum, Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania.

†We are indebted to Dr. Nobuo Yamaguchi of this laboratory for the performance of these assays.

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Seven to 10 days following the initial injection of unbound $Co^{57}B_{12}$ in 4 subjects, PAGJ-bound B_{12} was injected intravenously and similar measurements carried out for another week. In 2 subjects, 10 days following the initial injection of unbound $Co^{57}B_{12}$, autologous plasma-bound $Co^{57}B_{12}$ was administered intravenously and similar measurements carried out.

Hepatic radioactivity was measured by surface scintillation counting[10] with a heavily shielded flat faced 2 x 2 inch NaI crystal probe detector connected to a gamma spectrometer and scaler. The probe was positioned over the cutaneous projection of maximum hepatic dullness and strapped to the patient, who was placed supine on a firm surface with the right side slightly raised and supported by a sandbag. The position of the probe on the skin was marked by an indelible skin marker so that the same geometrical relationship of probe and liver could be maintained.

Arm radioactivity was detected by placing the hand and forearm of the individual tested in a large volume scintillation detector (Armac)* with six radially arranged photomultiplier tubes and coincidence circuitry to minimize background. This detector was coupled to another gamma spectrometer and scaler.

Circulating radioactivity was calculated as per cent of administered dose with blood volume derived from the weight of the subject.[25] Data obtained from hepatic and arm studies were recalculated to a uniform dose of radioactivity for purposes of comparison.

Results

Hepatic uptake of i.v. administered $Co^{57}B_{12}$ bound to PAGJ in 4 subjects and autologous human plasma in 2 subjects is shown in Table 1. For comparison, our previously reported results[11] with unbound B_{12} and NHGJ-bound $Co^{57}B_{12}$ are also shown. As may be seen, uptake of $Co^{57}B_{12}$ bound to PAGJ and NHGJ was more rapid than that of $Co^{57}B_{12}$ bound to plasma or injected unbound. At 60 minutes hepatic uptake of PAGJ-bound $Co^{57}B_{12}$ was significantly greater ($p<.05$) than for plasma-bound $Co^{57}B_{12}$ and unbound $Co^{57}B_{12}$. It was not significantly different, however, from NHGJ-bound $Co^{57}B_{12}$, indicating that the increased uptake induced by gastric juice B_{12} binders was quantitatively similar regardless of IF activity.

Since the geometry of hepatic surface scintillation counting differs in each subject, we calculated the ratio of hepatic radioactivity detected after the injection of bound B_{12} to that after the injection of unbound B_{12} in the same individual after identical time intervals. This permitted comparative treatment of the data with the exclusion of individual variations in counting geometry. The means of these ratios have been calculated (Table 2) and plotted for the gastric juice and plasma binders (Fig. 1). The line representing the mean ratio plasma-bound $Co^{57}B_{12}$/unbound $Co^{57}B_{12}$ remains near unity, indicating no difference in uptake between the two. In contrast, the mean PAGJ-bound $Co^{57}B_{12}$/unbound $Co^{57}B_{12}$ ratio was 1.43 at 1 hour. The difference compared with plasma-bound $Co^{57}B_{12}$/unbound $Co^{57}B_{12}$ was significant ($p<.01$) and indicates enhancement of hepatic uptake of B_{12} in man by PAGJ administered intravenously.

The mean PAGJ-bound $Co^{57}B_{12}$/unbound $Co^{57}B_{12}$ ratio of 1.43 was, however, not significantly different from the ratio of 1.72 found in our previous work for NHGJ-bound $Co^{57}B_{12}$/unbound $Co^{57}B_{12}$. Thus, enhancement of hepatic uptake of B_{12} by NHGJ with IF activity and PAGJ devoid of IF activity

*Packard Instrument Company, Inc., La Grange, Illinois.

Table 1.—Hepatic Radioactivity* in Man Following Intravenous Injection of $Co^{57}B_{12}$ Unbound and Bound to Pernicious Anemia and Normal Human Gastric Juice, and Autologous Plasma

	No. of Studies	10 min.	60 min.	6 hr.	24 hr.	7 days
PAGJ-B_{12}	4	6,888 ±791	10,855 ±1,918	11,876 ±3,405	13,178 ±3,705	17,709 ±5,623
NHGJ-B_{12}†	5	6,839 ±1,816	12,812 ±1,712	10,871 ±1,293	11,776 ±1,349	14,465 ±1,860
Plasma-B_{12}	2	8,449 ±1,087	8,475 ±895	10,146 ±850	14,081 ±1,483	10,614
Unbound B_{12}†	9	7,072 ±744	7,560 ±770	7,734 ±884	9,328 ±1,154	14,126 ±1,367

*Mean (with standard error) radioactivity (c.p.m.).
†These data are from previously reported work.[14]

is not significantly different during the early period after administration. Although there were no significant differences in the uptake before 24 hours, it may be seen in Figure 1 that at 7 days, while the uptake ratio of PAGJ-bound $Co^{57}B_{12}$ was 1.66, that of NHGJ-bound $Co^{57}B_{12}$ was near unity. This difference is significant at the 1 per cent level, indicating some late divergence in the hepatic turnover of PAGJ-bound B_{12} on one hand and NHGJ-bound B_{12} on the other.

Blood clearance of radio-B_{12} following the injection of B_{12} bound to PAGJ in 4 subjects and plasma in 2 subjects is shown in Figure 2. For comparison, our previously reported results[14] with B_{12} alone in 5 subjects and bound to NHGJ in 2 subjects are also shown. Clearance of radioactivity from the blood is significantly delayed during the first 24 hours for B_{12} bound to either PAGJ or NHGJ as compared to unbound B_{12} or B_{12} bound to plasma. As shown in Figure 3 at 24 hours, means (± standard error) of 18.8 ± 0.4 per cent of the injected dose of NHGJ-bound and 17.3 ± 3.1 per cent of the dose of PAGJ-bound B_{12} were detected in blood. These B_{12} levels were significantly greater ($p<.02$) than those observed after injection of plasma-bound B_{12} or unbound B_{12} (3.2 ± 0.5 per cent and 4.5 ± 0.5 per cent, respectively). Thus, despite its more rapid hepatic uptake, B_{12} bound to gastric juice with or without IF activity is retained in the circulation longer than B_{12} injected bound to plasma or unbound.

Changes in arm radioactivity after injection of bound and unbound B_{12} are shown in Tables 3 and 4. To minimize the error due to variations in counting geometry of the arms of different individuals, the ratio of arm radioactivity after the injection of B_{12} bound to PAGJ, NHGJ, or plasma to that after injection of unbound B_{12} was calculated for the same patients (Table 4). As may be seen, there are no significant differences at any given time between the ratios of PAGJ-, NHGJ-, or plasma-bound $Co^{57}B_{12}$ to unbound $Co^{57}B_{12}$. This finding indicates that binding B_{12} to gastric juice with or without IF activity or to plasma does not affect its uptake by arm tissue.

A further comparison of the change in the uptake ratios for NHGJ- and PAGJ-bound B_{12} in liver and arm are shown in Figure 4. In the arm, these

Table 2.—Ratio* of Hepatic Radioactivity Following Intravenous Injection of $Co^{57}B_{12}$ Bound to Pernicious Anemia and Normal Human Gastric Juice and Autologous Plasma to that of Unbound $Co^{57}B_{12}$ in the Same Individual after the Same Time Interval

	No. of Studies	10 min.	60 min.	24 hr.	6 hr.	7 day
PAGJ–B_{12}/unbound B_{12}	4	1.07 ±.10	1.43 ±.00	1.57 ±.38	1.72 ±.05	1.66 ±.[illegible]
NHGJ-B_{12}/unbound B_{12}†	5	0.93 ±.17	1.73 ±.12	1.44 ±.10	1.32 ±.12	1.[illegible] ±.[illegible]
Plasma-B_{12}/unbound B_{12}	2	1.06 ±.13	0.93 ±.08	1.02 ±.15	1.21 ±.33	1.1[illegible]

*Mean (with standard error) ratio.
†These data are from previously reported work.[11]

ratios come to a peak immediately after the injection of PAGJ-bound or NHGJ-bound B_{12} and decline thereafter. This differs from the changes in the uptake ratios in liver, which increase rapidly for the first hour after injection, reflecting the different manner in which B_{12} enters each of these tissues.

The relationship of arm to blood radioactivity was evaluated by plotting the mean ratio arm/blood radioactivity for each study group as a function of time (Fig. 5). For NHGJ- and PAGJ-bound B_{12} this ratio increased approximately threefold during the first 24 hours. On the other hand, it increased tenfold and sevenfold, respectively, during the same time period after the injection of B_{12} alone and bound to plasma. Thus, with the passage of time, the radioactivity detected in the arm after injecting B_{12} alone or bound to plasma represented radio-B_{12} in tissues rather than radio-B_{12} circulating in the blood of the arm. In contrast, NHGJ- and PAGJ-bound B_{12} are taken up less by arm tissue and remain longer in circulation.

Discussion

In the present study, binding of B_{12} to PAGJ enhanced hepatic uptake of B_{12}, after i.v. injection in man, over that seen with unbound B_{12}, whereas the binding of B_{12} to plasma did not. This effect of PAGJ during the first 24 hours after administration is similar to that observed previously by us in man with NHGJ.[13,14] Thus, human gastric juice B_{12} binders, with or without IF activity, enhance early hepatic uptake of B_{12} above that observed after the i.v. injection of B_{12} either unbound or bound in vitro to human plasma. This hepatic uptake effect, however, is not a function of binding alone, since binding to plasma did not increase hepatic B_{12} uptake.

The similar behavior of injected unbound B_{12} and plasma-bound B_{12} in the liver is in line with previously demonstrated similarities in their blood clearance.[22] Hall has shown that intravenously injected trace amounts of B_{12} are rapidly bound in vivo to the same binder, as similar amounts of B_{12} incubated with plasma in vitro.[22]

In interpreting our results with gastric juice, it is important to note that PAGJ for all practical purposes contains a single non-IF (tertiary) B_{12} binder[26-28] and that the B_{12} injected with it was over 95 per cent bound. In

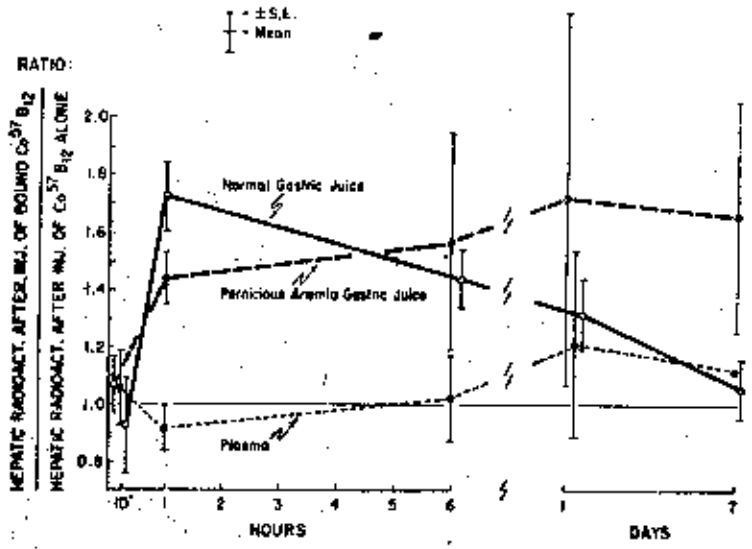

Fig. 1.—Ratio of hepatic radioactivity after the sequential injection of $Co^{57}B_{12}$ bound to normal and pernicious anemia gastric juice and plasma to hepatic radioactivity after injection of $Co^{57}B_{12}$ alone in the same individuals.

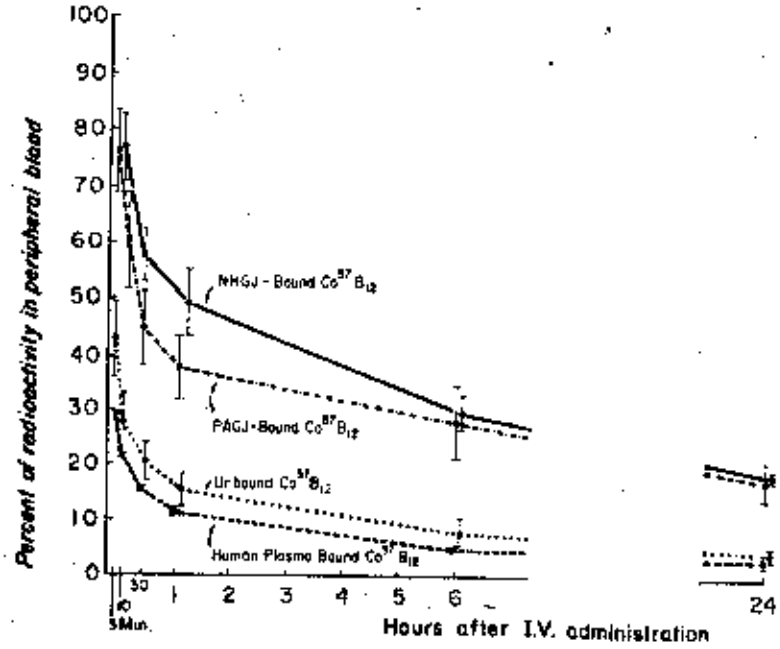

Fig. 2.—Blood clearance of unbound $Co^{57}B_{12}$ and of $Co^{57}B_{12}$ bound to normal and pernicious anemia gastric juice and plasma following intravenous injection.

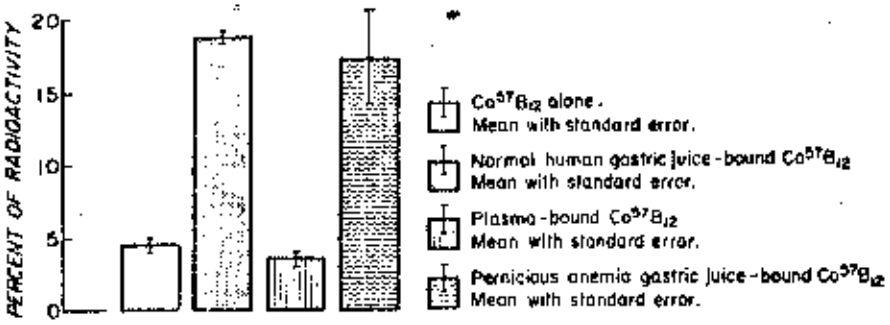

Fig. 3.—Per cent of administered radioactivity remaining in blood 24 hours after intravenous injection of $Co^{57}B_{12}$ alone and $Co^{57}B_{12}$ bound to normal and pernicious anemia gastric juice and plasma.

contrast, we consider the B_{12} binders of histamine-stimulated NHGJ used in our previous work[7,11] to be almost entirely IF-related, since it has been shown in this laboratory that the non-IF-related B_{12} binders are largely destroyed by acid incubation.[26,27] Although there is some disagreement on the latter point,[28] in any event maximum histamine stimulation of gastric juice secretion increases the output of IF-related binders, while the output of non-IF binders is decreased.[29] From these considerations, it may be concluded that both the IF binders of NHGJ and the non-IF binders of PAGJ must have been responsible for the enhancement of hepatic B_{12} uptake and the delay in its blood clearance. Although the reasons for the late difference in the hepatic turnover of PAGJ- and NHGJ-bound B_{12} are not clear, this may be due to differences in their metabolic pathways after entry into the hepatic cell or their differential secretion into bile. It has been shown that IIIFC may be secreted into bile after passage through the liver during perfusion of the isolated rat liver.[9]

The different behavior of gastric juice-bound and plasma-bound B_{12} does not support the contention of Simons[30] that a binder, "R", common to both, is responsible for enhancing B_{12} uptake by the liver; neither does it support the more recent contention of Toporek[31] that "R" binder is the same as "circulating IF." Although the "R" binders of both human gastric juice and plasma may possess certain immunologic and physicochemical similarities[30] and can enhance B_{12} uptake by rat liver homogenates,[32] they evidently have different biological activities under the conditions of our studies in man. Furthermore, as shown by Finkler and Hall,[33] there are distinct physicochemical and biological differences between the "R" binder-B_{12} complex of gastric juice and plasma in their characteristics on column chromatography and their uptake by HeLa cell cultures.

The delay in blood clearance of radio-B_{12} in man after i.v. injection of PAGJ-bound B_{12} also resembles our previous observations with NHGJ-bound B_{12}.[13,14] This contrasts with the rapid clearance of plasma-bound B_{12} found by Hall[22] and confirmed in the present work, which resembles the rapid clearance of unbound B_{12} found by earlier workers.[34] The slow disappearance of NHGJ- and PAGJ-bound B_{12} differ with the findings of Brody et al.,[11] who observed

Table 3.—Arm Radioactivity* in Man Following Intravenous Injection of $Co^{57}B_{12}$ Bound to Pernicious Anemia and Normal Human Gastric Juice, Autologous Plasma, and Unbound

	No. of Studies	5 min.	60 min.	6 hr.	24 hr.	7 days
PACJ-B_{12}	4	8,473 ±1,095	5,579 ±697	5,998 ±841	6,143 ±891	3,804 ±818
NHGJ-B_{12}	5	8,853 ±1,056	5,322 ±523	5,714 ±457	5,644 ±320	4,804 ±385
Plasma-B_{12}	2	4,190 ±926	4,089 ±929	4,571 ±1,053	4,107 ±461	3,108
Unbound B_{12}	9	5,872 ±626	4,382 ±515	5,349 ±471	5,353 ±455	4,083 ±344

*Mean (with standard error) radioactivity (c.p.m.).

Table 4.—Ratio* of Arm Radioactivity Following Intravenous Injection of $Co^{57}B_{12}$ Bound to Pernicious Anemia and Normal Human Gastric Juice, and Autologous Plasma to that of Unbound $Co^{57}B_{12}$ in the Same Individual and at the Same Time Intervals

	No. of Studies	5 min.	60 min.	6 hr.	24 hr.
PACJ-B_{12}/unbound B_{12}	4	1.55 ±.37	1.34 ±.29	1.11 ±.17	1.20 ±.18
NHGJ-B_{12}/unbound B_{12}	5	1.87 ±.23	1.08 ±.22	1.03 ±.03	0.98 ±.06
Plasma-B_{12}/unbound B_{12}	2	1.65 ±.87	1.59 ±.94	1.26 ±.47	0.77

*Mean (with standard error) ratio.

more rapid disappearance from the blood of injected B_{12} mixed with HIFC in patients with pernicious anemia and intestinal malabsorption. Comparison of results is difficult, however, since homologous binders were used in the present study and heterologous binders were used in their study. This difference is important since it has been shown in the rat that HIFC-bound B_{12} is cleared more rapidly from the blood than is B_{12} bound to homologous IF.[12] Since our findings also demonstrate a slower clearance of homologous IF-bound B_{12}, it may be that the more rapid clearance of HIFC-bound B_{12} in both rat and man is a characteristic of HIFC when used in heterologous systems.

Finally, the blood clearance data of PACJ- and NHGJ-bound B_{12} suggests that although B_{12} uptake at hepatic sites is enhanced, movement into the non-hepatic extravascular pool is reduced. An alternate explanation is that the apparent slow disappearance represents reentry of radioactivity into the circulation, still in a bound form, as evidenced by the high blood radioactivity at 24 hours.

The possibility that removal of injected gastric juice-bound B_{12} by the liver is mediated by the reticuloendothelial system has been suggested.[12] If this were true, its disappearance curve would be different, since removal of foreign materials injected into the circulation proceeds exponentially.[35] A more complete discussion of the evidence against the removal of gastric juice-bound B_{12}

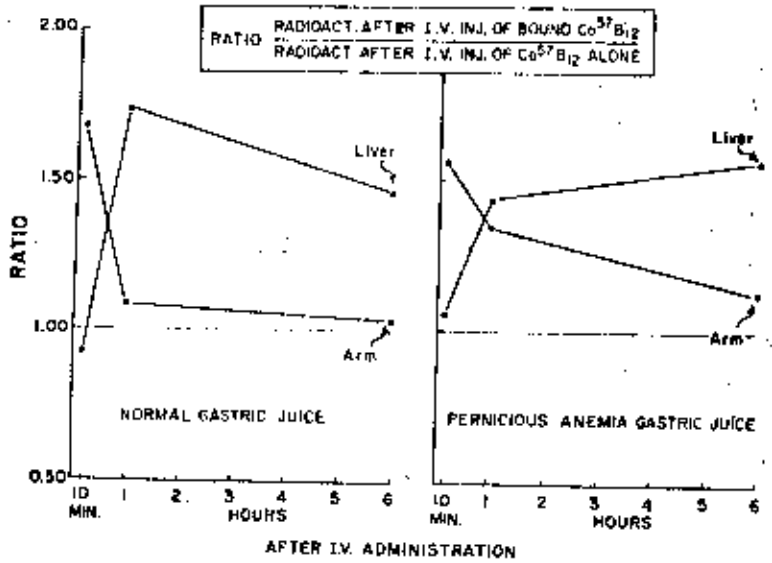

Fig. 4.—Comparison of the ratios of radioactivity after intravenous injection of $Co^{57}B_{12}$ bound to normal and pernicious anemia gastric juice to that after $Co^{57}B_{12}$ alone in liver and arm.

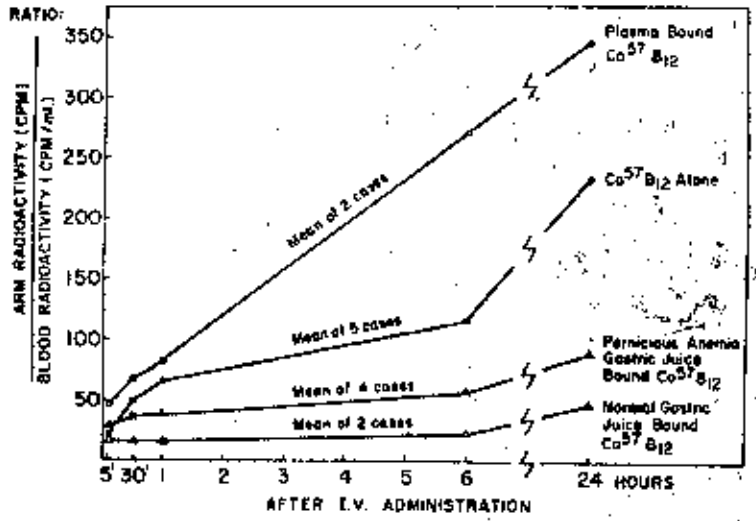

Fig. 5.—Relationship of arm to blood radioactivity after i.v. injection of $Co^{57}B_{12}$ alone and $Co^{57}B_{12}$ bound to normal and pernicious anemia gastric juice and plasma.

from the circulation as a foreign substance is given in Part II of this study.

Although arm radioactivity initially reflected blood radioactivity following injection of plasma bound-B_{12}, as time elapsed arm radioactivity no longer reflected blood radioactivity. This speaks for the passage of the injected plasma-bound B_{12} into nonhepatic extravascular sites. After injection of gastric

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juice-bound B_{12}, however, the ratio of arm to blood radioactivity remained relatively constant, indicating decreased distribution to extravascular sites in the arm under these conditions. Loss in the urine would not explain the more rapid disappearance of unbound B_{12}, since at the dosage levels used in this study urinary losses are not significant.[22,34] These findings are in accord with the work of Okuda and associates[6] and Schwartz et al.[36] in rats who have shown that the administration of B_{12} with IF was followed by enhancement of the uptake of B_{12} by liver, while injection of unbound B_{12} resulted in a greater distribution into a nonhepatic, extravascular pool.

The reason that plasma binding resulted in more rapid passage of the B_{12} into the extravascular pool is not clear. It implies that gastric juice B_{12} binders are more efficient in the transport of B_{12} from blood to liver than the plasma binder, transcobalamin II. This may be due to the different behavior of B_{12} at the liver cell membrane when bound to gastric juice or to plasma. It may also be due to the prolonged exposure of the liver to gastric juice bound B_{12} resulting from its greater retention in the circulation. On the other hand, the similar behavior of B_{12} bound to NHGJ and PAGJ may be due to similarities in molecular configuration, size, or to similar reactive groups on the binders.

Summary

$Co^{57}B_{12}$ bound to pernicious anemia gastric juice was administered intravenously to 4 normal individuals, and $Co^{57}B_{12}$ bound to autologous plasma was administered to 2 of these. Hepatic, arm and blood radioactivity were measured for one week and compared with that following the intravenous injection of unbound $Co^{57}B_{12}$. Pernicious anemia gastric juice increased hepatic uptake, delayed blood clearance and decreased arm uptake of $Co^{57}B_{12}$ as compared with $Co^{57}B_{12}$ injected unbound or bound to plasma. Thus, the B_{12} bound to gastric juice is retained longer in the intravascular space with greater uptake by liver.

These results suggest that gastric juice B_{12} binders, but not plasma B_{12} binders, enhance hepatic B_{12} uptake either by acting at specific receptor sites on the liver cell membrane or by retaining B_{12} longer in circulation. This property of gastric juice B_{12} binders is not related to IF activity but is rather dependent upon some structural or functional feature common to both IF-related and nonrelated B_{12} binders of gastric juice.

SUMMARIO IN INTERLINGUA

Vitamin B_{12} a Co^{57} ligate a succo gastric ab subjectos con anemia perniciose esseva administrate per via intravenose a 4 subjectos normal. Duo de istes recipeva B_{12} a Co^{57} ligate a plasma autologe. Lo radioactivitate hepatic, brachial, e sanguinee esseva mesurate durante un septimana e comparate con valores observate post le injection intravenose de non-ligate B_{12} a Co^{57}. Succo gastric ab anemia perniciose augmentava le acceptation hepatic, retardava le clearance sanguinee, e reduceva le acceptation in le bracio de B_{12} a Co^{57} in comparation con B_{12} a Co^{57} injicite sin ligation o ligate a plasma. Il seque que le B_{12} ligate a succo gastric es retenite plus longemente in le spatio intravascular, resultante in un plus grande acceptation hepatic.

Iste resultatos suggestiona que ligatores de B_{12} in succo gastric (sed non illos in plasma) promove le acceptation hepatic de B_{12} in consequentia del facto que illos age in specific situs receptori in le membrana del cellulas hepatic o del facto que illos retene B_{12} plus

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longemente in le circulation. Iste proprietate de ligatores de B_{12} in succo gastric non es relationate al activitate de factor intrinseco sed depende plus tosto del un o del altere characteristica structural o functional que es commun a ligatores de B_{12} in succo gastric del typo relationate e del typo non-relationate con factor intrinseco.

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Vitamin B_{12} Assay in Body Fluids

VITAMIN B_{12} is required as an essential growth factor by *Euglena gracilis* variant *bacillaris*[1]. No other substance has been found to stimulate the growth of this alga in the absence of B_{12}.

Assays of crystalline B_{12} (obtained by courtesy of Glaxo Laboratories) and liver extracts have been carried out, using a modification of the technique described by Hutner. About 1 μgm./ml. is the least amount stimulating growth, and, because of the greater sensitivity of this assay method over the *Lactobacillus* methods, it was considered more suitable for attempting the estimation of body fluid concentrations of vitamin B_{12}.

Normal human serum, human urine (unless dilute) and horse serum were found to stimulate growth of the *Euglena* when added to a basal medium containing no B_{12}. It is not practicable to detect less than the equivalent of about 10 μgm./ml. with the technique used, because of growth inhibition and coagulation of serum on heating if these fluids are more concentrated than in a final dilution of 1/8 or 1/10. The amount of growth obtained with sera was found to vary according to the degree of heating before inoculation. Preliminary work was done with dilutions of sera in distilled water heated at 70° C. for half an hour and added to the basal medium, and values equivalent to 20–350 μgm./ml. with a mean of 185 μgm./ml. were found in sera from forty patients with a variety of conditions. Twelve similar sera, heated at 100° C. for a half to one hour, were much more active, giving the equivalent of 350–750 μgm./ml. with a mean of 490 μgm./ml.,

and some assays of other sera have suggested activity equivalent to more than 1,000 μμgm./ml. Sera, however, heated at 100° C. for one and a half to two hours were less active than after one hour, while heating at 100° C. at about pH 10 for half an hour reduced the activity of twelve sera by from 60 to 95 per cent. As an example of the difference in growth activity after heating, a batch of horse serum heated for half an hour at 56° C. gave growth equivalent to 13 μμgm./ml., at 70° C. to 70 μμgm./ml. and at 100° C. to 220 μμgm./ml.

Heating of urine at 70° C. and cerebro-spinal fluid at 100° C. has not significantly altered assay results. The activity of the few cerebro-spinal fluids so far tested has been from nil to 16 μμgm./ml.—a very low result compared with sera—and that of urines has been from nil to 200 μμgm./ml.

Sera in the lowest practicable dilutions (heated at 70° C.) from four cases of pernicious anæmia, and urine from one, did not stimulate growth of the *Euglena*. (Urines from the other cases were not available for testing.) Two of these sera were later heated at 100° C. and by autoclaving, and some activity was found, but this was much less than with the normal sera so far assayed.

Sera from two cases of pernicious anæmia after intramuscular injection of B_{12} showed very marked activity which has gradually declined in serial assays continued over several weeks after injection. Heating of these sera also was necessary for full activity, suggesting that B_{12} circulates in a microbiologically inactive (an altered or combined) form after injection, as it appears to do when absorbed normally from the intestine. Two and a half hours after an injection, however, as much as 25 per cent of the total activity in one serum could be detected without heating.

This modification of Hutner's technique would therefore appear to detect B_{12} in body fluids as well as in liver extracts, particularly in view of the fact that the activity of serum is increased after parenteral injection of B_{12}. The possibility of some substance other than B_{12} present in body fluids stimulating or potentiating growth cannot yet be excluded; but the relatively poor growth with the pernicious anæmia sera so far tested, and the reduction of activity of normal serum at 100° C. at pH 10, suggest that the assay is specific.

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April 19.

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Evidence for the absorption of immunoreactive intrinsic factor into the intestinal epithelial cell during vitamin B_{12} absorption

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To investigate the distribution and nature of vitamin B_{12} in the subcellular fractions of the ileal epithelial cells during the process of absorption, $^{57}CoB_{12}$ bound to human intrinsic factor (IF) was incubated in isolated ileal loops of anesthetized guinea pigs and periodically for 120 minutes the animals were killed and the homogenate of the mucosa separated by differential centrifugation into (1) cell debris fraction containing large fragments of partially disrupted surface structures, nuclei, and other cell constituents, including some mitochondria, (2) mitochondrial fraction, (3) microsomes, and (4) cytosol. The particulate composition of each fraction was confirmed using electron microscopy. Radioactivity accumulated continuously during the observation period in the cell debris and mitochondria-rich fractions. Lesser amounts were found with the microsomes and in the cytosol. The nature of the $^{57}CoB_{12}$ in the cell debris and mitochondrial fractions was further studied by sonicating each fraction to solubilize the radioactivity and then removing the insoluble particulate by centrifugation at 10,000 g. Eighty to 94 per cent and 57 to 78 per cent of the solubilized radioactivity in the supernates of the cell debris and mitochondrial fractions, respectively, could not be extracted by albumin-coated charcoal, indicating that a major fraction of the $^{57}CoB_{12}$ was bound to a macromolecule. When these supernates were then incubated with antihuman IF antibody, 71 to 84 per cent and 54 to 69 per cent of the radioactivity in the cell debris and mitochondrial fractions, respectively, precipitated with the immunoglobins at 15 per cent Na_2SO_4 indicating that a major fraction of the absorbed $^{57}CoB_{12}$ was bound to a large molecule with the immunologic identity of IF. Although significantly less radioactivity in the supernates of these sonicated fractions precipitated at 15 per cent Na_2SO_4 in the absence of anti-IF antibody, it was still significantly more than precipitated when IF-$^{57}CoB_{12}$ in gastric juice was subjected to this salt concentration. This finding suggests that either a small fraction of the absorbed immunoreactive IF-B_{12} complex has been altered in some way, or that some of the absorbed $^{57}CoB_{12}$ becomes bound to a different protein which is insoluble at 15 per cent Na_2SO_4.

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The intestinal phase of vitamin B_{12} (B_{12}) absorption begins with the attachment of an intrinsic factor (IF)-B_{12} complex to the brush borders and microvillous membranes of the ileal mucosal cell.[1] In the guinea pig this attachment is mediated by a specific IF-binding macromolecular factor located on the surface of the intestinal mucosa.[2] More recent studies using the guinea pig have demonstrated that during absorption, B_{12} accumulated in the mitochondrial fraction of the mucosal epithelium.[3, 4]

Very little is known of the fate of IF during this phase of B_{12} absorption. Since B_{12} in blood is bound to non-IF proteins, at some point in the absorption process either the vitamin must separate from IF or the IF-B_{12} complex must be restructured. To gain further insight into this process, it would be important to determine whether B_{12} enters the ileal cell bound to IF. The guinea pig is well suited for the study of B_{12} absorption because its ileum accepts IF of many different species.[5] We have, therefore, determined the early subcellular distribution and nature of B_{12} in the guinea pig ileal mucosa during in vivo absorption of $^{57}CoB_{12}$ previously incubated with normal human gastric juice. The results of these studies indicate that a significant fraction of B_{12} absorbed into the ileal cell is localized in the mitochondria-rich fraction bound to a macromolecule with the immunologic identity of IF.

Methods

High specific activity $^{57}CoB_{12}$ (approximately 200 to 250 μCi per microgram) was purchased from the N. V. Philip-Duphar Company, Petten, Netherlands. Normal human gastric juice (NHGJ) obtained after augmented histamine stimulation[6] was used as the source of IF. Serum obtained from a patient with pernicious anemia was shown to contain binding type anti-IF antibody by radioimmunoassay,[7] and this antiserum was used for the immunoassay of IF by a method reported from this laboratory.[7] This radioimmunoassay is based on the observation that $^{57}CoB_{12}$ bound to IF and non-IF binders in human gastric juice will not precipitate at a concentration of 15 per cent Na_2SO_4. However, when IF is in turn bound by specific anti-IF antibody, the whole antibody-IF-$^{57}CoB_{12}$ complex will then precipitate at this salt concentration.[8] The unsaturated B_{12} binding capacity of the anti-IF antiserum and normal control serum was reduced by the addition of 2 ng. per milliliter of unlabeled B_{12}, an amount which exceeds the unsaturated B_{12} binding capacity of most normal human serums.[9]

In vivo guinea pig incubations. Following 72 hours of fasting, the terminal 25 cm. of small intestine in guinea pigs anesthetized with ether was isolated between 2 silk ligatures. Four milliliters of Krebs-Henseleit (K-H) buffer solution, pH 7.4, containing approximately 5 ng. of $^{57}CoB_{12}$ and sufficient NHGJ to bind to 80 to 90 per cent of the vitamin was injected into the isolated intestinal lumen of each of 3 animals. In general, 70 to 75 per cent of the $^{57}CoB_{12}$ in the NHGJ was bound to IF as determined by radioimmunoassay.[7] At predetermined intervals, from 7 to 120 minutes, the animals were bled by cardiac puncture and then killed. In one series of experiments the loops of intestine were isolated by nontraumatic clamps instead of ligatures, the incubation mixture was flushed out of the small intestine into the colon with 0.15M NaCl after 10 minutes' incubation, and the animals were then killed 120 minutes later.

Fractionation of ileal mucosa. All of the following steps were carried out at 4° C. The lumen of the isolated intestinal segment was opened longitudinally, washed thoroughly with K-H buffer, and the mucosa then scraped with a glass slide. The scrapings were washed several times with K-H buffer and then suspended in 0.25M sucrose and homogenized with 5 or 6 up-and-down strokes of a motor-driven Potter-Elvejhem pestle. The homogenate was centrifuged for 10 minutes at 600 g and the first sedimented fraction, termed cell debris, obtained. This fraction was washed twice with, and then suspended in 4 ml. of K-H buffer and stored at -20° C.

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The supernate separated from the cell debris was centrifuged for 10 minutes at 1,200 g, the sediment discarded, and this supernate centrifuged for 30 minutes at 10,000 g to obtain a second sediment, the mitochondrial fraction. This fraction was washed twice with, and suspended in 2 ml. of K-H buffer and stored at -20° C.

The supernate from the mitochondrial separation was centrifuged at 100,000 g for 60 minutes to obtain a third sediment, the microsomal fraction. The final supernate, or cell cytosol, was also obtained from this separation. The microsomes were washed twice with, and then suspended in 2 ml. of K-H buffer and stored at -20 C°.

The following studies were performed on each fraction:

1. Before the sediments were suspended and frozen in K-H buffer, a small sample of each was prepared for electron microscopy by a method previously described for intestinal tissue[10] to determine the completeness of separation of each of the subcellular fractions. The efficiency of the separation was further determined by measuring the alkaline phosphatase activity of the cell debris and mitochondrial fractions using p-nitrophenylphosphate as the substrate.[11]

2. The specific activity of $^{57}CoB_{12}$ in each subcellular fraction (counts per minute per milligram of protein), was determined by dissolving 0.1 ml. of each in 0.9 ml. of 1 N NaOH. The radioactivity of 1 aliquot was assayed using a well-type scintillation detector, and the protein concentration of a second aliquot was determined using the method of Lowry and co-workers.[12]

3. To determine whether the absorbed $^{57}CoB_{12}$ was either free or bound to a macromolecule, the cell debris and mitochondrial fractions were sonicated at 4° C. with a Branson sonifier by applying 10 second bursts for 3 minutes at the 5 watt setting. The disrupted sediment was then removed by centrifugation at 10,000 g for 30 minutes and the supernatant of each fraction analyzed as follows: (1) An aliquot of each fraction in a total volume of 1.3 ml. of K-H buffer was treated with a small spatula of dry albumin-coated charcoal,[13] and following centrifugation the supernate was assayed for radioactivity. Unbound $^{57}CoB_{12}$ is virtually all extracted by the coated charcoal while $^{57}CoB_{12}$ bound to a macromolecule remains in solution. (2) To further determine whether any of the $^{57}CoB_{12}$ was bound to IF, an aliquot of each sonicated fraction was incubated with 0.3 ml. of anti-IF antiserum and sufficient K-H buffer for a total volume of 1.3 ml. A similar reaction mixture containing normal serum instead of anti-IF antiserum was run as control. After 5 minutes' incubation 1.3 ml. of 30 per cent Na_2SO_4 was added to each mixture to precipitate immunoglobins and other proteins insoluble at 15 per cent Na_2SO_4. After 20 minutes' standing to insure complete precipitation, the mixture was centrifuged and the supernate assayed for radioactivity. The level of statistical significance of these experiments was determined using Student's t test.

Results

The distribution of $^{57}CoB_{12}$ in each subcellular fraction and cytosol is shown in Fig. 1, where specific activity is plotted as a function of time. The greatest accumulation of radioactivity occurred in the cell debris and mitochondrial fractions for the 2 hours of observation. Microsomal radioactivity was much lower and plateaued after 1 hour. The activity of the cytosol was also much lower but appeared to increase slightly with time.

The simultaneous increase in radioactivity of the cell debris and mitochondrial fractions suggested that the mitochondrial activity may have been an experimental artifact due to contamination with elements from the cell debris fraction. To rule out this possibility 3 different controls were applied: First, a comparison was made between the ratio of cell debris to mitochondrial specific activity obtained (1) after continuous ileal incubation of NHGJ-$^{57}CoB_{12}$ mixture for 2 hours and (2) after incubation of the NHGJ-$^{57}CoB_{12}$ mixture in the ileum for 10 minutes after which it was flushed out of the lumen and the animal then

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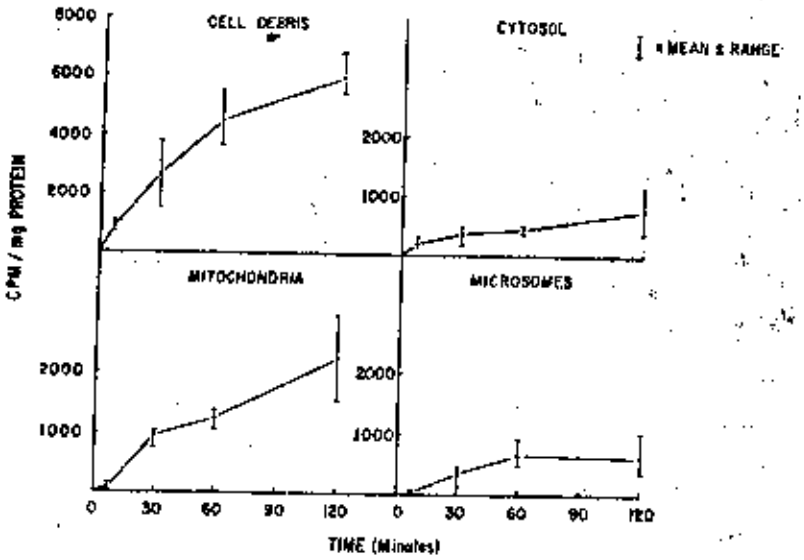

Fig. 1. Accumulation of $^{57}CoB_{12}$ in the cell fractions of guinea pig ileum. Each point and vertical line represents the mean and range of values, respectively, for 3 animals.

killed 2 hours later. The results are shown in Table I. It is evident that with continuous incubation, the cell debris contained significantly more radioactivity than the mitochondria as indicated by the higher ratio of specific activities. If the observed mitochondrial radioactivity was a result of contamination from cell debris, similar ratios of activity should have been obtained in both experiments.

Secondly, electron microscopic analysis showed that with the exception of variable but low contamination by microsomal elements and lysosomes, the mitochondrial fraction contained no other cellular components (Fig. 2). In particular, no mitochondrial fraction was ever seen to contain intact surface epithelial structures (i.e., microvilli or microvillous membranes). The cell debris fraction on the other hand, did contain an abundant quantity of both surface structures (brush borders) and mitochondria (Fig. 3).

Finally, because surface structures contain greater alkaline phosphatase activity than mitochondria,[14] an aliquot of each reaction was analyzed for this enzyme. The ratio of cell debris to mitochondrial activities was 4.2 ± 1.7 (mean ± S.D.). Thus, the cell debris contained the expected fourfold greater activity for this enzyme than did the mitochondria.[14]

The results of experiments to determine whether the $^{57}CoB_{12}$ in the cell debris and mitochondrial fractions was free or bound are shown in Figs. 4 and 5, respectively. The major portion of radioactivity of each fraction was bound to a macromolecule as determined by the charcoal absorption method. When sonicates of each fraction were then incubated with anti-IF antiserum, almost as much radioactivity as was previously determined to be bound, precipitated at 15 per cent Na_2SO_4 indicating that a major fraction of the $^{57}CoB_{12}$ was bound to a macromolecule with the immunologic identity of IF.[7]

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Table I. Comparison of ratio of specific activity of cell debris to mitochondria $^{57}CoB_{12}$ obtained for continuous and pulse incubation of NHGJ$^{57}CoB_{12}$ in ileal loops

Experiment	Specific activity* (c.p.m./mg.)		Cell debris / Mitochondria
	Cell debris	Mitochondria	
Continuous:†			
1	5,407	1,632	3.31
2	6,795	2,950	2.30
3	5,580	2,161	2.58
			M ± S.E.M. = 2.73 ±0.30§
Pulse:‡			
1	239	432	0.55
2	219	590	0.37
3	365	807	0.45§
4	641	481	1.33
5	684	886	0.08
			M ± S.E.M. = 0.67 ±0.17§

*Determined 120 minutes after installation of NHGJ-$^{57}CoB_{12}$ into ileal loops.
†NHGJ-$^{57}CoB_{12}$ kept in isolated ileal loop for total 120 minute incubation period.
‡NHGJ-$^{57}CoB_{12}$ flushed out of ileal loop after 10 minutes, and animals killed 120 minutes later.
§Mean ± S.E.M. Significance of difference between these groups is $p < 0.001$.

When, as a control, normal serum was substituted for anti-IF antiserum, significantly less cell debris and mitochondrial radioactivity precipitated at 15 per cent Na_2SO_4. However, the per cent $^{57}CoB_{12}$ which did precipitate was significantly more than precipitates when NHGJ containing $^{57}CoB_{12}$ is subjected to this salt concentration (Table II).[7, 8] Sonicated mitochondria prepared from the ileum of guinea pigs not previously exposed to NHGJ bind little $^{57}CoB_{12}$, and that which is bound does not precipitate at a concentration of 15 per cent Na_2SO_4 whether incubated with anti-IF antiserum or normal serum (Table II, Experiments 3 and 4).

Discussion

The most striking finding of the present study was the accumulation in the mitochondrial fraction of ileal absorptive cells of B_{12} bound to immunoreactive IF. This result extends the work of previous investigators who have shown that the IF-B_{12} complex attaches to the microvillous membranes of the hamster ileal cell[1] and that B_{12} accumulates in the mitochondria of guinea pig ileum epithelium.[5, 6] Although electron microscopic analysis of the subcellular fractions demonstrated that the major component of the 10,000 g fraction was mitochondria, lysosomal elements were also present. At this time we cannot exclude the possibility that the bound B_{12} was localized in these lysosomes.

In the present study, IF-bound B_{12} also accumulated in the cell debris fraction. This finding is to be expected because this fraction of the mucosa was shown

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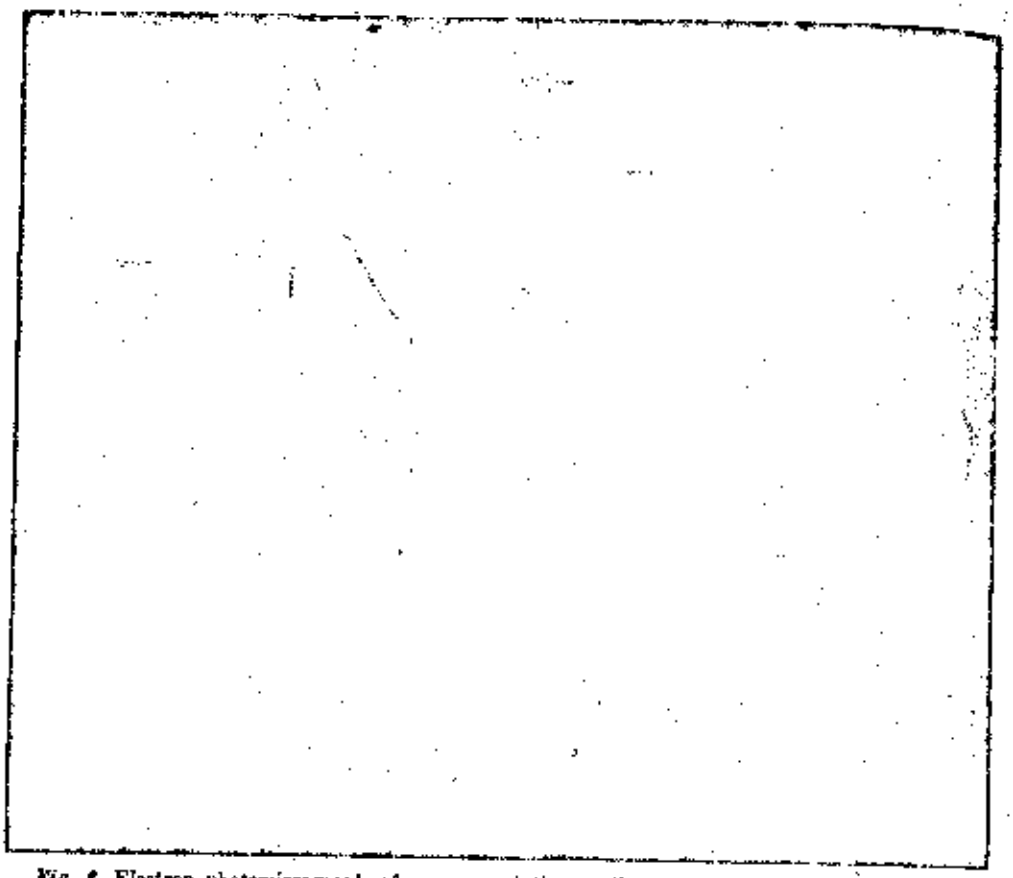

Fig. 2. Electron photomicrograph of a representative portion of the mitochondrial sediment. In addition to mitochondria, a few scattered lysosomes (large arrows) and microsomes (small arrows) are also seen. (×28,000.)

on electron microscopy to contain numerous elements of both brush borders and mitochondria (Fig. 3). Although we could not identify by electron microscopy contamination of the mitochondria by brush border elements, we do appreciate the possibility that nonrecognizable fragments of microvillous membranes may have still been present in this fraction. However, we believe our control experiment which compared the ratio of cell debris to mitochondrial radioactivity obtained after continuous and pulse incubations of NHGJ-$^{57}CoB_{12}$ in the ileal loops indicates that such contamination could not have been significant (Table I).

The significance of the small amount of $^{57}CoB_{12}$ in the microsome and cytosol fractions will require further investigation. At this time, the consistently low levels of radioactivity in each of these fractions precluded any precise analysis.

It should be noted that after in vivo incubation some of the $^{57}CoB_{12}$ in the mitochondrial fraction precipitated at 15 per cent Na_2SO_4 when normal serum was substituted for anti-IF antiserum. This suggests either that some of the

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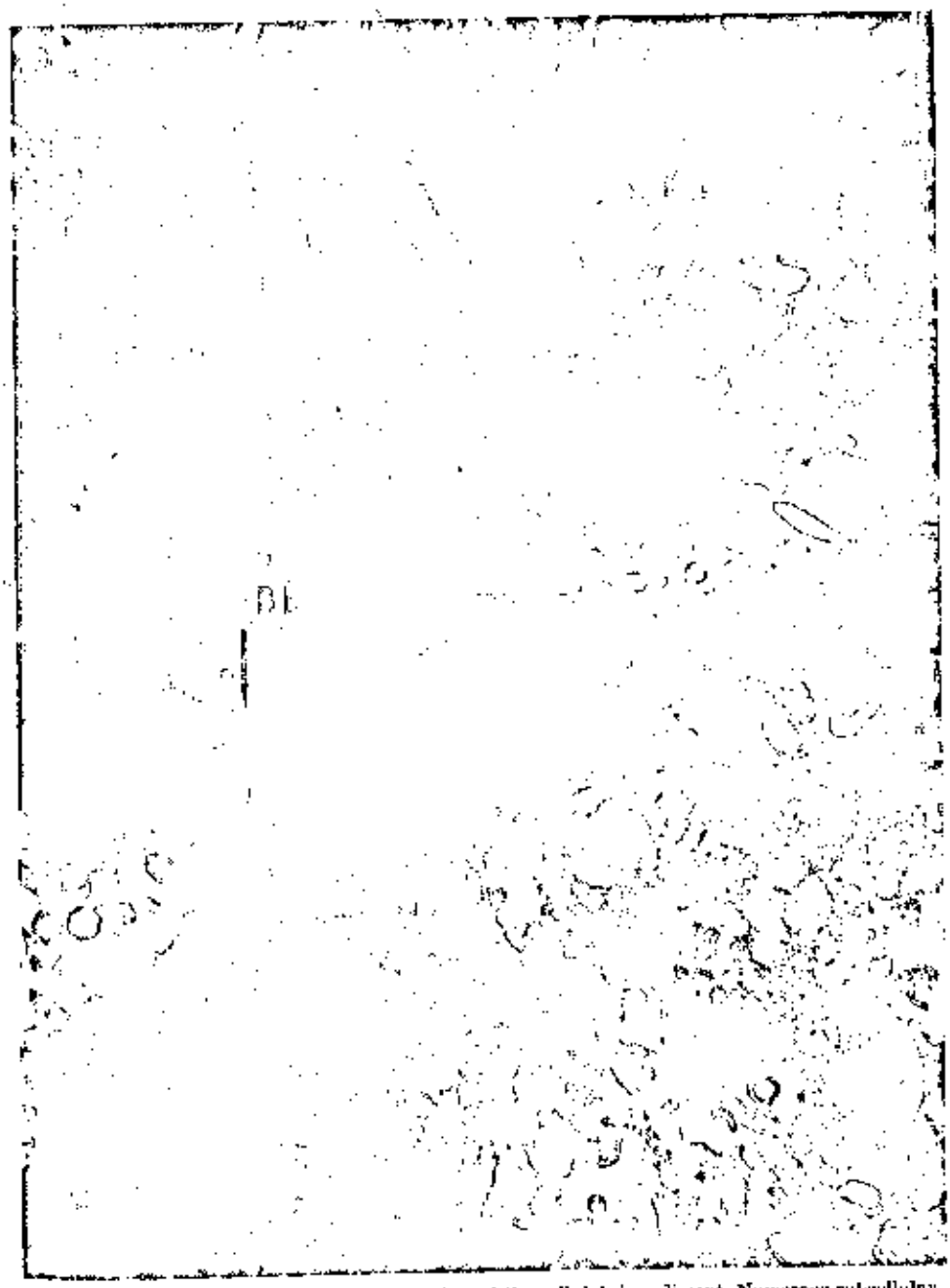

Fig. 5. Electron photomicrograph of a portion of the cell debris sediment. Numerous subcellular elements may be seen, including a fragment of brush border (BB) from an intestinal epithelial cell. (×30,000.)

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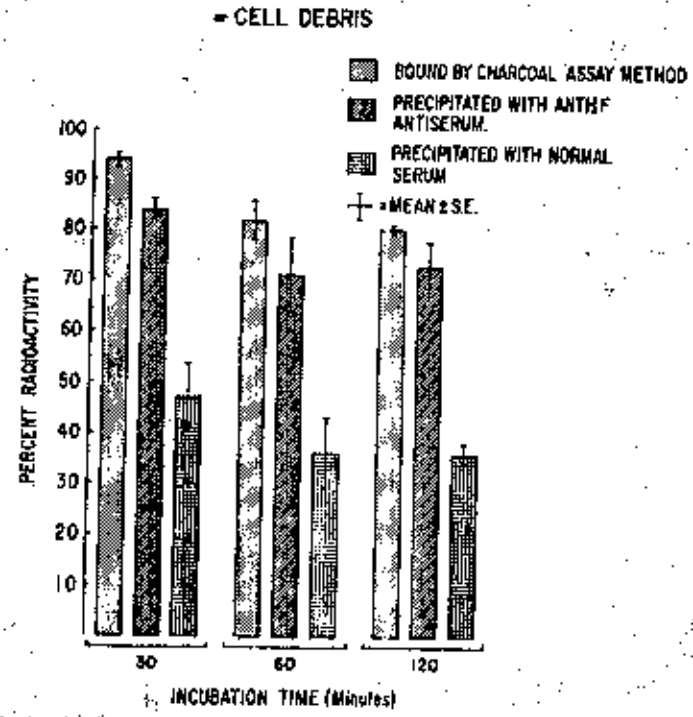

Fig. 4. Analysis of the solubilized $^{57}CoB_{12}$ in the cell debris fraction after sonication. The ordinate is the per cent of the total radioactivity which, (1) was bound as determined by the coated charcoal assay, (2) precipitated at 15 per cent Na_2SO_4 after incubation with anti-IF antiserum, and (3) precipitated at 15 per cent Na_2SO_4 after incubation with normal serum. The height of each bar and the vertical line are the mean and S.E.M., respectively, for 3 animals. The significance of the difference in the means between the groups precipitating at 15 per cent Na_2SO_4 with anti-IF antiserum and normal serum is $0.001 < P < 0.01$ for each of the indicated time periods.

vitamin was bound to another protein insoluble at this salt concentration, or that some of the IF-$^{57}CoB_{12}$ in this fraction has been altered in such a way as to precipitate in the absence of anti-IF antiserum. In contrast, neither IF-B_{12} in NHGJ nor B_{12} bound by mitochondrial sonicates in vitro precipitate at 15 per cent Na_2SO_4 (Table II). The precipitation of the IF-B_{12} complex at 15 per cent Na_2SO_4 in the absence of antiserum was previously observed in this laboratory when NHGJ-bound B_{12} was incubated with ileal mucosa in vivo or in vitro.[5] This change in the solubility property of IF-B_{12} was interpreted to be the result of binding to the receptor protein on the microvillous membranes of the brush borders. The similar precipitation of some of the mitochondrial $^{57}CoB_{12}$ in this study suggests the possibility that this surface IF-binding factor may have also entered the cell, perhaps serving in the transport of IF-B_{12} into the cell.

Although the present set of experiments indicate that IF is absorbed together with B_{12} in the guinea pig, Hines, Rosenberg, and Harris[18] concluded from their own experiments in the same animal that IF did not enter the intestinal cell.

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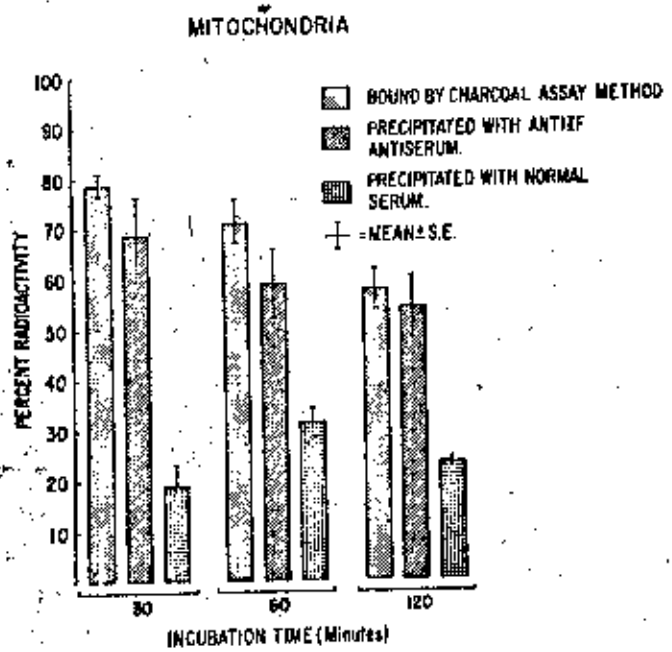

Fig. 5. Analysis of the solubilized $^{60}CoB_{12}$ in the mitochondrial fraction after sonification. The ordinate is the per cent radioactivity which, (1) was bound as determined by the coated charcoal assay, (2) precipitated at 15 per cent Na_2SO_4 after incubation with anti-IF antiserum, and (3) precipitated at 15 per cent Na_2SO_4 after incubation with normal serum. The height of each bar and the vertical line are the mean and S.E.M., respectively, for 3 animals. The significance of the difference in the means of the groups precipitating at 15 per cent Na_2SO_4 with anti-IF antiserum and normal serum is $0.001 < P < 0.01$ for each of the indicated time intervals.

In their experiments, sequential incubation of IF-$^{60}CoB_{12}$ followed by free $^{57}CoB_{12}$ in everted sacs of guinea pig ileum showed that immunoreactive IF could again bind the $^{57}CoB_{12}$ after facilitating the absorption of the $^{60}CoB_{12}$ previously bound to it. Such findings are not necessarily at variance with our present studies identifying IF in the mitochondria of cells absorbing B_{12}. Levine and co-workers[16] have demonstrated that the epithelium of everted sacs of rat intestine rapidly loses structural integrity. Similar changes in the everted sac of guinea pig ileum could expose intracellular IF, free of absorbed $^{60}CoB_{12}$, to the incubation solution containing $^{57}CoB_{12}$.

The results of this study do not support the speculation of Mackenzie and Donaldson[17] that digestion of the IF-B_{12} complex occurs at the cell surface. From our data, it appears more likely that dissociation of B_{12} from IF occurs intracellularly. Preliminary work from this laboratory has, in fact, indicated that the IF-B_{12} complex in the mitochondrial fraction following in vivo absorption undergoes gradual dissociation at 37° C.[18]

Our findings are more consistent with the hypothesis put forward by Grasbeck[19] that IF-B_{12} enters the cell by carrier-mediated transport after attachment of the complex to a surface receptor site. This mechanism would further

Table II. Properties of $^{57}CoB_{12}$ after incubation with mitochondrial sonicates or NHGJ

Experiment	*No. of experiments*	*Per cent radioactivity bound (M ± S.E.M.)*	*Per cent radioactivity precipitated at 15 per cent Na_2SO_4 (M ± S.E.M.)*
1. NHGJ -$^{57}CoB_{12}$* + Normal serum	3	—	0.6 ± 0.66
2. NHGJ -$^{57}CoB_{12}$* + Anti-IF antiserum	3	—	70.3 ± 4.9
3. $^{57}CoB_{12}$† + Mitochondrial sonicate	2	20.2 ± 7.75	—
4. $^{57}CoB_{12}$‡ + Mitochondrial sonicate + either Anti-IF antiserum or Normal serum	2	—	1.25 ± 1.25
	2	—	0

*A 10 pg. quantity of $^{57}CoB_{12}$ bound to diluted NHGJ was incubated with 0.3 ml. of normal serum or anti-IF antiserum made up to a total volume of 1.3 ml. with K-H buffer. After 30 minutes' incubation, 1.3 ml. of 30 per cent Na_2SO_4 was added and the radioactivity determined in the supernatant following centrifugation of the precipitate.

†A 10 pg. quantity of free $^{57}CoB_{12}$ contained in 0.9 ml. of K-H buffer was incubated with 0.1 ml. of mitochondrial sonicate for 30 minutes. A scoop of dry albumin-coated charcoal was added, shaken, and the radioactivity in the supernate determined following centrifugation of the charcoal.

‡A 10 pg. quantity of free $^{57}CoB_{12}$ contained in 0.9 ml. of K-H buffer was incubated with 0.1 ml. of mitochondrial sonicate for 30 minutes. Either anti-IF or normal serum (0.3 ml.) was then added, followed in 5 minutes by 1.3 ml. of 30% Na_2SO_4. The radioactivity remaining in the supernate was determined following centrifugation of the precipitate.

explain the changes in the solubility properties of B_{12} bound to IF on surface structures[2] as well as in the mitochondrial fraction. According to this hypothesis, the alteration of the solubility of the IF-B_{12} complex is due to its coupling to the surface receptor which then functions in the transport of IF-B_{12} to the interior of the cell. The concept of a "transport piece," derived from the receptor site, carrying a macromolecule into the cell might be compared to the hypothesis advanced by Tomasi and co-workers[20] to describe the mechanism by which IgA immunoglobins appear in the external secretions.

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Some Aspects of the Role of Vitamin B_{12} In Nucleic Acid Synthesis In a Neoplastic C3H Mouse Cell Strain. II. Incorporation of Precursors [1]

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SUMMARY—The effects of vitamin B_{12} and folate on the incorporation of certain ^{14}C-labeled nucleosides into the nucleic acids of the neoplastic C3H mouse cell strain, NCTC 5905, were studied, as well as the effect of B_{12} on adenine incorporation. In medium that contained thymidine, the radioactivity incorporated from this into DNA-thymidine and the amount of DNA synthesized did not decrease when B_{12} was omitted. When B_{12} was left out of medium that contained uridine, cytidine, or deoxycytidine, incorporation from these into DNA-thymidine was reduced 50–60% and the amount of DNA synthesized was less. With deoxyuridine, both incorporation and DNA synthesis were decreased 20% in the absence of B_{12}. If, however, the folate content of medium that contained B_{12} was reduced from 0.25 to 0.025 μg/ml, there were much greater decreases in incorporation as follows: deoxycytidine 80, deoxyuridine 90, cytidine 90, and uridine about 100%. In the absence of B_{12}, labeled cytidine appeared to be incorporated into DNA-deoxycytidine and DNA-thymidine entirely as the intact nucleoside. It was concluded that the stimulating effects of B_{12} on DNA synthesis could be ascribed at least in part to an enhanced conversion of deoxycytidine and deoxyuridine to DNA-thymidine precursors.—J Nat Cancer Inst 44: 91–97, 1970.

IN GROWTH STUDIES with strain NCTC 3681, a neoplastic C3H mouse cell strain from which the strain used in this study, NCTC 5905, also neoplastic, was derived, we found previously that pyrimidine nucleoside and purine requirements for continuous proliferation were related to both the folate and vitamin B_{12} content of the medium (*1, 2*). Pyrimidine nucleoside requirements for DNA-thymidine synthesis in both 3681 and 5905 cells were also related to the medium folate content (*3*). It has been suggested by Loughlin and coworkers (*4*) and Jaenicke (*5*), Stahlberg (*6*), and Barker (*7*) that a B_{12} function may be associated with certain methyl-group transfer reactions as well as with folate-dependent reactions in mammalian cells, as for example, in the synthesis of tetrahydrofolate which is catalyzed by the B_{12}-

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containing 5-methyltetrahydrofolate-homocysteine transmethylase (*8*) [*see also* Smith (*9*)]. Also, Blakley and Vitols (*10*) demonstrated that the ribonucleotide triphosphate reductase of *Lactobacillus leichmanii* requires a B_{12} coenzyme for activity. Bolinder and Reichard studied the effect of B_{12} deficiency on the *in vitro* conversion of deoxyuridine and cytidine to DNA-thymidine and DNA-deoxycytidine in chick embyro mince (*11*). In the studies we report here we examined the effect of B_{12} on the utilization by NCTC 5905 cells of adenine and certain pyrimidine nucleosides for the synthesis of nucleic acids to learn whether any of the reactions involved in certain stages of nucleic acid synthesis were stimulated by B_{12} or required B_{12}.

MATERIALS AND METHODS

The cell strain NCTC 5905 is a subline of strain NCTC 3681 (*12*) which was derived from minced 12-day C3H/HeN mouse embryos and had been grown in chemically defined medium NCTC 135 (*2*) for about 7 years. NCTC 5905 cells were derived from strain 3681 by subculture in medium NCTC 141 (*2*) to which 0.25 μg/ml folate (5.7×10^{-7} M), 10 μg/ml deoxyadenosine (4.0×10^{-5} M), and 10 μg/ml thymidine (4.2×10^{-5} M) were added. Stock cultures were grown in T-60 flasks containing 6 ml of culture fluid. They were subcultured every 7 days and the culture fluid was renewed on the 2d and 4th days.

For the incorporation studies, 5905 cells were planted in the modified medium 141 described above supplemented with a ^{14}C-labeled precursor and, as indicated in the tables, supplemented or not with B_{12} (10 μg/ml, 7.3×10^{-6} M). When a pyrimidine nucleoside other than thymidine was added to the medium, thymidine was omitted. When ^{14}C-labeled glycine was present, deoxyadenosine was sometimes left out. When adenine (5 μg/ml, 3.7×10^{-5} M) was present, deoxyadenosine was omitted. The medium was renewed on the 2d day after the cells were planted, and the cells were harvested on the 4th day except as noted in table 3.

The cells were washed with saline and dissolved in a small volume of 0.3 N LiOH by incubation at 37°C for 2 hours (*13*). A fraction of the solution was removed for determination of DNA. The remaining solution was acidified to approximately 0.1 N by addition of perchloric acid. Two volumes of ethanol were added. After 18 hours at 4°C, the supernatant solution was removed, the precipitate washed with ethanol-water, and the supernatant containing the acid-soluble and RNA nucleotides neutralized and evaporated to dryness. The precipitate was extracted for 30 minutes at 100°C with 10% NaCl at about *p*H 8. Two volumes of ethanol were added to the neutralized saline extract. After 18 hours at 4°C, the precipitated DNA was collected by centrifugation, washed with ethanol-water, and dried. The DNA was dissolved in water and samples were removed for measurement of radioactivity and determination of DNA.

In some experiments with ^{14}C-glycine as a purine precursor, the saline-washed cells were digested with pronase before alkaline extraction. The pronase digest was acidified to 0.2 N with perchloric acid at 0° and the precipitate was washed with cold acid. The precipitate was then dissolved in 0.3 N LiOH and digested.

The supernatant containing the total acid-soluble and RNA nucleotides of the cell sample was applied to paper for chromatography. The lithium salts were extracted from the paper (*14*) before development.

The DNA was converted to deoxynucleosides by digestion with deoxyribonuclease followed by a period of digestion with snake venom (*Crotalus adamanteus*) phosphodiesterase and 5′-nucleotidase. The digests were evaporated to dryness and the deoxynucleosides then isolated by paper chromatography.

The chromatographic solvents used for isolation of ribonucleotides were: isobutyric acid-0.5 N NH_4OH (40:20), *p*H 3.6, and isopropanol-concentrated HCl (65:16.4), water to 100 ml. Solvents for purine deoxynucleoside isolation were *n*-butanol-water (86:14); *n*-butanol-concentrated NH_4OH-water (86:4:10), ammonia atmosphere; and water, *p*H 10.5. For pyrimidine deoxynucleosides the solvents were ethyl acetate-90% formic acid-water (60:5:35); *n*-butanol-concentrated NH_4OH-water (86:4:10), ammonia atmosphere; and isopropanol-HCl.

After chromatography the ribonucleotides and deoxynucleosides were located on the paper strips

with a Baird-Atomic radiochromatogram scanner and visually under ultraviolet light. The compounds were eluted from the paper. Radioactivity was measured in a windowless gas-flow counter. Ribonucleotides were identified and determined by ultraviolet absorption measurements, and deoxynucleosides were identified and determined by ultraviolet absorption measurements and microbiological assay with the organism *L. acidophilus R26* ATCC 11506 (*15*).

The vitamin B_{12} content of cells was determined microbiologically by the method of Hutner *et al.* (*16*) as modified by Bonelli and Rinaldini (*17*). The deoxyriboside content of the B_{12}-containing cell extract was determined by the *L. acidophilus* assay.

DNA was determined by the Burton modification (*18*) of the diphenylamine reaction.

^{14}C-uridine, ^{14}C-deoxycytidine, ^{14}C-thymidine, and ^{14}C-cytidine, uniformly labeled, and adenosine-8-^{14}C were purchased from Amersham/Searle Corporation, Des Plaines, Illinois. Glycine-2-^{14}C and adenine-8-^{14}C were purchased from Schwarz Bioresearch, Orangeburg, New York, and thymidine-6-^{14}C and deoxyuridine-2-^{14}C from New England Nuclear Corporation, Boston, Massachusetts.

RESULTS

The effects of vitamin B_{12} on the synthesis of DNA by 5905 cells and on the incorporation of certain labeled precursors into DNA and RNA are shown in table 1. When thymidine and deoxyadenosine or thymidine and adenine were the only nucleoside or purine precursors of DNA present in the culture medium, addition of B_{12} had no effect on the amount of DNA synthesized from the 2d–4th day after planting. However, when the medium contained the precursors deoxyadenosine and deoxyuridine, deoxyadenosine and cytidine, deoxyadenosine and deoxycytidine, or deoxyadenosine and uridine, there was a stimulation of DNA synthesis when B_{12} was added to the medium. The effects of B_{12} on the relative extent of utilization of certain ^{14}C-labeled precursors for synthesis of DNA and DNA-thymidine by 5905 cells are also shown in table 1 as well as the relative specific activities of some DNA nucleosides and the RNA nucleotides. The percent of counts per minute (cpm) incorporated into DNA and DNA-thymidine from ^{14}C-deoxyuridine, ^{14}C-uridine, ^{14}C-cytidine, and ^{14}C-deoxycytidine increased when B_{12} was added. The addition of B_{12} had no effect on the utilization of thymidine for DNA-thymidine synthesis or of adenine for DNA synthesis.

In table 2, data for 5905 cells from table 1 are compared with results obtained previously in a study (*3*) of some effects of folate on the incorporation of radioactivity from certain precursors into the DNA-thymidine of NCTC 3681 cells. When vitamin B_{12} was added there was approximately a twofold increase in the incorporation of radioactivity from labeled uridine, cytidine, and deoxycytidine into the DNA-thymidine of 5905 cells in medium containing 0.25 μg/ml of folate and a 20% increase for labeled deoxyuridine. However, the extent of incorporation from these precursors was sixfold to fiftyfold greater than in 3681 cells that were carried in medium containing B_{12} in which the folate content was 0.025 μg/ml rather than 0.25 μg/ml.

NCTC 5905 cells utilized cytidine for synthesis of the pyrimidine nucleosides of DNA by reduction of the intact precursor when B_{12} was left out of the medium. As shown in table 3, there was radioactivity in the deoxyribose of both the DNA-deoxycytidine and DNA-thymidine that were formed. The specific activities of the deoxyadenosine produced by transfer of the labeled DNA-pyrimidine deoxyribose to unlabeled adenine were the same as would be expected if all the cytidine used for the DNA-deoxynucleosides were converted by reduction of the intact precursor.

When adenine-8-^{14}C (table 1) or adenosine-8-^{14}C was added to the culture medium, both the RNA nucleotides and the DNA nucleosides were labeled to the same extent (Rotherham, unpublished). The incorporation of radioactivity into both DNA and RNA purines from ^{14}C-glycine was decreased in the presence of either deoxyadenosine or adenine (table 4). However, in experiments similar to those described in table 1, column 1), in which thymidine-2-^{14}C and unlabeled deoxyadenosine were replaced by uniformly labeled ^{14}C-thymidine and unlabeled adenine, no radioactivity could be detected in the DNA-deoxyadenosine. When the medium contained cytidine-^{14}C (U) and either

TABLE 1.—Effect of vitamin B_{12} on the incorporation of certain labeled precursors into the nucleic acids of NCTC 5905 cells

	B_{12}	Precursors (10 μg/ml)					
		1) Thymidine* -2-^{14}C	2) Deoxyuridine* -2-^{14}C	3) Deoxycytidine* -^{3}C (U)	4) Uridine* -^{14}C (U)	5) Cytidine* -^{3}C (U)	6) Adenine† -8-^{14}C
μmoles DNA-P 2d day	+	0.39	0.45	0.43	0.38	0.57	0.57
	−	.40	.37	.40	.30	.47	.52
μmoles DNA-P 4th day	+	.91(.52‡)	1.02(.57)	.90(.47)	1.06(.68)	1.05(.48)	1.04(.47)
	−	.86(.46)	.82(.45)	.64(.24)	.52(.23)	.49(.02)	1.03(.51)
Total added cpm × 10^{-6}	+	9.4	13	6.0	3.9	7.9	5.9
	−	9.5	12	5.7	3.9	7.9	5.4
Percent of total cpm§ in DNA 4th day	+	3.3	2.1	3.8	5.8	5.2	12
	−	3.5	1.7	1.7	2.5	1.9	13
Relative specific activity‖ DNA-thymidine	+	1500	850	1300	1000	1000	
	−	1800	860	720	720	780	
DNA-deoxycytidine	+			380	1100	1400	
	−			250	1100	1100	
DNA-adenine	+						2700
	−						2800
DNA-guanine	+						2100
	−						2400
RNA-uridine monophosphate	+				1200	1000	
	−				1300	1100	
RNA-cytidine monophosphate	+				1300	1300	
	−				1300	1300	
RNA-adenosine monophosphate	+						2700
	−						2900
RNA-guanosine monophosphate	+						2400
	−						2600
Percent of total cpm§ in DNA-thymidine 4th day	+	3.3	2.1	2.9	2.8	2.2	
	−	3.5	1.7	1.3	.98	.79	
Number of paired experiments		4	7	9	6	8	5

*With unlabeled deoxyadenosine (10 μg/ml, 4.0×10^{-4} M).

†With unlabeled thymidine (10 μg/ml, 4.2×10^{-5} M).

‡Increase, 2d–4th day.

§The percent of the total added cpm found in the DNA or DNA-thymidine was calculated from the cpm in the isolated DNA after correction for recovery. The percent of cpm in DNA-thymidine was obtained from the ratio of specific activities of DNA-thymidine to DNA-deoxycytidine and the percent cpm in DNA.

‖Specific activity × 10⁶/total added cpm = relative specific activity. RNA nucleotides include acid-soluble nucleotides.

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TABLE 2.—Comparison of the incorporation of certain precursors into the DNA-thymidine of NCTC 5905 and NCTC 3681 cells* with respect to the B_{12} and folate contents of the culture media

Labeled precursor	Cell strain	Folate (μg/ml)	B_{12} (μg/ml)	Percent of total cpm in DNA-thymidine, 4th day
Cytidine	3681	0.025*(5.7 × 10^{-8} M)	10(7.3 × 10^{-6} M)	0.13
	5905	.250	0	0.79
	5905	.250	10	2.20
Uridine	3681	.025	10	0.05
	5905	.250	0	0.98
	5905	.250	10	2.80
Deoxycytidine	3681	.025	10	0.43
	5905	.250	0	1.30
	5905	.250	10	2.90
Deoxyuridine	3681	.025	10	0.12
	5905	.250	10	1.70
	5905	.250	10	2.10
Thymidine	3681	.025	10	2.10
	5905	.250	0	3.50
	5905	.250	10	3.30
Adenine	5905	.250	0	(12)†
	5905	.250	10	(13)

*NCTC 3681 cells were carried in medium containing .025 μg/ml folate and 10 μg/ml B_{12}, whereas NCTC 5905 cells were carried in medium containing 0.25 μg/ml folate from which B_{12} was omitted. For the incorporation studies with 5905 cells, B_{12} was added or not, as shown. See (3) for data on 3681 cells.

†Percent of total cpm in DNA, 4th day.

unlabeled deoxyadenosine or unlabeled adenine, the specific activities of the DNA-deoxyadenosine were low (table 3).

The vitamin B_{12} content of 5905 cells of stock cultures grown in the absence of B_{12} in medium 141, with thymidine and deoxyadenosine present, was 6.7 × 10^{-8} μmoles/μmole DNA-P or less. This increased to 2.6 × 10^{-6} μmoles/μmole DNA-P on the 3d day after the cells were planted in the same medium with B_{12} added. In either medium the content of deoxyribosyl compounds in the cell extract used for the B_{12} determination was 2–4 μg/μmole DNA-P.

DISCUSSION

We have concluded from our results that the synthesis of DNA and DNA-thymidine in 5905 cells in medium containing 0.25 μg/ml of folate was stimulated by vitamin B_{12}. It appears that this could not be attributed to an effect on the utilization of thymidine for synthesis of DNA-thymidine nor to an effect on the use of adenine for synthesis of DNA-deoxyadenosine and DNA-deoxyguanosine. If the culture medium contained thymidine, the radioactivity incorporated from this into DNA-thymidine, or into DNA purines from ade-

TABLE 3.—Utilization of uniformly labeled ^{14}C-cytidine for synthesis of DNA-nucleosides of NCTC 5905 cells in the absence of vitamin B_{12}*

Labeled deoxynucleoside	Specific activities cpm × 10^{-6}/μmole		
Experiment No.	1	2	3
Deoxycytidine			
a) Isolated from DNA	1.30	1.50	—
b) Of deoxyadenosine formed by tranfer of deoxyribose to unlabeled adenine†	0.72	0.85	—
Thymidine			
a) Isolated from DNA	0.88	1.30	—
b) Of deoxyadenosine formed by transfer of deoxyribose to unlabeled adenine	0.53	0.75	—
Deoxyadenosine			
a) Isolated from DNA	—	0.055	0.023

*Cells were planted in medium 141 supplemented with 0.25 μg/ml folate, 10 μg/ml cytidine (0.09 × 10^6 cpm/ml), and 10 μg/ml deoxyadenosine. The medium was renewed on the 2d and 4th day and the cells were harvested for isolation of DNA on the 7th day. In experiment 3, deoxyadenosine was replaced by adenine.

†The deoxyribose was transferred from the ^{14}C-labeled DNA-deoxycytidine or DNA-thymidine to unlabeled adenine by incubation with a crude extract of *L. acidophilus* R26 (ATCC 11506) according to the method of McNutt (19). The products were isolated by paper chromatography.

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TABLE 4.—Dilution by deoxyadenosine and adenine of incorporation of radioactivity from glycine-2-^{14}C into RN[illegible] and DNA purines of NCTC 5905 cells

Unlabeled precursor	Total cpm × 10^{-4}	Relative specific activities			
		DNA-deoxyadenosine	DNA-deoxyguanosine	RNA-adenosine monophosphate	RNA-guanosine monophosphate
None	10.0	180	210	180	180
Deoxyadenosine	11.0	10	20	10	20
Adenine	12.0	10	10	10	10

nine, and the amount of DNA synthesized did not decrease when B_{12} was omitted. A possible explanation for the observed effect of B_{12} on DNA-thymidine synthesis is that the conversion of deoxycytidine and deoxyuridine to DNA-thymidine precursors is indirectly stimulated by B_{12}. This would account for the decreased synthesis of DNA and decreased incorporation into DNA-thymidine from uridine, cytidine, and deoxycytidine when B_{12} was absent from the medium.

From the data described, together with those presented in (2), we concluded that both the utilization of cytidine for DNA-deoxycytidine and DNA-thymidine and the reduction and methylation reactions involved can proceed in the absence of B_{12}. But the possibility that B_{12} may stimulate these reactions has not been excluded.

Some free deoxyribose was probably available to the cells in all experiments in table 1. Deoxyadenosine was a constituent of the media in all experiments listed except when adenine was added, and thymidine was then added together with adenine. From the data in tables 1 and 4 and from incorporation studies (20), it is clear that either adenine itself or the adenine of deoxyadenosine or adenosine was extensively used in the presence of thymidine for both DNA and RNA purines, whether or not B_{12} was present. But, even though deoxyribose may have been released from thymidine by the cells, it appeared not to be used for DNA-deoxyadenosine synthesis. The extent of incorporation of the ribose of cytidine into DNA-deoxyadenosine was also low.

Recently we examined a second strain of cells, NCTC 6324, derived from strain NCTC 3681 in the same way as strain NCTC 5905, but 60 weeks later. Cultures of NCTC 6324 cells, unlike 3681 cells (2), could not be subcultured continuously in medium 141 that was supplemented with 0.25 μg/ml folate and with adenine and cytidine or adenine and deoxyuridine in place of deoxyadenosine and thymidine. Since 5905 cells used cytidine for DNA-thymidine synthesis in the absence of B_{12}, it appears from this single observation that we may also need to consider the possibility that, in the two strains of cells derived from 3681, at least two cell types are included. One may require B_{12} for utilization of cytidine and/or deoxyuridine for DNA-thymidine synthesis and one may not.

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Further Studies on the Binding of Vitamin B_{12} to the Cell Wall of a B_{12}-Requiring *Lactobacillus*

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The vitamin B_{12}-binding property of *Lactobacillus leichmannii* ATCC 7830 has been studied. The organism could bind 0.52 μg of B_{12} per mg of cells. With regard to the cellular site for B_{12} accumulation, three-quarters of the B_{12} bound to the cell was found in the crude cell wall fraction, and the remaining one-quarter was found in the particulate (ribosome) fraction. After receiving enzymatic treatments with ribonuclease, lipase, and trypsin, the wall fraction retained three-fifths of the initial B_{12}. The possibility of cross-contamination of the wall and particulate fractions was excluded by measuring the contents of ribonucleic acid and hexosamines in each fraction. The B_{12}-binding activity of the wall was destroyed by pretreatment of the wall with pepsin, Pronase, or trypsin. However, once bound to the wall, the B_{12} was not released by the same treatments. These facts suggest that B_{12} is bound to a polypeptide in the wall on which these enzymes act and that, once bound, B_{12} somehow inhibits the enzymatic actions as described earlier with *L. delbrueckii* no. 1. A B_{12}-polypeptide complex was isolated by treatment with 0.2 N HCl from walls to which B_{12} had been bound. The complex was then purified. The complex moves as a single band on polyacrylamide gel electrophoresis. Its molecular weight was estimated around 21,500 with microheterogeneity on a Sephadex G-75 column. The mode of B_{12} binding was found to be similar to that of *L. delbrueckii*.

We have previously shown (13) that vitamin B_{12}-requiring lactobacilli characteristically take up and preserve large amounts of B_{12} in cells from surrounding media. This property was analyzed by using *Lactobacillus delbrueckii* no. 1, a B_{12}-requiring strain. The organism takes up about 8×10^4 molecules of B_{12} per cell, 130- to 160-fold as many as those required for normal cell proliferation of this organism. The accumulation of B_{12} by the cell occurs whether the organism is growing or resting with no exogenous energy supply, and the accumulated B_{12} can be utilized for subsequent growth. The cellular site of B_{12} accumulation was shown to be located principally in the cell wall where a B_{12}-binding principle exists (17–19). A B_{12} complex was isolated from the wall and analyzed for its nature (20, 21). On the other hand, Beck and his co-workers, who studied the binding of B_{12} to the cell of *L. leichmannii* ATCC, 7830, stated that B_{12} is bound almost exclusively in the ribosomes (8, 9). On the basis of the similarities in taxonomical characteristics of *L. leichmannii* and *L. delbrueckii* (12, 16), we suggested (17, 18) that B_{12} may be bound first to the wall in *L. leichmannii* as well; the suggestion was supported by Kinoshita et al. (10, 11) with *L. leichmannii* ATCC 4797. However, they did not perform a quantitative analysis of B_{12} distribution within the cell, i.e., what percentage and amount of B_{12} is bound to the wall (maximum binding capacity of the isolated wall) as we did with *L. delbrueckii* no. 1 (17, 18).

This paper describes the results of such studies with *L. leichmannii* ATCC 7830 and some properties of a B_{12} binder in its wall which are somewhat different from those of *L. delbrueckii* no. 1. B_{12} complexes (B_{12} binders to which B_{12} is bound) were isolated from the wall and ribosomes of *L. leichmannii* by treatment with 0.2 N HCl and purified. A comparative study was also made for B_{12}-binding capacities of cells and isolated walls of several strains belonging to *L. delbrueckii* and related species.

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MATERIALS AND METHODS

Cultivation of organism. *L. leichmannii* ATCC 7830 was mainly used. The organism was grown stationary at 37 C in inoculation medium for *L. leichmannii* (Nissui Seiyaku Co., Tokyo, Japan), harvested by centrifugation at the early stationary phase of growth, washed twice with 10 mM tris(hydroxymethyl)aminomethane (Tris)-succinate buffer, pH 7.2, containing 5 mM magnesium acetate (TSM), and subjected to further studies. When the B_{12}-binding capacity of the whole cell was examined, cells were washed once with distilled water instead of TSM.

Fractionation of cellular components. Washed cells of *L. leichmannii* were suspended in TSM and disrupted by treatment either with a sonic oscillator (10 kc) as described previously (18) or with a French pressure cell. With this organism, the two methods of disruption gave somewhat different yields for the wall (sedimentable at 27,600 × *g* for 20 min) and particulate (sedimentable at 107,000 × *g* for 120 min) fractions; therefore, the method employed is specified in each experimental section. Cellular components were separated by differential centrifugation as follows. Walls were sedimented at 27,600 × *g* for 20 min and washed twice with TSM. The supernatant solution and washings from the walls were combined and again subjected to centrifugation at the same speed for 20 min. The precipitate obtained was omitted from the present study. The supernatant solution was centrifuged at 107,000 × *g* for 120 min, resulting in its separation into the particulate (ribosomal) and soluble fractions.

Other organisms which were used in the experiments summarized in Table 1 were disrupted by passing through a French pressure cell; walls were obtained as with *L. leichmannii*.

All the fractionation procedures were carried out at 0 to 4 C.

Treatments of walls with enzymes or HCl, or both. Methods are described below for small-scale experiments which were conducted to examine the effect of treatments with various enzymes and HCl solutions on the B_{12}-binding activity of the wall of *L. leichmannii*.

To investigate the distribution of B_{12} within the cell of *L. leichmannii*, cells which had bound B_{12} were disrupted sonically and fractionated as above. The crude wall preparation received enzymatic treatments with ribonuclease and lipase under the conditions described below, except that the relative concentrations of ribonuclease and lipase to the preparation were approximately 7 and 2.5 times higher, respectively, and the volume of reaction mixture was small. After these treatments, trypsin (about 0.1 mg per mg of walls) was added to the above mixture, which was then incubated with stirring overnight at 37 C. In every enzymatic treatment, drops of toluene were added as a preservative. The treated walls were washed three times with TSM (RLT-wall). To obtain a B_{12} complex from the wall, the walls which were washed with TSM as above were washed once more with 0.05 M phosphate buffer (pH 7.0) and suspended in the same buffer at an approximate concentration of 25 mg of walls/ml in a final volume of 150 ml. After addition of 5 mg of B_{12}, the suspension was incubated with stirring for 20 min. The suspension then was added to 5 mg of ribonuclease and 100 mg of lipase and was incubated with stirring for 24 hr at 37 C. Drops of toluene were added as above. After the incubation the walls were washed twice with distilled water by centrifugation (RL-wall) and suspended in distilled water. HCl was added to the suspension to give a concentration of 0.2 N and an approximate RL-wall concentration of 15 mg per ml. The suspension then was incubated with stirring for 42 hr at 37 C. The B_{12} was found as a B_{12} complex in the supernatant solution after this treatment. The residual wall fraction was sedimented by centrifugation and washed with a small volume of distilled water (RLH-wall); the washing was combined with the above supernatant solution. The yield of the RLH-wall was about 68% of that of the RL-wall. In other words, 32% of the latter fraction was solubilized into the supernatant solution in which the B_{12} complex was contained.

Electron microscopy. Electron micrographs (Fig. 1) of whole cells, crude walls, and enzymatically treated walls of *L. leichmannii* were taken on carbon-coated preparations with a Hitachi HU-11 DS electron microscope (Tokyo).

Determination of chemical marker substances. Total sugars were estimated by the anthrone method (24) with glucose as standard. Ribonucleic acid (RNA) was estimated by the method of Ceriotti (3) with yeast RNA (Sigma Chemical Co., St. Louis, Mo.) as standard. Total hexosamines were estimated by the method of Boas (2), with glucosamine hydrochloride as standard, after hydrolysis of the cellular component with 3 N HCl in a sealed tube at 105 C for 20 hr and subsequent evaporation of HCl.

Determination of B_{12}. Vitamin B_{12} was determined either by bioassay with *L. leichmannii* ATCC 7830 by using B_{12} assay medium USP (Difco Laboratories, Detroit, Mich.) or from radioactivity by using ^{60}Co-labeled B_{12}, which was counted with a well scintillation counter. The concentration of a standard solution of B_{12} was determined from its optical density at 361 nm.

Purification of B_{12} complex. Purification of a B_{12} complex, released from the RL-walls by treatment with 0.2 N HCl, was carried out as follows. The solution of the 0.2 N HCl treatment and the washing from the treated walls were combined and neutralized with 10 N NaOH. White precipitates which appeared upon neutralization were removed by centrifugation. The supernatant solution was dialyzed in a cellophane bag against 0.01 M phosphate buffer, pH 7.0, containing 0.1 M NaCl (PBN) at 4 C for 48 hr. Then, the dialyzed material was placed onto a CM-cellulose column equilibrated with the same solution. The column was eluted first with PBN and then with a linear gradient concentration of phosphate buffer, pH 7.0, containing a constant concentration of 0.1 M NaCl. Subsequent to the purification process, optical densities at 280 and 361 nm were measured as described previously (20).

Gel filtration. Sephadex G-75 (Pharmacia, Uppsala, Sweden) in PBN at 4 C was used with a column

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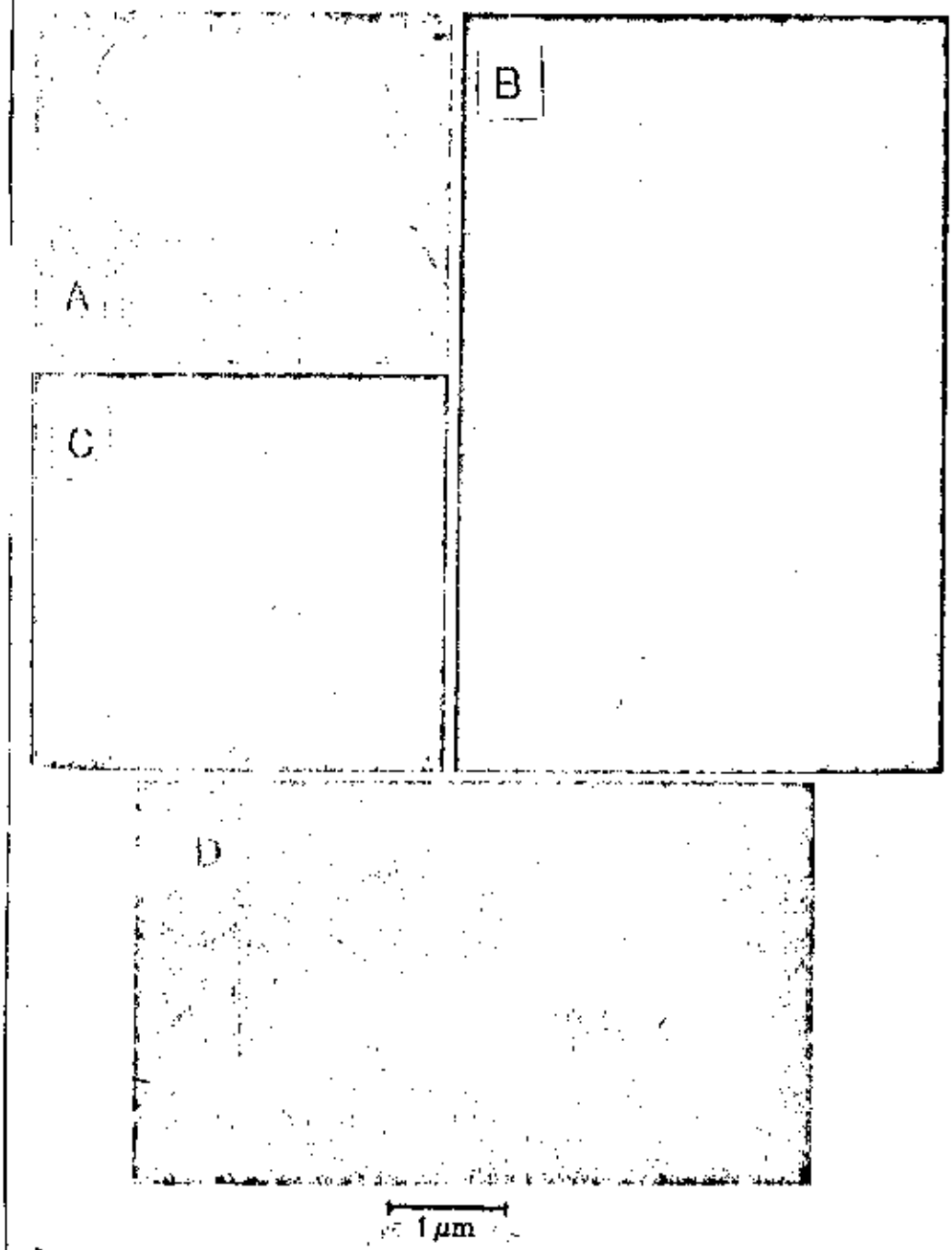

FIG. 1. *Electron micrographs of intact cell (A), crude wall (B), B_{12}-RL-wall (C), and RLH-wall (D) preparations.*

2 by 78.5 cm). The B_{12} complex in the equilibration buffer (1.5 ml) was layered on the top and eluted. The elution profile was monitored with a Uvicord monitor (LKB-Produkter, Stockholm, Sweden) at 280 nm. The void and total volumes were determined by using blue dextran 2000 and H_2O, respectively. Pepsin, soybean trypsin inhibitor, and cytochrome *c* were used as reference standards in molec-

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ular weight determination of the B_{12} complex, as described by Andrews (1) and Whitaker (22).

Disc electrophoresis. Polyacrylamide gel electrophoresis of the B_{12} complexes was performed at pH 4.0 as described by Reisfeld et al. (15), except that the direction of electric current was reversed.

Absorption spectra of the B_{12} complexes. Absorption spectra of the purified B_{12} complexes in PBN were recorded by using a Beckman DK-2 spectrophotometer.

Chemicals. Chemicals were obtained from the following sources: vitamin B_{12} (cyanocobalamin), Roussel-Uclaf, Paris, France; ^{60}Co-labeled B_{12}, Radiochemical Centre, Amersham, England; blue dextran 2000, Pharmacia; cytochrome c (horse heart, type III), pepsin (two times crystallized), ribonuclease A (bovine pancreas, five times crystallized), trypsin (bovine pancreas, two times crystallized, type-1), and trypsin inhibitor (soybean, type 1-s), Sigma Chemical Co., St. Louis, Mo.; lipase MY (10,300 units/g), Meito Co., Tokyo; Pronase P, Kaken Kagaku Co., Tokyo; papain (two times crystallized, suspension), Worthington Biochemical Corp., Freehold, N.J. Hog intrinsic factor was generously supplied by L. Ellenbogen of Lederle Laboratories.

RESULTS

B_{12}-binding capacities of cells and walls of some lactobacilli in relation to their B_{12} requirements. A previous paper (13) has shown that the cells of B_{12}-requiring lactobacilli such as *L. delbrueckii* and *L. leichmannii* possess a specific activity to take up and preserve B_{12} from surrounding media. This study is extended to other strains belonging to these species. Figure 2 shows the binding of B_{12} to resting cells of *L. leichmannii* ATCC 7830 at increasing concentrations of B_{12}. The B_{12} bound to the cells was not released by washings with distilled water or buffers at pH values within physiological range. The maximum binding ability of this organism was 0.52 μg per mg of cells (dry weight) under the conditions described. The results with other organisms (Table 1) confirm the previous findings that the cells of B_{12}-requiring strains alone can bind large amounts of B_{12}. Unexpectedly, however, the isolated crude walls of these strains could not bind as large amounts of B_{12} as were bound to the walls of *L. delbrueckii* no. 1 (17, 18) and *L. leichmannii* ATCC 7830 (Table 1). The amounts of B_{12} estimated from radioactivity and by bioassay using *L. leichmannii* ATCC 7830 were entirely equal.

The cellular B_{12}-binding activity of *L. leichmannii* was destroyed partially by heating of cells for 10 min at 60 C (residual activity = 27%) and almost completely by heating at 100 C (residual activity = 3%).

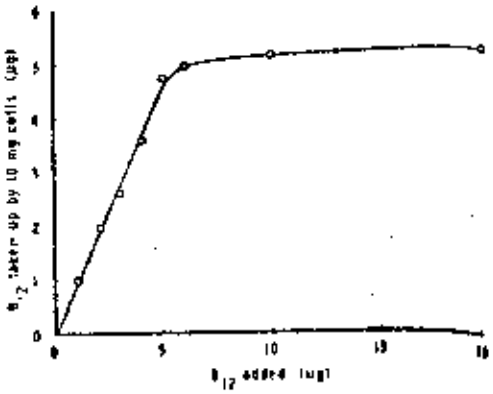

FIG. 2. *Amounts of B_{12} taken up by Lactobacillus leichmannii cells at increasing concentrations of B_{12}. A cell suspension (10 mg of cells, dry weight) was added to solutions with various B_{12} concentrations in total volumes of 10 ml. The reaction mixture was placed, with occasional stirring, in the dark at room temperature for 30 min. Then, the cells were collected by centrifugation and washed with distilled water. The radioactivity was measured, and was calculated as the quantity of B_{12}.*

Distribution of B_{12} in *L. leichmannii* cells. To investigate the cellular site for B_{12} accumulation, *L. leichmannii* cells grown in medium which had been supplemented with a large amount of B_{12} were disrupted, fractionated, and analyzed for B_{12} contents. Contents of carbohydrate, RNA, and hexosamines were also analyzed to check cross-contamination between the wall and ribosomal fractions. As shown in Table 2, the contents of hexosamines in the crude wall and particulate (ribosomal) fractions differed greatly, indicating that wall fragments may not significantly contaminate the particulate (ribosomal) fraction. When the crude wall fraction was treated with ribonuclease, lipase, and trypsin, RNA became undetectable in it; the facts exclude the possibility of the contamination of the fraction by ribosomes. A large part of the B_{12} which had been contained in the crude fraction remained after the enzymatic treatments (RLT-wall). The quantity of cell wall decreased to 18%, however, after the enzymatic treatments. This was caused by the rapid autolysis of the isolated walls of this organism in the buffer used for the enzymatic treatments. [This autolytic property differs greatly from *L. delbrueckii* no. 1 (20).] Thus, the decreased quantity does not necessarily indicate the removal of materials other than the wall, but rather represents removal of the wall itself.

Properties of B_{12} binder in *L. leichmannii*

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TABLE 1. *B_{12}-binding capacities of cells and walls of lactobacilli*[a]

Strain	B_{12} requirement[b]	B_{12} bound to cells [μg/mg (dry wt)]	Yield of crude walls (% of cells)	B_{12} bound to crude walls [μg/mg (dry wt)]
Lactobacillus delbrueckii				
AHU 1056	No	0.019		
ATCC 9649	Yes	0.190	45.7	0.016
ATCC 11978	No	0.016		
ATCC 11979	No	0.051	23.7	0.008
Hybgo-jözö	Yes	0.150	22.9	0.036
IAM 1085	No	0.013		
IAM 1149	Not determined	0.260	50.3	0.440
IAM 1174[c]	Yes	0.220	30.4	0.050
IAM 1197[c]	Yes	0.240	41.0	0.196
IAM 1928	No	0.019		
IFO 3534	Yes	0.155	26.8	0.100
L. casei subsp. rhamnosus				
ATCC 11443[d]	No	0.020		
L. leichmannii				
ATCC 7830	Yes	0.520	21.2–27.4	0.880

[a] The organisms were grown at their optimal temperatures in appropriate media, harvested, washed, and fractionated as *L. leichmannii*. B_{12}-binding capacities of cells and isolated walls were measured as in Fig. 2.

[b] B_{12} requirement was determined by using B_{12} assay medium USP.

[c] IAM 1174 formerly referred to as *L. delbrueckii* no. 2; IAM 1197 formerly *L. delbrueckii* no. 3.

[d] Formerly *L. delbrueckii* ATCC 11443.

TABLE 2. *Distribution of B_{12} within the cell*[a]

Cellular component	Dry wt (g)	Total sugars (mg)	Ribonucleic acid (mg)	Hexosamines (mg)	B_{12} (μg)
Whole cell	1.24 (100)[b]	31.8	319.8	13.7	292.9
Crude walls	0.44 (35.5)	16.8	81.8	11.6	214.8
Particulate	0.25 (20.2)	8.4	108.0	0.3	70.6
Soluble part	0.55 (44.3)	6.6	130.0	1.8	7.5
RUT-walls	0.08 (6.5)		0		131.0

[a] Cells grown in 2 liters of inoculation medium for *L. leichmannii* containing 1 mg of B_{12} were harvested at the early stationary phase of growth, washed with 10 mM Tris-succinate buffer (pH 7.2) containing 5 mM magnesium acetate, and fractionated as described in Materials and Methods after repeated sonic treatments (18). A part of the crude wall fraction received enzymatic treatments (RUT-walls).

[b] Values in parentheses are percentages.

walls. Properties of the B_{12} binder in the wall of *L. leichmannii* were investigated by using several hydrolytic enzymes or HCl solutions, or both. Table 3 shows that B_{12}, once bound to the wall, was not significantly liberated by treatments with the enzymes. Treatments with 0.2 N HCl resulted in the disappearance of B_{12} from the wall in the presence or absence of pepsin. Supernatant solutions which were derived from these 0.2 N HCl treatments contained nondialyzable B_{12}, suggesting that the B_{12} was in bound form with the binder liberated from the wall. When the wall was treated with enzymes such as pepsin, Pronase, or trypsin before the binding of B_{12}, the ability of the wall to bind B_{12} was almost completely lost (Table 4). The same was true for the wall treated with 0.2 N HCl in the presence or absence of pepsin. However, in the case of 0.2 N HCl treatment without pepsin, the supernatant solution contained a nondialyzable B_{12} binder. The difference in results given in Tables 3 and 4 may be interpreted as follows. B_{12} is bound to the same, or very close, position in the wall as that on which enzymes such as pepsin, Pronase, or trypsin act; thus, once bound, B_{12} could not be liberated by treatments with these enzymes because of steric hindrance by

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TABLE 3. *Liberation of B_{12} from the wall by various treatments*[a]

Treatment	Dry wt after treatment (mg)	B_{12} in treated walls (μg)	Nondialyzable B_{12} in supernatant solution (μg)
0.02 N HCl, pepsin	14.9	36.6	
0.2 N HCl, pepsin	15.2	1.0	20.0
0.02 N HCl, none	36.9	37.2	
0.2 N HCl, none	28.8	2.4	31.4
P buffer, papain	19.0	41.7	
P buffer, Pronase	16.6	23.8	3.8
P buffer, trypsin	16.4	25.4	3.7
P buffer, lipase	15.3	41.2	
P buffer, ribonuclease	15.4	40.6	
P buffer, none	16.0	41.7	
Distilled water, 0 C, none	46.8	40.1	

[a] Crude walls to which B_{12} had been fully bound were washed with distilled water and resuspended in HCl, 0.05 M phosphate buffer (P buffer), *p*H 7.0, or distilled water. Where indicated, enzymes (2 mg each, except 1 mg of ribonuclease) were added in final volumes of 7.5 ml. Cysteine hydrochloride (4 mg) was added in the papain reaction mixture. The reaction mixtures were incubated at 37 C for 48 hr in the dark, except that the tube containing the distilled water suspension was placed at 0 C for the same period. Drops of toluene were added as a preservative. The treated walls were collected by centrifugation and washed with distilled water, and radioactivity was measured. Dry weights were also determined. The supernatant solutions were dialyzed against distilled water at 4 C for 72 hr, and radioactivity remaining in the cellophane bags was measured. Free B_{12} was dialyzed as a reference.

TABLE 4. *B_{12}-binding abilities of walls treated with various agents*[a]

Treatment	Dry wt after treatment (mg)	B_{12} bound to treated walls (μg)	B_{12} bound to supernatant solution (μg)
0.02 N HCl, pepsin	11.4	1.0	1.2
0.2 N HCl, pepsin	15.5	1.0	1.9
0.02 N HCl, none	34.5	21.5	
0.2 N HCl, none	30.1	0.9	12.5
P buffer, papain	18.3	21.0	
P buffer, Pronase	15.0	0.4	1.8
P buffer, trypsin	15.7	0.6	1.5
P buffer, lipase	14.4	19.4	
P buffer, ribonuclease	15.7	17.3	
P buffer, none	13.4	21.0	
Distilled water, 0 C, none	40.3	28.0	

[a] Crude walls were first treated with enzymes as described in the footnote to Table 3. They were collected by centrifugation, washed with distilled water after the above treatments, and then treated with 6 μg of B_{12} in a total volume of 7.5 ml with 0.05 M phosphate buffer (P buffer), *p*H 7.0. Radioactivity was measured for B_{12} bound to the walls, as in Fig. 2. The supernatant solutions of enzymatic or HCl treatments were put in cellophane bags and dialyzed against distilled water at 4 C for 16 hr until they reached neutral *p*H. Then, 60 μg of B_{12} was added to each, with subsequent incubation for 1 hr at room temperature and dialyzation against distilled water at 4 C for 72 hr. The remaining radioactivity was measured.

the B_{12}. An alternative possibility is the conformational change of a polypeptide, which contains a B_{12}-binding portion in the wall, upon binding with B_{12}. The change prevents the proteolytic actions on the B_{12}-binding portion which is usually sensitive to these enzymes. The enzyme could digest the B_{12}-binding portion before the binding of B_{12}. Treatment with 0.2 N HCl could liberate a B_{12} complex from the wall retaining B_{12}, or a B_{12} binder from the wall retaining no B_{12}. Similar phenomena have been observed with *L. delbrueckii* (19). Lipase and ribonuclease had no significant effect on the B_{12}-binding activity of the wall.

The wall which was incubated only in phosphate buffer showed a remarkable loss of materials sedimentable by centrifugation, indicating that autolysis took place. However, the amount of B_{12} which had been bound to the wall was not reduced by this decrease in the quantity of the wall (Table 3); neither was the B_{12}-binding ability significantly affected by the decrease (Table 4). The facts suggest that the part of the wall that is susceptible to autolysis has no relation to the B_{12}-binding site.

Physicochemical properties of the B_{12} complex isolated from the wall. An elution pattern of the B_{12} complex on CM-cellulose column chromatography is shown in Fig. 3. Although the B_{12} complex was eluted as a single peak, the ratio of optical densities at 361 nm (absorption owing to B_{12} moiety) and 280 nm (polypeptide moiety) differed slightly at the top and at both extremities of the peak, suggesting that the B_{12} complex was microheterogeneous. The B_{12} complex could be purified by rechromatography, which was subsequently concentrated by using a small column of CM-cellulose and dialysis. This showed a single band on polyacrylamide gel electrophoresis (Fig. 4). The final yield of the B_{12} complex was 1.7 to 2.7 mg/g of cells (dry weight). The B_{12} complex was insoluble in salt-free water and soluble in an NaCl solution above 0.05 M concentration. The B_{12} complex contained neither

carbohydrate nor RNA, as revealed by the anthrone (24) and orcinol (3) methods, by subtracting values attributable to the B_{12} moiety from measuring directly those values in the B_{12} binder. The B_{12} binder was obtained by separation of the B_{12} complex into B_{12} and the binder by using the procedures of Gräsbeck et al. (5). The B_{12} complex consisted of B_{12} and polypeptide. On Sephadex G-75 column chromatography, the B_{12} complex was eluted just where soybean trypsin inhibitor was eluted (Fig. 5). Since the molecular weight of soybean trypsin inhibitor is 21,500 (23), that of the B_{12} complex should be around this value. Minimum molecular weight of the B_{12} complex, estimated by measuring the content of B_{12} from an optical density at 361 nm and the content of protein by the method of Lowry et al. (14), agreed well with this value. Microheterogeneity in the ratio of optical densities at 280 and 361 nm was also observed in this chromatographic elution pattern.

Resemblance of the absorption spectra (Fig. 6) of the B_{12} complex to those of B_{12} (cyanocobalamin) over the range from 330 to 600 nm clearly indicates that the complex contained B_{12}. Slight shift of peaks around 361 and 550 nm toward shorter wavelengths was observed for the B_{12} complex. The increased peak at 278 nm of the B_{12} complex indicates involvement of polypeptide in this complex. Vitamin B_{12} showed a shift of the peak at 361 to 368 nm when KCN was added at a final concentration of 0.1 M (pH 11.0), whereas the B_{12} complex (peak at 360 nm in this case) did not. This fact suggests that the position for entry of the sec-

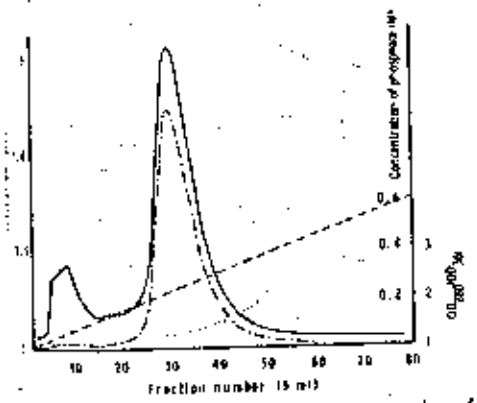

FIG. 3. *CM-cellulose column chromatography of the B_{12} complex of the wall. A B_{12} complex solution (0.01 M phosphate buffer (pH 7.0) containing 0.1 M NaCl (PBN; 145 ml with optical densities: 280 nm = 0.346, 361 nm = 0.196) was placed on a CM-cellulose column (1.2 by 13 cm) equilibrated with PBN. The elution was performed as described in Materials and Methods. Symbols: optical density at 280 nm (—) and at 361 nm (- - -); ratio of optical densities at 361 and 280 nm (· · ·); concentration of phosphate buffer (- - -).*

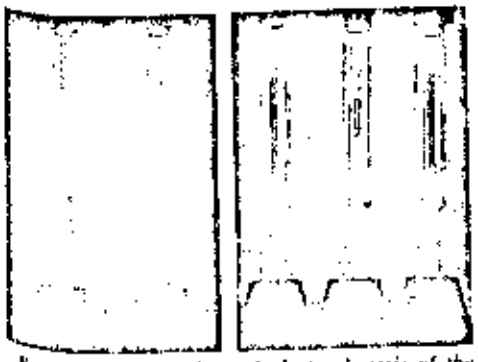

FIG. 4. *Polyacrylamide gel electrophoresis of the purified B_{12} complex of the wall. Before staining (left), the color of the band was red. After staining with amido black (right), the color of the band was blue-black.*

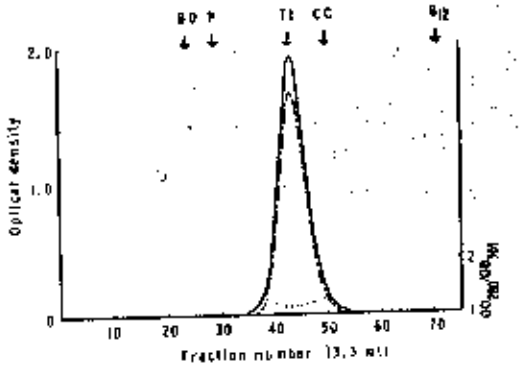

FIG. 5. *Gel filtration on Sephadex G-75 of the purified B_{12} complex of the wall. Symbols: optical density at 280 nm (—) and at 361 nm (- - -); ratio of optical densities at 361 and 280 nm (· · ·). Arrows indicate where blue dextran (BD), pepsin (P), trypsin inhibitor (Tr), cytochrome c (CC), and free B_{12} (B_{12}) were eluted as peaks.*

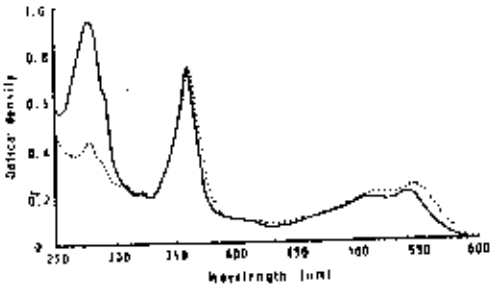

FIG. 6. *Absorption spectrum of the purified B_{12} complex of the wall. B_{12} complex (—), B_{12} (- - -).*

ondary cyano group in the B_{12} moiety was not available. This may have been a result of the involvement of this position for binding directly to the polypeptide moiety, or of some conformational changes of the B_{12} moiety, caused upon binding to the polypeptide, which hindered the entrance of more cyano group to form dicyanocobalamin with the B_{12} molecule. The same phenomenon has been observed with the B_{12} complex of *L. delbrueckii* (20). When the *p*H of the B_{12} complex solution was brought to *p*H 12.9 with Gräsbeck's buffer consisting of NaOH, glycine, and NaCl (5), the peak at 360 nm shifted to 368 nm upon addition of KCN, indicating that the B_{12} moiety received the secondary cyano group. The addition of Gräsbeck's buffer alone did not cause any shift of the peak at 360 nm of the B_{12} complex or at 361 nm of B_{12}. Neutralization of the alkaline KCN solution (*p*H 12.9) of the B_{12} complex caused a shift of the peak at 368 nm back to 360 nm. Therefore, the B_{12} complex should be dissociated into free B_{12} and polypeptide at *p*H 12.9 to be ready to receive a cyano group, as was revealed more clearly by using the procedures of Gräsbeck et al. (5). This method consisted of eluting the B_{12} complex through an alkali-equilibrated Bio-Gel P-10 column. The detached polypeptide and B_{12} eluted separately from the column. The free polypeptide thus obtained could bind B_{12} again at neutral *p*H.

Effect of B_{12} binders on B_{12} binding to *L. leichmannii* and other bacterial species. The B_{12} binder, isolated from *L. leichmannii* walls, or hog intrinsic factor was added together with B_{12} to the cell suspensions of *L. leichmannii* or other bacterial species having no B_{12}-binding activity. *L. leichmannii* cells took up B_{12} in the presence of the B_{12} binder as well as in its absence. On the other hand, hog intrinsic factor, used as a reference, interfered with the B_{12} binding to the cells (Table 5). *Escherichia coli* cells took up large amounts of B_{12} in the presence of the B_{12} binder isolated from *L. leichmannii*, suggesting that B_{12} was bound to *E. coli* cells through intermediation of the B_{12} binder. The amount of B_{12} taken up by cells such as *L. delbrueckii* and *Streptococcus faecalis* with the addition of B_{12} binder was no greater than the amount taken up by these cells with no addition of B_{12} binder.

Properties of the B_{12} complex isolated from ribosomes. A B_{12} complex quite similar to that of the wall was obtained from the *L. leichmannii* particulate (ribosomes) by treatment with 0.2 N HCl using the same procedures employed for isolation of the B_{12} complex from the wall.

TABLE 5. *Effect of B_{12} binders on binding of B_{12} to bacterial cells*[a]

Organism	B_{12} bound to cells in the presence of		
	No binder (μg)	B_{12} binder[b] (μg)	Hog IF[c] (μg)
Escherichia coli no. 215[d]	0.15	4.35	
Lactobacillus delbrueckii AHC 1056	0.17	0.08	
L. leichmannii ATCC 7830	3.52	2.88	0.015
Streptococcus faecalis ATCC 8043	0.03	0.08	

[a] The organisms were grown at their optimal temperatures in appropriate media, harvested, and washed as *L. leichmannii*. A washed-cell suspension (10 mg dry weight) was added to a reaction mixture containing 5 μg of B_{12}, 1 μmole of phosphate buffer (*p*H 7.0), and the B_{12}-binder derived from *L. leichmannii* walls or hog intrinsic factor, in a final volume of 10 ml. The reaction mixture had been allowed to stand for 10 min before addition of the cell suspension. After 30 min of incubation, the cells were collected by centrifugation and washed with the same buffer, and radioactivity was measured as in Fig. 2.

[b] Obtained from *L. leichmannii* walls retaining no B_{12} by treatment with 0.2 N HCl and used in an amount sufficient to bind 5.2 μg of B_{12}.

[c] Hog intrinsic factor in an amount sufficient to bind 5.2 μg of B_{12}.

[d] A B_{12}-requiring mutant (6).

The B_{12} complex from ribosomes was eluted as single peaks on CM-cellulose or Sephadex G-75 column chromatography, or both, but it was microheterogeneous, as judged from the ratio of optical densities at 280 and 361 nm, at the top and at both extremities of the peak as was B_{12} complex from the wall. However, it moved as a single band on polyacrylamide gel electrophoresis. The absorption spectrum of the B_{12} complex and the molecular weight (ca. 21,500) estimated from the chromatographic elution on the Sephadex G-75 column were quite similar to those of the wall.

DISCUSSION

Kashket and Beck (7) questioned whether the B_{12}-binding ability of our previous cell wall preparation of *L. delbrueckii* no. 1 (17, 18) may have been attributable to a B_{12}-binding protein detached from ribosomes and co-precipitated with the wall preparation during cell fractionation. They stated that we did not show that our wall preparation was free from contamination by ribosomal materials. Their statements are not exact. We washed the wall

preparation several times by centrifugation under conditions in which ribosomes were not precipitated and successively treated the preparation with trypsin, ribonuclease, and lipase (17, 18, 20). These treatments may have cleaved off materials other than the wall. Electron micrographs also showed that our wall preparation was free from electron-dense materials, namely, cytoplasmic components. Moreover, approximately two-thirds of the B_{12} bound to the cell was released into the surrounding medium when it was transformed to the protoplast. This fact provided additional evidence that the wall possesses B_{12}-binding ability.

Rashket and Beck (7) did not isolate the cell wall fraction nor did they examine the B_{12} binding ability of their strain, *L. leichmannii* ATCC 7830. The results of the present investigation clearly indicate that the wall is a "primary reservoir" of B_{12} (17, 18) in this organism. Three-fourths of the B_{12} bound to the cell was found in the crude wall fraction. This amount of B_{12} was not decreased significantly by treating the fraction with ribonuclease, lipase, and trypsin (Table 2). The possibility of contamination of the wall fraction by ribosomes or co-precipitated ribosomal B_{12} binders is safely excluded by measuring the contents of B_{12}, RNA, and hexosamines in the wall and ribosomal (particulate) fractions. The fact that the ratio of B_{12} contents in the wall and particulate fractions was 3:1 and that of hexosamines was 39:1, together with the finding that the enzymatically "purified wall" fraction (RLT-wall) retained three-fifths of the initially bound B_{12} and no RNA, emphasizes the B_{12}-binding capacities of the wall and of the ribosomes. The ribosomes (particulate) were responsible for approximately one-fourth of the B_{12} bound to the cell. It appears quite unlikely that most of the ribosomal B_{12} binders were released during cell fractionation procedures and bound to the wall fraction. In fact, the B_{12} bound to ribosomes could not be released by washings under the conditions routinely employed. The wall fraction obtained from *L. leichmannii* cells containing no B_{12} also possessed the B_{12}-binding capacity (Table 1).

B_{12} was shown to bind, in the wall, to a polypeptide on which pepsin, Pronase, and trypsin act (Tables 3 and 4).

The previous findings that B_{12}-requiring lactobacilli have sufficient specific activity to take up large amounts of B_{12} were also confirmed in the present study (Table 1). However, it was found that the cellular capacities for B_{12} binding cannot be accounted for by the B_{12}-binding activity of the walls alone in these lactobacilli strains; some other cellular site, probably ribosomes, may exist as a principal site for B_{12} accumulation. This situation may be interpreted as an evolutional cellular differentiation of these bacterial strains into walls and ribosomes with respect to the B_{12}-accumulating sites. The fact that quite similar B_{12} binders were obtained from the wall and ribosomes of *L. leichmannii* may support this interpretation.

A B_{12}-polypeptide complex (the B_{12} binder to which is bound B_{12}) was isolated from the wall and purified. Its molecular weight was estimated to be around 21,500. A B_{12}-polypeptide complex obtained from the ribosomes was quite similar to that obtained from the wall with respect to absorption spectrum, electrophoretic mobility, and molecular weight (ca. 21,500).

The B_{12} binder that was isolated from the wall of *L. leichmannii* retaining no B_{12} did not significantly affect binding of B_{12} to the cell of the same organism when added to the reaction mixture for B_{12} binding (Table 5). The possibility that ribosomal B_{12} binders were bound to the wall preparation, thus giving an apparent B_{12}-binding ability to the preparation, can safely be excluded if we examine carefully the values obtained. The amount of B_{12} bound to the cell was 3.52 μg at pH 7.0 (0.1 M phosphate buffer) in the absence of B_{12} binder (control), whereas this amount was 2.88 μg in the presence of the B_{12} binder. If ribosomal B_{12} binders were responsible, the latter value should exceed the control value of 3.52 μg. It should be remembered that cellular fractionation was carried out at a pH at which B_{12} binding was not increased if "released B_{12}-binders" were present. Hog intrinsic factor completely inhibited B_{12} binding to *L. leichmannii* cells, as described by Gräsbeck (4). In contrast, the fact that the B_{12} binder derived from the wall did not significantly reduce the B_{12} binding to *L. leichmannii* cells is compatible with previous findings that a B_{12}-polypeptide complex derived from *L. delbrueckii* no. 1 wall can be utilized by *L. delbrueckii* for growth (17, 20).

In view of the present findings, the wall of *L. leichmannii* ATCC 7830 plays a role as a "primary reservoir" of B_{12} as proposed earlier with *L. delbrueckii* (17, 18), because B_{12}, once bound to the wall, should enter the cytoplasm to exhibit its biological functions. The mechanism by which B_{12}, once bound to the wall, is transported to the ribosomes (17) awaits further studies.

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PEDIATRIC PHARMACOLOGY AND THERAPEUTICS

A survey of American experience with vitamin B_{12} therapy of neuroblastoma

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THE PEDIATRIC solid tumor subcommittee of the Leukemia Chemotherapy Cooperative Study Group B has been aware of general interest in the published work of Bodian[1,2] in which continued good experience was cited with the use of vitamin B_{12} in the treatment of neuroblastoma. Except for a negative comment by Dargeon,[3] no reference could be found in the American literature to support or to refute Bodian's data. In conversation with various investigators, it became apparent that a substantial number of children with neuroblastoma have been treated with vitamin B_{12} alone or in conjunction with x-ray and other chemotherapeutic agents. Because the number of patients in any of the individual series studied has been small, the results have not been reported. A questionnaire was sent to 90 investigators throughout this country with known experience and interests in this field. Of these, 71 responded and 20 had used vitamin B_{12} in the treatment of 103 children with neuroblastoma. Their replies to this questionnaire form the basis of this report and represent a pool of the American experience with this agent.

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METHODS

The questionnaire on the following page was employed.

The results of the data obtained here were collated from the response of 20 investigators who reported the treatment of 103 children with neuroblastoma. These children received vitamin B_{12} either as the sole chemotherapeutic agent or combined with x-ray or other therapy. The dosage schedule used was 1 mg. of vitamin B_{12} intramuscularly daily or every other day. Vitamin B_{12} therapy was "similar in dosage and duration to that reported by Bodian."[2] Only 6 investigators gave information concerning extent of metastases, bone marrow involvement, and the stage of disease when B_{12} therapy was initiated. In this group, 16 children received vitamin B_{12} as the sole agent early in the disease, while 10 were given the vitamin as sole chemotherapy late in the disease; 37 children were treated by vitamin B_{12} in conjunction with surgery and x-radiation soon after diagnosis, while 40 were given the vitamin with other modalities late in the disease. A breakdown of the data was made for age at onset of disease. For 20 children of the reported 103, no age was given. Arbitrarily, these 20 children were classified as "over 2

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Dear Dr.:

The Pediatric Solid Tumor Subcommittee of the Leukemia Chemotherapy Cooperative Study Group B is interested in the recently published work of Dr. Martin Bodian (*Archives Dis. Child.*, Dec., 1963). The author elaborates on his experience with Vitamin B_{12} in the treatment of neuroblastoma and reports better results than that usually obtained with conventional therapy. We have not been able to find any references in the American literature to lend either statistical support or to refute his data. In our conversations with various individuals, we know that some work with Vitamin B_{12} in the treatment of neuroblastoma has been done in this country and not published because of the small number of patients so treated. We would like to collect as much data as possible to evaluate whether a pilot study utilizing this agent is or is not indicated.

We are aware of your known interest in this area and would greatly appreciate your answers to the following questions:

A) Have you used Vitamin B_{12} as the sole therapeutic agent in any phase of the treatment of neuroblastoma?

Yes_____ No_____

B) Have you used Vitamin B_{12} in conjunction with other chemotherapeutic agents?

Yes_____ No_____

C) Was your protocol for Vitamin B_{12} therapy similar in dosage and duration to that reported by Dr. Bodian? If not, what dosage was used and what was the duration of therapy?

D) Number of patients treated? _____

E) Number of patients under 2 years _____ Under 1 year _____ Over 2 years _____

F) Effects:

1) No effect at all (beneficial or otherwise). Number_____?
2) Beneficial effect (subjective improvement). Please amplify:
3) What objective benefits were seen? (e.g. regression of tumor size, etc.)?
4) Was any toxicity noted? Yes_____ No_____ Type_____

G) Are you now using Vitamin B_{12} either as sole therapeutic agent or in conjunction with other agents? Yes_____ No_____

H) We would appreciate your comments concerning this type of therapy in neuroblastoma.

I) Do you think it worthwhile to treat any patient with Vitamin B_{12} as cancer therapy?

Thank you for your cooperation. We will send you a brief summary of the collated data when it has been put in its final form. Your help in this matter is greatly appreciated.

years" of age. Since no benefit was noted in any of these 20 children treated with B_{12}, the effect of this arbitrary assignation was to bias the results in favor of a B_{12} effect. We have assessed "beneficial effect," "responding," or "no effect" from the report of the cooperating investigator, but criteria used in the reporting institutions were not explored. In general, a beneficial effect was reported on the basis of a measurable regression of the tumor, and clinical improvement evaluated by an investigator experienced in childhood cancer chemotherapy.

RESULTS

Table I summarizes the results of the survey.

The majority of patients treated with vitamin B_{12} had undergone previous surgical procedures (biopsy, partial excision, or complete excision). Of the 20 patients treated with vitamin B_{12} alone, 2 patients under 2 years of age demonstrated regression of tumor size. Of the 83 who received concomitant x-ray therapy, a beneficial effect was noted in 12 (14.5 per cent). Of this group, 10 patients were under 2 years of age. In 8 of

Table I

	Type of treatment									
	All	B_{12} with and without x-ray therapy			B_{12} alone			B_{12} with x-ray		
	All ages	0-1 year	0-2 years	Over 2 years	0-1 year	0-2 years	Over 2 years	0-1 year	0-2 years	Over 2 years
No. patients	103	13	35	68	2	5	15	11	30	53
No. patients responding	14	5	12	2	1	2	0	4	10	2
% response	13.6	38.5	34.3	2.9	50.0	40.0	0.0	36.3	33.3	3.8

the children benefited, vitamin B_{12} and x-ray therapy comprised initial treatment, while another previously untreated child responded to an operation, and nitrogen mustard in combination with vitamin B_{12}.

Two patients in whom vitamin B_{12} was used alone and 3 in whom x-ray therapy was combined with vitamin B_{12} demonstrated widespread metastatic disease prior to treatment and experienced regression of the metastatic lesions following therapy; these patients have remained well for follow-up periods of 4 to 9 years. One of these children was noted to have a massive thoracic neuroblastoma which regressed under therapy to one half the original size. Surgical biopsy at this time revealed a mature ganglioneuroma. On the other hand, no evidence of improvement was noted in 12 children with widespread neuroblastoma treated with vitamin B_{12} and x-irradiation who were reported to this survey by the Memorial Hospital group in New York City.

DISCUSSION

Excluding leukemia and lymphoma, tumors arising from the sympathetic nervous system, i.e., neuroblastoma, are the most common of the malignant tumors of children and account for about 10 per cent of childhood cancer.[2, 4] These tumors constitute a rare example of malignant undifferentiated tissue which in the course of time alone or of therapy can mature to a more differentiated form, i.e., ganglioneuroma,[5, 6] or can regress spontaneously.[5, 4] Survival in this disease has been reported to average 4 months in the untreated patient and less than 1 year in most treated patients,[2, 5] but "apparent cure" has been reported.[2, 10] The initial treatment of primary neuroblastoma is surgical removal of the tumor when possible.[9] Total extirpation of the primary growth is frequently impossible, yet long remissions have followed even partial resection.[10] Although the radiosensitivity of any one neuroblastoma cannot be predicted, most such tumors are responsive for varying periods. Since disseminated disease is frequent, and clinically undetectable metastasis likely, the addition of chemotherapeutic agents to surgical procedures and radiation therapy is advised. A few years ago nitrogen mustard and antifolic acid compounds were advocated[3] while, more recently, beneficial results have been reported with cyclophosphamide[11-14] and vincristine.[15, 16]

In 1959, Bodian[1] first reported tumor regression or arrest in 15 of 28 patients with neuroblastoma treated with vitamin B_{12}. In 1963, Bodian[3] again reported benefit with vitamin B_{12} in 40 of 73 children so treated. The results of our survey do not confirm Bodian's observations. The over-all response in this survey was 14 of 103 patients, 13.5 per cent. The response rate for children under one year of age was 5 of 13, or 38.4 per cent. Although the number of patients treated with vitamin B_{12} as the sole chemotherapeutic agent is small, a beneficial response in 2 of 20 children would not appear to be significant in view of the known incidence of spontaneous remissions and tumor maturation. A beneficial effect was observed in 12 of 83, or 14.5 per cent of those children treated with a combination of vitamin B_{12} and x-ray therapy. It is impossible to determine whether the beneficial

Table II. A comparison of various treatment schedules for neuroblastoma

	Per cent benefited				Ratio: Patients under 2 years/total no. of patients treated
	All ages	0-1 year	0-2 years	Over 2 years	
I. *Bodian*[1, 2]					
A. Surgery and x-ray treatment	32	56	–	–	9/25*
B. Surgery, x-ray, and B_{12}	44	75	76	9	38/73
II. *Literature*					
A. Surgery alone[10]	39	70	60	0	27/41
B. Surgery and x-ray[7]	30	–	–	–	73†
C. Surgery, x-ray,[5] and/or combination (450 patients)‡	23	–	40	7.5	158/292
III. *This survey*					
Surgery, x-ray and/or combination, and B_{12}	13.5	38	34	3	35/103

*Numerator represents number of children under 1 year of age; no data given for total number of children less than 2 years of age.
†Not classified as to age.
‡Total number of patients was 450; however, ages are recorded for only 292 of these patients.

effect was attributable to vitamin B_{12}, x-ray, the combination of these agents, or other supporting measures. The rate of improvement in this group is again comparable to that reported for x-ray treatment alone and/or in conjunction with other chemotherapeutic agents.[4-6] A comparison of results from various treatment regimens is summarized in Table II.

The survival rate for patients under 2 years of age treated with vitamin B_{12} in this survey is comparable to the survival rate in the same age group obtained by Sutow[5] from his review of the literature (Table II). There is no evidence of increase in benefit when vitamin B_{12} is added to the treatment regimen of patients in comparable age groups. When Sutow analyzed the data for patients under 2 years of age with no skeletal metastases, he obtained a survival rate of 61.5 per cent and he stated that "statistically, no differences in survival rate were apparent among those receiving surgical treatment alone, radio-therapy alone, and combined surgery and x-ray." Survival rates and response to various agents must be correlated with the age of the patient at symptomatic onset of the disease and with the presence or absence of skeletal metastases.

Because of the differences in response rates noted by this survey and others (Table II) as compared to that of Bodian, an attempt was made to analyze the patients in Bodian's series in terms of the above biologic factors. Of the 32 survivors, 29 were under 2 years of age and only 3 children had skeletal metastases. However, of a total of 11 children under 2 years of age with bone metastases, only 3 survived; this was a survival rate of only 27 per cent for this small group with widespread disease. There was no apparent enhancement by the vitamin B_{12} therapy. Of additional interest is the finding of the adrenal area as the site of origin of this tumor in only 39.7 per cent of the patients, whereas in the pre-vitamin B_{12} group, the frequency of the adrenal as site of origin of this tumor was noted to be 68 per cent. Bodian himself has called attention to the better prognosis in pelvic and thoracic neuroblastoma. Therefore, among 29 of the 38 patients under 2 years of age, only 3 survivors were noted of the 11 children with skeletal metastases and there were only 9 survivors of the 16 children with primary adrenal tumor. We would suggest that the seemingly increased survival rate noted in Bodian's group of vitamin B_{12}-treated patients may reflect a sample of better-risk patients. When further analyzed in these

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terms, the improvement rates appear to be similar to those obtained in other series without the use of vitamin B_{12}.

SUMMARY

A survey was undertaken to evaluate the experience of American investigators in childhood cancer chemotherapy with vitamin B_{12} in the treatment of neuroblastoma. The results indicate that no increase in the remission rate was observed either when this agent was used alone or in conjunction with x-ray or other chemotherapeutic agents in patients with advanced disease.

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Whole-Body Counting Studies of Retention and Accessibility of Radioactive Vitamin B_{12}[1,2]

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ANALYSES OF PLASMA (1), liver (2–4), and whole-body radioactivity (5–8) after administration of radioactive cyanocobalamin (CNCB), have all been used to estimate the biologic half-life (T½) of vitamin B_{12} in man. In addition, the minimum daily requirement, daily physiologic requirement, and vitamin B_{12} kinetics have also been studied with these techniques. Although the critical requirement of stability of the labeled CNCB has apparently been satisfied (9), a controversy about the extent of final equilibrium reached between labeled CNCB and other body pools of vitamin B_{12} does persist. Heyssel et al. (7, 10) have stated that subsequent to 200 days after administration of ^{60}Co-CNCB whole-body radioactivity diminishes in a manner that can be expressed as a single exponential function. Heinrich's (6) results were qualitatively similar, but Reizenstein et al. (5, 11) found that the rate of loss of ^{60}Co-CNCB was still decreasing up to 250 days after administration. The first part of this paper will document our results with whole-body counting in patients followed for up to 9 years after administration of ^{60}Co-CNCB. Data will be presented to show that the rate of loss of ^{60}Co-CNCB can, and usually does, decrease with time.

Evidence will also be presented to indicate that both ^{58}Co-hydroxocobalamin (HOCB) and ^{60}Co-CNCB biologic T½'s respond identically to the administration of large amounts of both nonradioactive cobalamins.

MATERIALS AND METHODS

A ^{60}Co-CNCB of specific activity 1 μc/μg was used in all but *patients B* and *S*, from whom it was 2 μc/μg. The specific activity of ^{60}Co-HOCB (used in *patients B* and *S*) was 18 μc/μg. See Table I for the amounts of tracer administered.

The Brookhaven National Laboratory (BNL) whole-body gamma spectrometer is of the single crystal type with the patient in the standard chair position. The counter and counting geometry have been described in detail in previous publications (12, 13). During the year from July 1965 to July 1966 the 8 x 4 inch crystal was replaced by an 11½ x 4 inch crystal. Appropriate cross calibrations were made by counting patients prior to the change and shortly thereafter. Photopeak counts were used throughout these studies. Counting statistics were sufficiently good to use 10-min counts and still obtain rather small standard errors, as seen in Tables II and III. Decay corrections were made at monthly intervals for the long-term studies.

In order to determine whether differences exist in the biological T½ of ^{60}Co-CNCB at different time periods after administration, whole-body counts (WBC) were partitioned into specific time periods and each of these was analyzed individually. The divisions of time used in the analyses were selected by dividing the whole period of time into approximately equal parts, or by utilizing the time periods during which the patients were available for study. Biological

[1] From Medical Research Center, Brookhaven National Laboratory, Upton, New York.

[2] This work performed under the auspices of the United States Atomic Energy Commission.

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TABLE I

Tracer administration data on the 10 patients in the long-term vitamin B_{12} study

Patient	Diagnosis	Amount and Route ^{60}Co-CNCB Administered	Administration Date
BI.	Polycythemia vera	3 μc iv	8/27/57
DE	Atherosclerosis	2 μc iv	2/20/60
AB	Hodgkin's disease	3 μc iv	10/2/57
GL	Polycythemia vera	0.3 μc im	1/24/63
RO	Polycythemia vera	0.5 μc im	1/24/63
CA	Polycythemia vera	0.5 μc im	1/24/63
B	Pernicious anemia	1.0 μc im (2 μc ^{58}Co-HOCB im)	6/12/62 6/19/62
S	Pernicious anemia	2.4 μc im (5 μc ^{58}Co-HOCB im)	4/17/62 4/17/62
C	Pernicious anemia	0.8 μc im 0.8 μc im	6/7/63 6/24/63
M	Multiple sclerosis	2 μc iv	1/31/64

T½'s were excluded if they were made from less than six points. The biologic T½'s, standard errors, correlation coefficients, and *P* value determinations of the resultant slopes were made with a least squares linear regression analysis programmed for the CDC 6600 computer. Only those WBC subsequent to *day 240* are reported, thereby excluding any data that might be considered within the early turnover period.

A similar least squares program for the BNL MERLIN computer was used for the earlier studies on *patients B* and *S*. The initial slopes in this study were made with less than six points.

Patients

Patients from the clinic and hospital of the Medical Research Center, BNL, were utilized in this study. Informed consent was obtained from all patients. Whole-body counting was begun in early 1963 on *patients BI., DE,* and *AB*. These were 5.5, 3, and 5.7 years, respectively, after administration of ^{60}Co-CNCB for other investigative purposes. *Patient BI.* had polycythemia vera treated with phlebotomy, sp, and busulfan, and has been in complete remission since 1960. He received 1,000 μg CNCB intramuscularly, twice weekly, for 2 months 7.5 years after administration of ^{60}Co-CNCB. *Patient DE* had generalized atherosclerosis, arteriosclerotic heart disease, but was a hematologic normal. Four years after the ^{60}Co-CN he received 1,000 μg CNCB twice daily fo days. *Patients AB, RO,* and *CA* receive therapeutic doses of vitamin B_{12}. *Patient* had Hodgkin's disease, *patient RO* polycyth vera in remission, and *patient CA* polycyth vera requiring only occasional phlebot *Patient GL* has juvenile polycythemia vera covered at age 9), treated only with phlebotomy. During the time of study he gained 50 lb in weight.

Patient B had pernicious anemia in remission at the time of study. He received intramuscular injections of ^{60}Co-CNCB and ^{58}Co-HOCB 1 week apart. Two months later he received 1,000 μg CNCB twice daily for 10 days,

TABLE II

Whole-body retention data on four patients who received no vitamin B_{12} other than tracer ^{60}Co-CNCB

Patient	Months after ^{60}Co-CNCB Administration	Biologic T½	se	Correlation Coefficient	*P* Value
AB	50-68	15.96	2.20	0.991	>0.0304
	71-78	22.20	2.80	0.936	
	90-99	24.49	2.49	0.891	
GL[a]	13-24	10.56	0.28	0.987	>0.0008
	25-36	15.75	4.10	0.788	
	37-46	14.00	0.67	0.961	
RO	8-17	21.41	1.24	0.944	>0.0014
	18-27	21.55	0.98	0.975	
	28-38	29.38	1.59	0.918	
CA	12-30	20.78	0.63	0.950	>0.0060
	31-46	25.36	1.34	0.921	

[a] *Patient GL* was a juvenile and had a significant change in body mass during this study. His results are included for interest only.

TABLE III

Whole-body retention data on six patients who received nonradioactive vitamin B_{12} during the course of study

Patient	Administration of Vitamin B_{12}[a]		Months after ^{60}Co-CNCB Administration	Biologic T½	SE	Correlation Coefficients	P Value
			Part A				
BL	Large dose acutely	→	66–76	19.99	1.48	0.976	>0.0001
			84–90	29.89	0.80	0.909	
			91–97	19.60	0.67	0.986	
			99–112	27.06	1.00	0.968	
DK	Large dose acutely	→	36–44	21.83	3.72	0.890	>0.4217
			45–51	25.22	1.48	0.993	>0.0000
			52–56	16.22	0.93	0.975	
			Part B				
B	Small dose regularly	→	9–23	13.52	0.28	0.975	>0.0000
		→	24–33	17.63	0.44	0.981	
		→	34–45	18.25	0.55	0.921	>0.0001
		→	46–55	30.04	3.70	0.846	
S	Small dose regularly	→	10–25	19.24	0.33	0.976	>0.0000
		→	26–36	29.97	0.91	0.929	
		→	37–46	29.18	1.89	0.888	>0.3049
		→	47–55	33.37	3.06	0.876	
C	Large dose regularly	→	10–25	22.93	0.43	0.998	>0.0000
			26–42	15.77	0.26	0.978	
M	Large dose regularly	→	18–32	5.01	0.02	0.999	

Large dose = 1,000 μg/injection.
Small dose = 100 μg/injection.
[a] Refers to period of administration of vitamin B_{12}.
[b] See section on patient material for details.

and 2 months after that 1,000 μg HOCB twice daily for 10 days. After a period of several months without therapy he received 100 μg HOCB every 3 weeks. *Patient S* had pernicious anemia in remission at the time of study. He received ^{60}Co-CNCB and ^{60}Co-HOCB on the same day. Three and four months later he received 1,000 μg CNCB twice daily for 17 days and 1,000 μg HOCB twice daily for 10 days, respectively. Subsequently, he has been given 100 μg CNCB every 3 weeks. *Patient C* had pernicious anemia in relapse when she received two injections of ^{60}Co-CNCB. After extensive vitamin B_{12} therapy in July 1963 she received no CNCB for about 2 years. Since July 1965 she received 1,000 μg CNCB every 3 weeks. *Patient M* has multiple sclerosis and no hematologic abnormality. Several months after receiving ^{60}Co-CNCB she was started on a regimen of 500 μg CNCB every 2 weeks.

RESULTS

Change in Biologic T½ after Extended Periods of Time

Table II presents the data from the four nonpernicious anemia patients who received no further parenteral CNCB. The longest time period after administration of ^{60}Co-CNCB, analyzed in this group is 90–99 months and the shortest 8–17 months.

Except for GL, from 37 to 46 months, the T½ of these patients increased significantly with time.

In addition, *patient BL* (Table III, *part A*) had a similar pattern of increasing T½ before receiving large doses of CNCB when followed 66–90 months after ^{60}Co-CNCB administration. There is no significant change, however, in the T½'s of *patient DE* before administration of large amounts of CNCB.

Effect of Large Doses of CNCB on Biologic T½ Long after Administration of ^{60}Co-CNCB

Patients BL and DE (Table III, *part A*) received massive amounts of CNCB 7.5 and 4 years after ^{60}Co-CNCB, respectively. In both instances there was a highly significant decrease in the biologic T½ during the next 6-month period. In *patient BL* this decreased T½, thereafter, reverted toward the previous T½.

Effect of Large Dose of Vitamin B_{12} Soon after Administration of Tracer B_{12}

The whole-body retention of ^{58}Co-HOCB and ^{60}Co-CNCB before, during, and after large excesses of CNCB and HOCB in *patient B* (and *S*) is shown in Fig. 1 and the biologic T½'s for *patients B* and *S* are presented in Table IV. The last injection of HOCB took place 4.5 months after tracer injection. With one possible exception, there is no evidence that the equivalent time slopes of ^{58}Co-HOCB and ^{60}Co-CNCB are significantly different. The exception is that of *patient B*—slopes 1 and 6 (Table IV), and in these instances there were too few points to fit significant curves. Massive doses of CNCB and HOCB markedly decreased the biologic T½ of both the ^{58}Co-HOCB and ^{60}Co-CNCB. Nearly identical changes of biologic T½ were found after three or four series of injections. The fourth pair of slopes (4 and 9 in *patient S*), while not identical, are not significantly different on statistical analysis. Massive doses of HOCB or CNCB cause similar reductions in the biologic T½ of both labeled vitamin B_{12} analogues.

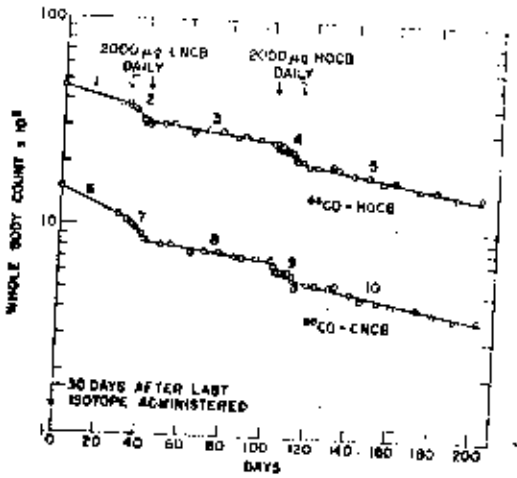

FIG. 1. Graphic depiction of whole-body retention of ^{58}Co-hydroxocobalamin and ^{60}Co-cyanocobalamin in *patient B*. Graph of *patient S* is very similar.

TABLE IV

Comparison of biologic T½ of ^{58}Co-HOCB and ^{60}Co-CNCB

Slope	^{58}Co-HOCB T½, Days	Vitamin B_{12} Load, 2,000 μg/Day	Slope	^{60}Co-CNCB T½, Days	P Value
Patient B					
1	128	Injections of	6	61	0.[illegible]
2	26	← CNCB →	7	24	>0.[illegible]
3	241	Injections of	8	214	>0.[illegible]
4	22	← HOCB →	9	20	>0.[illegible]
5	150		10	128	>0.[illegible]
Patient S					
1	133	Injections of	6	130	>0.10
2	63	← CNCB →	7	63	>0.10
3	218	Injections of	8	158	>0.10
4	38	← HOCB →	9	71	0.23
5	263		10	288	0.44

← and → refer to period of administration of vitamin B_{12}.

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Effect of Chronic Administration of Large and Small Doses of Vitamin B_{12}

Two patients with pernicious anemia in remission (*B* and *S*) (Table III), both of whom received large doses of CNCB and HOCB prior to the start of this long-term section of the study (see above), received regular injections of 100 μg HOCB or CNCB every 3 weeks. In these patients a considerable increase in biologic T½ was observed over periods up to 55 months after injection of tracer (Table III, *part B*).

Patient C (Table III, *part B*) received 1,000 μg CNCB every 3 weeks rather than the 100 μg given to *patients B* and *S*. Whole-body counting showed a decrease in T½ which was highly significant. *Patient M* (Table III, *part B*), who received 500 μg CNCB every 2 weeks, had a very short T½ (5.01 months). Unfortunately, the only comparison figures for this individual are in the months shortly after injection. Even at that time, however, when she was receiving no additional CNCB, her biologic T½ was already 13.5 months. The T½ of 5.01 months thus indicates a markedly decreased T½ associated with the larger doses of CNCB.

DISCUSSION

The most important point of this paper is the demonstration that no single, final biologic T½ of ^{60}Co-CNCB is obtained when the last points of whole-body retention are expressed as an exponential function. Over the period of the present study (~9 years), the biologic T½ appears to change continuously as a function of the length of study, and there is no "final exponential function." The data on which these statements are based were obtained from *patients AB, RO, GA,* and *BL* and *LE*, before they received large loads of CNCB. All but the last showed significant changes of biologic T½ when followed for as long as 112 months after tracer administration. These patients were on normal diets, received no additional vitamin B_{12}, and were presumably in a steady state with respect to their intake and output of vitamin B_{12}. The demonstration of a continuously changing slope of ^{60}Co-CNCB total-body retention, therefore, implies that the tracer compound did not come into final equilibrium with the total-body pool of vitamin B_{12}. That this disequilibrium does exist is not entirely surprising, since its existence was postulated for many years (14, 15). The evidence for the disequilibrium of administered CNCB must be considered when computing pool sizes or making compartmental models of vitamin B_{12} metabolism.

The counting geometry employed was constant throughout the study in all patients except *GL*, a juvenile who gained considerable height and weight during the observation period. Since he gained body mass, his vitamin B_{12} mass probably increased also, and he was not necessarily in a steady state. The counting geometry also changed because of increase in body mass. His data, therefore, are included for interest only.

Another possible explanation for the continually changing slope of the WBC might be in terms of the redistribution of the labeled compound within the body, resulting in a change in the counting geometry and hence in counting efficiency. Redistribution is known to occur in the period immediately after administration of the isotope, but seems unlikely over the long period of this study. The counting periods begin at 240 days after administration of the tracer, thus eliminating any initial redistribution effects. In addition, if the biologic T½ of the liver ^{60}Co-CNCB is different from the rest of the body, one would expect hepatic surface-counted T½'s to differ grossly from total-body T½'s. The T½ data of Glass (3, 4) and Schlosser (2) on hepatic surface radioactivity are quantitatively similar to previously described WBC data for similar time periods. Furthermore, the data of Grasbeck et al. (16) on the

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specific activity of B_{12} in organs of rats, and observations of Willigan (17) in rats do not support the hypothesis of significant late redistribution in the steady state.

The change in the scintillation crystal detector of the whole-body counter during the study had no effect on the slope of the regression curves. The counting rates were normalized by cross calibration of the detectors and by counting patients before and after the change. The only result of changing detectors was a twofold increase in counting efficiency. Most of the counting was actually completed prior to the change in crystals. The ratio of photopeak counts to Compton scatter of ^{60}Co, as measured by Heyssel (10), was also determined in the present study in several patients and found to be unchanged during the period of study.

Most of the data of Heyssel and Heinrich are on orally administered CNCB, whereas the present study was performed with parenterally administered CNCB. Therefore, our results might differ if there were evidence that whole-body retention varied as a result of the different methods of administration. Available evidence indicates that the mode of administration does not affect the turnover rate (18).

The ^{60}Co is probably excreted in the urine with the cobalt atom still firmly attached to the corrin moiety (18, 20). If the ^{60}Co atom was split off and excreted in the ionic form, as is suspected in fecal excretion (19, 20), the results would not be significantly altered because of the rather short biologic $T\frac{1}{2}$ of cobalt in the body (21, 22). If the excretion pattern were different and a catabolic product had a different biological $T\frac{1}{2}$, or if the ^{60}Co label remained in the body in any other biologic form, the results would not be interpretable.

Despite the evidence presented for isotopic disequilibrium, it is apparent, from our data and from that of others (2), that significant amounts of labeled cobalamin can be readily mobilized by administration of large quantities of nonradioactive vitamin B_{12}. The mobilization is more effective when the vitamin B_{12} is administered shortly after the labeled compound, but, contrary to other reports (23), it can also be done at much later periods. The results presented in Table IV and Fig. 1 on *patients B* and *S* demonstrate this mobilization phenomenon at times up to 4 months after the tracer injection.

Patients BH. and *DE* were given high body burdens of CNCB, 90 and 51 months after administration of ^{60}Co-CNCB (Table III, *part A*). There were highly significant decreases in biologic $T\frac{1}{2}$ compared with the previous control periods. In *patient BH.* the reversion toward the previous $T\frac{1}{2}$, after CNCB was stopped, possibly reflects the expansion of body B_{12} stores and, at the same time, an absolute decrease of the amount of tracer.

Pernicious anemia *patients B* and *S* (second study) received relatively small triweekly or monthly injections of 100 μg vitamin B_{12}. These 100-μg doses are equivalent to about 3 μg/day. Their WBC slope, as expected, did not differ significantly from the normals already presented. *Patient G*, who received 1,000 μg vitamin B_{12} triweekly, and *patient M*, who received 500 μg vitamin B_{12} biweekly, had $T\frac{1}{2}$'s which were consistently shorter than the normals over comparable periods of time after the tracer. This observation indicates that treatment of patients with pernicious anemia with large amounts of vitamin B_{12} changes the biologic $T\frac{1}{2}$ and invalidates any measurement of "true" biologic $T\frac{1}{2}$.

At this point it is desirable to compare the biologic $T\frac{1}{2}$'s of the final slope obtained to those measured by other investigators, with the realization that the biological $T\frac{1}{2}$'s represent just one portion of a multiexponential curve. For the five nonpernicious anemia patients (excluding *GL*)

who had received no parenteral CNCB, the values for biologic T½ ranged from 21.5 to 29.9 months with a mean of 26.9 and a median of 25.1 months. Glass (3), in similar patients and using hepatic surface counting, found an average ^{60}Co-CNCB T½ of 11.4 months with a range of 4.7 to 20.8. He later reported similar findings for HOCB (4) but with a T½ averaging 24 months for two patients. Hall (1), studying the biologic T½ of plasma disappearance in four patients, found T½'s of 16-24 months. The remainder of this comparison involves whole-body counting. Heinrich (6) reported one patient with a biologic T½ of ^{60}Co-CNCB equal to 45 months. Heyssel's (7, 10) most recently published results show an average of three normals equal to 20.7 months, with a range of 15.8 to 27.1 months. Reizenstein et al. (5, 11) do not present values for the biologic T½ in their patients, but from the curves of their recently published reports the T½'s are estimated to be considerably longer than those reported here.

The second part of this study was designed to demonstrate how HOCB and CNCB are retained as a function of time in the same individual. Preliminary results of these studies have been published (24). From the results (Table IV) it is apparent that the biologic half-lives of the two vitamin B_{12} analogues are very similar, if not identical. The similarity in their retention curves is not affected by the administration of massive loads of CNCB and HOCB. The fact that CNCB can influence the biologic T½ of ^{60}Co-HOCB, and likewise HOCB can alter ^{60}Co-CNCB, lends credence to evidence that, once within the body, CNCB is converted into a hydroxo compound (25, 26). The present studies, however, were begun a relatively short time after the tracer injection (the last was 4 months), and Glass and Lee (4) have reported a dissimilarity in HOCB and CNCB retention in two patients when followed for 1 year.

SUMMARY

1) The rate of disappearance of ^{60}Co-cyanocobalamin (CNCB) from the body as measured by whole-body counting was found to change continuously as a function of time after administration.

2) The administration of large doses of vitamin B_{12} (500–1,000 μg) any time after ^{60}Co-CNCB administration caused the biologic T½ of ^{60}Co-CNCB to decrease.

3) The administration of small doses (100 μg) of vitamin B_{12} caused no detectable change in biologic T½ of ^{60}Co-CNCB.

4) The administration of large doses of CNCB caused a decreased biologic T½ of ^{60}Co-hydroxocobalamin (HOCB) and the administration of large doses of HOCB caused a decreased biologic T½ of ^{60}Co-CNCB, and both analogues had similar biologic T½'s over the study period.

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Biologic Turnover Rate of Cyanocobalamin (Vitamin B_{12}) in Human Liver

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The availability of γ-emitting radioactive cyanocobalamin (vitamin B_{12}) Co^{60} with a relatively slow rate of radioactive decay has made possible an estimate of the biologic turnover rate of cyanocobalamin in the liver of man. There are several previously reported observations which suggest that this rate might be much slower for cyanocobalamin than for some of the other water-soluble vitamins, such as thiamine, nicotinic acid (niacin), and ascorbic acid. The interval between total gastrectomy and the development of megaloblastic anemia is usually several years.[1,2,3] Studies in pernicious anemia have shown that the interval between cessation of therapy and appearance of relapse will be greater than 6 months in 50 to 70% of the cases and in excess of 12 months in 25% of the cases.[4,5] Pitney and Beard[6] measured the serum cyanocobalamin level of a patient for 18 months after total gastrectomy, and they found that the level fell to one-half of the original value in about 9 months.

The liver is a large organ with a relatively high concentration of cyanocobalamin, and after the ingestion of radioactive cyanocobalamin there is a readily measurable quantity of radioactivity over the area of the liver.[7] According to Glass, Gellin, and Stephenson,[7] the radioactivity in the liver 90 days after the parenteral injection of cyanocobalamin Co^{60} was 86% to 14% of the peak radioactivity, which was observed 5 days after the injection. The rate of disappearance of this radioactivity in excess of the rate of physical decay of Co^{60} is a measure of the biologic half-life of hepatic cyanocobalamin. Implicit in the preceding statement is the assumption that the Co^{60} detected over the liver is physiologically active as cyanocobalamin and not an inactive degradation product containing cobalt.

This is a report of observations on the rate of biologic disappearance of radioactivity deposited in the liver after the ingestion of cyanocobalamin Co^{60}. Data are also presented indicating that such radioactivity has the solubility and chromatographic characteristics of cyanocobalamin when extracted from homogenized liver with various solvents.

Methods

Observations were made on six male patients with pernicious anemia and one healthy man. The patients were receiving intramuscular injections of 30μg. to 100μg. of cyanocobalamin every three or four weeks as maintenance therapy. All subjects studied had received cyanocobalamin Co^{60} orally several months prior to the beginning of these observations. The patients with pernicious anemia were given intrinsic factor to promote absorption of cyanocobalamin Co^{60}. Radioactivity was measured with a conventional scintillation detector and scaler. Correction for radioactive decay of the Co^{60} in the liver was made by counting a reference sample of Co^{60} on each day that a subject was observed for hepatic radioactivity. The ratio $\frac{\text{liver counts per minute}}{\text{reference sample counts per minute}}$ at the time of the

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initial recording of liver radioactivity was designated as 100% hepatic radioactivity. Subsequent values for per cent hepatic radioactivity were calculated by the following formula:

$$\frac{\dfrac{\text{Liver counts per min. on day } T}{\text{Reference sample counts per min. on day } T}}{\dfrac{\text{Liver counts per min. on day } 0}{\text{Reference sample counts per min. on day } 0}} \times 100$$

The values computed by means of this formula were plotted on semilogarithmic paper, as in Figure 1.

To determine if the radioactivity observed over the liver of the patients represented intact cyanocobalamin Co^{60}, the following studies were made: A 1500 gm. liver, from a patient who had received cyanocobalamin Co^{60} 11 months prior to death due to chronic lymphocytic leukemia, was homogenized in a high-speed mixer,* digested with pepsin,* extracted with a mixture of cresol and carbon tetrachloride, and then reextracted with water*. The aqueous extract was passed through Amberlite IRA 400 resin* and extracted again with cresol-carbon tetrachloride and then with water. Aliquots from the several steps in this extraction procedure were tested for radioactivity and cyanocobalamin content.

Assays of cyanocobalamin activity were made by use of Lactobacillus leichmannii as the test organism.[11] †

The final aqueous extract of liver was subjected to ascending chromotography on Whatman No. 1 paper with use of 2:4 lutidin and water (14:10) as a developing agent in an atmosphere of ammonia.

Results

The biologic half-life of hepatic radioactivity after the oral ingestion of cyanocobalamin Co^{60} approximates 400 days. The

* Omnimixer, Ivan Sorval.

† These assays were furnished by P. Derse and L. Hein, of the Wisconsin Alumni Research Foundation.

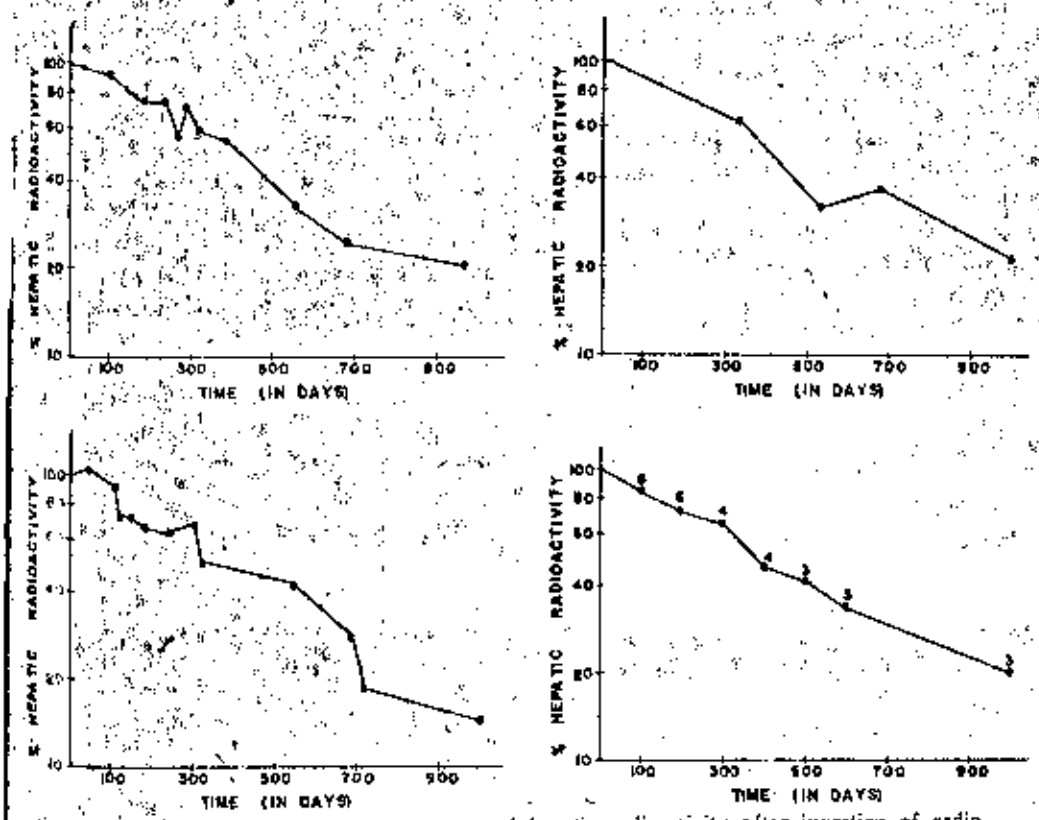

Fig. 1.—Biologic rate of disappearance of hepatic radioactivity after ingestion of radioactive cyanocobalamin. Radioactive decay has been eliminated from this figure. Upper, two patients who had pernicious anemia and were receiving maintenance injections of cyanocobalamin. Lower left, disappearance rate in a healthy man. Lower right, composite curve (five patients and one normal subject). The numbers above the respective points indicate the number of persons observed for that length of time.

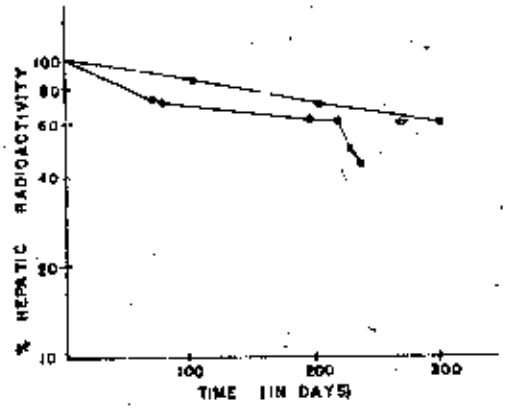

Fig. 2.—See legend for Figure 1. The dotted-line portion of the lower curve represents the rapid fall in hepatic radioactivity when the patient received cyanocobalamin in a dosage of 1 mg. intramuscularly twice a day for seven days (see text). The upper curve is the composite curve from the lower right portion of Figure 1.

rate of disappearance in the normal subject was similar to that seen in the patients with pernicious anemia receiving maintenance therapy (Fig. 1).

In one patient with pernicious anemia the decline in hepatic radioactivity was observed over a period of 217 days, during which time the patient was receiving maintenance cyanocobalamin therapy. For the next seven days he received 1 mg. of cyanocobalamin intramuscularly twice a day. The abrupt fall in liver radioactivity following the injection of this huge quantity of cyanocobalamin shows that even after 200 days the liver radioactivity can be diminished promptly by "flushing" injections of nonradioactive cyanocobalamin (Fig. 2). Doscherholmen and Hagen[12] have made analogous observations. Such data do not constitute positive evidence that the radioactivity disappearing from the liver is in the form of intact cyanocobalamin molecules, though such may be the fact.

The Table contains data which show that the radioactivity of the liver possessed the solubility characteristics of cyanocobalamin, because there is parallelism between radioactivity and microbiological cyanocobalamin activity during the several stages of extraction of the liver cyanocobalamin. Further

Extraction of Cyanocobalamin from Liver

Sample	Volume, Ml.	Cyanocobalamin,* μg./Ml.	Total Cyanocobalamin, μg.	CPM ‡/μg. of Cyanocobalamin
Original liver †	1,500	1.00	1,620	3.7
Extract 1	4,000	0.37	1,480	3.9
Extract 2	6,000	0.20	1,200	4.2
Extract 3	600	1.77	1,062	4.4
Extract 4	130	6.39	831	[illegible]
Extract 5	154	2.42	377	[illegible]
Extract 6	10.5	27.3	286	[illegible]

Extract 1. Filtrate from papain-hydrolysed liver
Extract 2. Supernate after zinc defecation
Extract 3. Aqueous supernate after cresol-carbon tetrachloride extraction
Extract 4. Aqueous extract after salting out with Na[illegible]
Extract 5. Eluate after passing through anion exchange [illegible]
Extract 6. Aqueous supernate after second cresol-carbon tetrachloride extraction

* L. leichmannii assay.
† CPM, counts per minute.
‡ The figure in Column 2 represents grams, and that in Column 3, micrograms per gram.

evidence that the radioactivity represents intact cyanocobalamin Co^{60} is the fact that the radioactivity and red color extracted from the liver showed a chromatographic mobility similar to that of a sample of crystalline cyanocobalamin.

Summary

The biologic half-life of hepatic radioactivity from cyanocobalamin (vitamin B_{12}) Co^{60} is approximately 11 to 14 months. The radioactivity in the liver of a patient who died 11 months after receiving cyanocobalamin Co^{60} had the solubility characteristics and chromatographic mobility of cyanocobalamin. This evidence indicates that the biologic half-life of cyanocobalamin in the human liver is of the order of magnitude of one year.

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EFFECT OF NUTRITION ON THE PRODUCTION OF ACUTE DISSEMINATED ENCEPHALOMYELITIS IN MICE*

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PLATE 28

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The experimental production of acute disseminated encephalomyelitis in mice rests upon the uncertain base of a complex empiricism, and consequently may be categorized as a kind of phenomenology. Historically, the development of this particular phenomenology has proceeded from a recognition of the consequences of injection into hosts of homologous and heterologous brain material (1, 2), the properties of adjuvants in enhancing the pathological effects of such injections (3, 4), the more precise chemical definition of the injected brain material incitant (5–8), the efficacy of various modes of injection (9), the role of hypersensitivity (10, 11), with enhancement by *Hemophilus pertussis* vaccine (12), and the recognition of the genetic control of host susceptibility with a consequent Mendelian analysis of resistant and susceptible mouse genotypes (11, 13). During the course of the genetic analysis evidence appeared which suggested that still another facet of this expanding phenomenology was open to experimental attack; namely, the effect of host nutrition on the capacity to elicit the disorder. It is the purpose of the present paper to report some experiments which indicate that the susceptibility of some known mouse genotypes to the experimental production of acute disseminated encephalomyelitis can be manipulated by nutritional means.

Preliminary Observations of a Nutritional Effect on Susceptibility

A genetic analysis of the difference in susceptibility to experimental acute disseminated encephalomyelitis (ADE) existing between BSVS mice (100 per cent susceptible) and BRVR mice (100 per cent resistant) has been reported previously (13). This report suggested that the resistance of the BRVR mice was due to two equipotent genetic factors either of which, in single dosage, by simple dominance, was sufficient to confer resistance. It was noted at the time that a sample of 41 mice obtained in the backcross of the F_1 to the susceptible parental line (BSVS) exhibited an excessive frequency of susceptibles, 37 per cent instead of the pre-

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dicted 25 per cent. A repetition of this test with an additional 40 backcross mice gave essentially the same results, *i.e.* 14 of the 40 were susceptible, a frequency of 35 per cent. The correspondence between the two independent tests suggested that the discrepancy from theory was possibly a real one and an attempt was made to analyze the situation further.

Analysis of the distribution of the susceptibles within the test samples failed to reveal any correlation with individual litters but did suggest that susceptibles were randomly distributed among all the litters. The hypothesis was next advanced that the observed excess of susceptibles was due to the incomplete penetrance of the genetic resistance factor, specifically in those instances in which the factor was in single dosage. In the instance of an F_1 backcross to the recessive parental stock in a two equipotent factor system, theory predicts that among the resultant genotypes the dominant factor (resistance, in this case) will appear in double dosage with a frequency of 25 per cent, in single dosage, 50 per cent, and fail to appear in the remaining 25 per cent. This latter genotype would be the anticipated completely susceptible type. Actually 35.8 per cent susceptibles had appeared in our tests. The excess of 10.8 per cent susceptibles was assumed to have been derived from among the 50 per cent of the backcross mice which had the factor for resistance in single dosage. The resistant phenotypes of this group was thus reduced from 50 to 50 per cent minus 10.8 per cent, or 39.2 per cent. The penetrance of the single dose resistance factor would thus be $39.2/50 \times 100$, or 78.4 per cent.

Differences in the genotypic background of the backcross mice as compared with the F_2 generation do not permit a direct prediction of the frequency of susceptibles to be expected in the F_2 mice on the assumption of 78.4 per cent penetrance of the single dose resistance factor. However, on this assumption a simple calculation shows that the frequency of susceptibles to be expected would be 11.65 per cent. As previously reported (13) a test of 231 such F_2 animals revealed an incidence of 6.49 per cent. The probability of experiencing a discrepancy of this size by chance alone is greater than 0.05, but less than 0.1. Thus no clear decision can be made from the data either for or against the hypothesis that penetrance of the resistance factor was less than complete.

In a more direct examination of the hypothetical role of penetrance in these phenomena an attempt was made to affect the degree of penetrance through manipulation of the nutritional environment of the mice. The ideal mouse genotype for this examination would be one with the resistance factor in single dosage. Such stocks were not directly available and their preparation a matter of great practical difficulty because of the historical occurrence of the resistance factor in quadruple dosage in the analyzed, resistant parental homozygote. Instead, use was made of the 50 per cent incidence of this single dose genotype among the progeny of the F_1 backcross to the BSVS stock. For this purpose male F_1 hybrids of the BRVR × BSVS cross were backcrossed to BSVS dams and the weanling progeny reared in individual screen-bottomed cages for a period of 4 weeks on the following diets, with litters and sexes evenly distributed between two groups. Group 1, consisting of 23 mice, received a diet of natural foodstuffs (diet 100) composed of 66 per cent whole ground wheat, 33 per cent whole dried milk, and 1 per cent NaCl. Group 2, consisting of 21 mice, received a so called synthetic diet (diet 191) composed of vitamin-free casein, glucose, salts, fat, and vitamins. (The precise composition of the diets which will be repeated later and the history of the introduction of diets 100 and 191 in mouse nutrition studies have been described in previous publications (14).) The young adult mice thus prepared were then tested for susceptibility to ADE as described before (11). The results are presented in Table I.

The results provided two comparisons: First, the frequency of susceptibles on the natural foodstuff diet (diet 100), 30.4 per cent, was not significantly different from the frequency (35.8 per cent) encountered in the previous tests in which the animals had been on a dietary regimen of commercial fox chow pellets plus supplementary bread and milk ($P > 0.7$). Secondly, the synthetic diet (191) lowered the frequency of susceptibility. This was only at the

level of suggestion in the comparison of the effects of diet 100 with that of diet 191 ($0.1 > P > 0.05$), but upon combining the experience on the two diets composed of "natural" materials and comparing the frequency of susceptibles (34/102, or 33.3 per cent) with that encountered with diet 191, it was evident that the difference was real ($P < 0.02$) and that the nutrition of the mouse could indeed influence susceptibility to acute disseminated encephalomyelitis.

The observed nutritional effect on the frequency of susceptibility, however, now raised problems of interpretation and analysis. If the change of the backcross mice to a synthetic diet had been accompained by a *decrease* in penetrance of the single dose resistance factor it would have been expected that susceptibility would have been *increased* from the 33 per cent hitherto experienced on natural diets. The observed 4.8 per cent was, of course, just the reverse. On the other hand, if the shift to the synthetic diet had been accompanied by an *increase* in the penetrance of the single dose resistance factor, susceptibility would have been expected to fall only to the theoretical 25 per cent point;

TABLE I

Susceptibility to ADE of Backcross Mice ((BRVR × BSVS) × BSVS) on Two Different Diets

Diet	No. tested	No. susceptible	Susceptible
			per cent
Natural diet 100	23	7	30.4
Synthetic diet 191	21	1	4.8

but the observed decrease in susceptibility to 4.8 per cent was greater and suggested the additional involvement of the homozygous susceptibles furnished by genetic recombination in the backcross population. The hypothesis was thus developed that the homozygous susceptible genotype was phenotypically dependent upon the nutritional elements of a diet of natural foodstuffs and that the change to the simplified synthetic diet resulted in a diminution of susceptibility to an insusceptible phenotype. This hypothesis was capable of direct test using the homozygous susceptible BSVS stock.

Methods and Materials

Mice.—The mouse genotypes used in these nutritional investigations were (*a*) the completely susceptible BSVS strain (44 generations of brother-sister inbreeding), (*b*) the completely resistant BRVR strain (33 generations of brother-sister inbreeding), and (*c*) a backcross population prepared by crossing BRVR × BSVS and mating the hybrids back to the parental BSVS stock. For experiments, mice were taken as weanlings, divided by litter and sex into dietary groups and housed individually in screen-bottomed cages in an air-conditioned room at 80°F., 50 per cent relative humidity, and with fluorescent light for 12 hours alternating with complete darkness for 12 hours. After being reared on the experimental diets for 4 weeks the mice were tested for susceptibility to ADE as described below. During this test period the mice remained on the experimental diets.

Diets.—The diets used were of two main types, "natural" and "synthetic." Their composition is listed below. Distilled water was supplied *ad libitum*.

"Natural" diets:

Diet 100

	gm.
Ground whole wheat	66
Dried whole milk	33
NaCl	1
Total	100

Diet 115? (A stock diet for general management)

Fox Chow (Ralston Purina Co., St. Louis) ..... *ad lib.*

Whole wheat bread moistened with whole milk, supplied fresh daily ..... *ad lib.*

"Synthetic" diets:

Diet 191

		gm.
Casein (labor. vitamin-free)		18.0
Glucose (cerelose)		72.35
Salts W-2[1]		4.0
l-Cystine		0.2
Water-soluble vitamins		
Thiamine hydrochloride	mg. 2.5	
Riboflavin	5.0	
Pyridoxine hydrochloride	2.5	
Ca pantothenate	10.0	
Niacin	25.0	
Choline chloride	100.0	
Para-aminobenzoic acid	5.0	
Inositol	100.0	
Fat-soluble vitamins, in cottonseed oil (Wesson)		0.25
β carotene	mg. 0.72	5.0
Vitasterol (170 I.U.)	0.33	
2-Methyl-1, 4-naphthohydroquinone diacetate	1.7	
Tocopherol acetate		
Total		100.0

Using diet 191 as a basal diet permitted the addition of various supplements, displacing cerelose as necessary. These supplements will be described in the text for the appropriate experiment.

Production of ADE in Mice.—The empirical basis of the production of ADE in mice has been subject to a continuing process of revision in recent years (15, 11, 12). During the investigations reported here one such revision was adopted so that, in course, two techniques were employed. For brevity these two methods will be referred to as the SC_4 method and the IC_2 method and will be described in the chronological order of their development and use.

SC_4 Method.—Mouse brain proteolipide A + B (8, 5) was injected subcutaneously in a series of 6 weekly injections. The injection material was prepared in a modified Freund type adjuvant as follows:

1.4 gm. proteolipide A + B (dry weight basis)

50 ml. 0.85 per cent NaCl

[1] For composition of salts W-2 see reference 14.

20 mg. merthiolate
250 mg. killed, dried tubercle bacilli, type H37Rv
50 ml. socanol (liquid petrolatum, heavy (U. S. P.), distributed by Socony-Vacuum Oil Co., New York)

These materials were homogenized for 2 minutes in a Waring blendor and the white suspension, having the consistency of light cream, was stored in the ice chest for use. With time the tubercle bacilli tended to sediment out, but were easily resuspended by shaking. The emulsion itself has shown no tendency to separate when kept for periods as long as 6 months, with no detectable deterioration in encephalitogenic activity. The weekly subcutaneous dose per mouse was 0.3 ml.

All animals were examined daily for manifest, chiefly neurological,[3] signs of ADE already described in detail (15). If they showed no sign 12 to 15 days after the sixth injection, their brains were removed and examined for histopathological lesions. Positive signs were also confirmed by pathological findings. Animals exhibiting no signs, but with characteristic lesions upon histopathological examination of the central nervous system were counted as positive reactors. Attention is drawn to the fact that in a varying number of animals, up to 10 per cent, objective signs of illness may be overlooked, or be absent, yet characteristic lesions can be observed in the CNS (16). Finally all animals were scored as either positive reactors, or non-reactors; *i.e.*, as susceptible or resistant.

IC_2 Method.—This method, a modification of the SC_6 method above, was devised to shorten the time necessary to produce ADE and to conserve the preparations of the encephalitogenic proteolipide A + B (12). Briefly, this involved the shift from the subcutaneous to the intracutaneous route, thus enhancing the hypersensitivity reactions of the animals, and for further similar enhancement, added a prior intraperitoneal injection of *B. pertussis* vaccine. In this way, the number of injections were reduced from 6 to 2. Specifically, 4 days before injection of proteolipide all animals received, intraperitoneally, an injection of 0.1 ml. of *B. pertussis* vaccine (Lederle; phase 1, 60,000 million/ml.). The following day the hair was removed from the backs of the animals with an electric shaver, and on the 4th day the mice were injected, intracutaneously in the shaved sites, with a total dose of 0.3 ml. of the proteolipide emulsion described above. The 0.3 ml. was divided into 3 or more blebs through a 1-inch 21 gauge needle. 10 days later a second, similarly divided dose of proteolipide emulsion was injected. The animals were subsequently examined daily for signs of ADE. Such signs usually appeared 7 to 10 days following the 2nd injection of proteolipide. On the 21st post injection day the experiment was terminated. Histopathologic examination of brains of the animals then followed as in the SC_6 method, with similar scoring of positive reactors and non-reactors.

Histopathological Studies.—Approximately 30 sections obtained from various parts of each examined brain were stained with hematoxylin-eosin and were studied for histopathological changes characteristic for ADE (15). Briefly, the outstanding changes in the brain and spinal cord, the chief tissues which were affected, were characteristic and comprised a productive inflammation of the blood vessels involving all the vascular coats, especially an infiltration with monocytic, glial, and compound granular cells and plasmocytes with only few polynuclear leucocytes. The infiltration spread out perivascularly into the surrounding parenchyma but the involved areas were surrounded by apparently normal tissue. Glial infiltrations and proliferation and degeneration of glial cells were seen and demyelination was not extensive or

[3] In an early observation (15) certain signs of respiratory distress, such as "wheezy respiration," were reported but in later, other and different stocks of mice, this was not observed. It is inferred that the respiratory sign is not referrable to a specific effect of ADE; rather, this phenomenon was evoked under the stress of manipulation (see text, *infra*, discussion of *Pasteurella* infection).

commonly noted. Generally, the cerebellum, the white matter, meningeal, and the subependymal areas revealed these changes of an acute disseminated encephalomyelitis (Figs. 1 and 2); for other details, see reference 15.

Statistical Method.—Tests for the significance of experimental results as departures from purely chance events were conducted by estimation of χ^2 from fourfold contingency tables using the correction of Yates for small numbers.

The Effect of Diet on the Phenotypic Susceptibility of BSVS Mice

At the time of testing the hypothetical nutritional dependence of the phenotypic susceptibility of BSVS mice to ADE, consideration was given to possible supplements which might be added to the "synthetic" diet and which might be

TABLE II

Dietary Effects on Phenotypic Susceptibility to ADE of BSVS Mice

Group	Diet		No. of mice	Average weight at test	Lesion score			Total susceptible	Susceptible
	No.	Description			Mild	Moderate	Marked		
				gm.					*per cent*
1	100	Wheat, milk	10	21.3	3	2	4	9	*90*
2	191	Synthetic, basal	19	22.8	4	0	0	4	*21*
3	1000	Basal plus biotin, B_{12}, folic acid	10	22.3	2	3	2	7	*70*
4	1157	Fox chow, bread, milk	17	22.3	3	7	7	17	*100*

implicated in the dietary effect already observed in the instance of the backcross mice; *i.e.*, the difference in effect between "natural" and "synthetic" diets. For example, the vitamin content of synthetic diet 191, reflecting the state of knowledge at the time of its original formulation (14), does not contain supplements of biotin, folic acid, or vitamin B_{12}. The present availability of these vitamins in pure form made it possible to test their supplementary effects directly.

56 weanling BSVS mice were weighed and divided by litter and sex into 4 groups as indicated in Table II. Each mouse was caged individually and the following diets were supplied: Group 1, diet 100, a "natural" diet of whole wheat and whole dried milk; Group 2, diet 191, a "synthetic" diet; Group 3, diet 1000, the basal diet 191 plus supplements, per kilogram, of 1 mg. of biotin, 10 mg. folic acid, and 50 μg. of crystalline vitamin B_{12}; Group 4, diet 1157, a stock management diet of commercial fox chow pellets plus bread and milk. The animals were reared on these diets for 4 weeks and weighed weekly. At that time the mice were tested for susceptibility to ADE by the SC_4 method. Upon histopathological examination of the brains

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of the animals, the severity of the observed lesions was graded on a crude scale of "negative" (non-reactor), "mild," "moderate," and "marked." Results are presented in Table II.

These results provide verification for the hypothesis that the phenotypic susceptibility to ADE of the BSVS genotype is indeed nutritionally dependent. The 100 per cent susceptibility again observed on the stock management diet of fox chow, bread, and milk (diet 1157) was reduced to 21 per cent by the synthetic diet, diet 191, ($\chi^2 = 19.87$, $P < 0.001$). The supplementation of diet 191 with biotin, folic acid, and vitamin B_{12} resulted in a restoration of the frequency of susceptibility to 70 per cent ($\chi^2 = 4.75$, $P < 0.05$). The use of the "natural" diet of whole wheat and whole dried milk, diet 100, resulted in a further increment of susceptibility to the 90 per cent level. These differences in susceptibility, nutritionally arranged, were not reflected in the usual criterion of nutritional adequacy, gain in body weight, for all the diets used were adequate in this sense as indicated by the close correspondence in body weights achieved during the 4 weeks the weanling mice were being reared to young adulthood.

The severity of the neurological lesions produced parallelled, in general, the frequency of susceptibility produced, *i.e.*, when susceptibility was reduced to a frequency of 21 per cent by diet 191 only 4 of 19 mice showed any neurological lesions and these were all scored as "mild"; whereas on stock diet 1157, with a susceptibility frequency of 100 per cent, only 3 of 17 were scored as "mild" and the remaining 14 were scored as "moderate" or "marked."

The Effect on Susceptibility of Single Additions of Biotin, Folic Acid, and Vitamin B_{12} to the Synthetic Basal Diet

The finding that a multiple supplement of biotin, folic acid, and vitamin B_{12} was capable of partially restoring the reduced susceptibility produced by a synthetic diet to the 100 per cent susceptibility supported by a stock diet now raised several problems. Since the restoration of susceptibility was only partial the possibility arose that this was a reflection of the quantitative amounts supplied in the multiple supplement, and that increasing these amounts might further increase the degree of restoration of susceptibility. A failure in the latter instance would suggest that possibly other qualitative items might be needed further to restore susceptibility to the 100 per cent level. Another consideration was the possibility that the action of the multiple supplement was analyzable in terms of its three members, the effect being attributable to one or two of these three, or to combinations thereof. Consequently, the following experiment was performed to examine the effect of enhancement of susceptibility by (*a*) increasing tenfold the supplement to diet 191 of biotin, folic acid, and vitamin B_{12}, and (*b*) supplying these three vitamins singly to diet 191 at the new high level. The experiment was expanded to include, as a technical aspect, a test of the extension of the prefeeding period from 4 to 8 weeks as a

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means of increasing the effect of the synthetic diet, diet 191, in its failure to support phenotypic susceptibility. In this experiment the test for susceptibility to ADE was shifted from the SC_6 method to the IC_3.

TABLE III

Effect of Single and Combined Supplements of Biotin, Folic Acid, and Vitamin B_{12} on the Diminished Susceptibility of BSVS Mice Fed a Simplified Synthetic Diet

Group	Diet		Pre-feed	No. of mice		Av. weight at test		Pasteurellosis deaths		Females for test	Lesion score			Total susceptible	Susceptible
	No.	Description		♂	♀	♂	♀	♂	♀		Mild	Moderate	Marked		
			wks.			gm.	gm.								per cent
1	191	Synthetic, basal	4	10	10	22.1	19.5	10	0	10	0	0	0	0	0
2	1000	Basal plus B_{12}, biotin, folic acid, (low)	4	10	10	23.3	19.8	9	0	10	4	1	1	6	60
3	1153	Basal plus B_{12}, biotin, folic acid, (10 × low)	4	10	10	22.8	19.6	9	0	10	4	0	0	4	40
4	1154	Basal plus folic acid, 10 × (100 mg./kilo)	4	10	9	22.7	18.0	9	0	9	2	1	1	4	44
5	1155	Basal plus B_{12}, 10 × (500 μg./kilo)	4	20	0*	22.8	—	18	—	—	—	—	—	—	—
6	1156	Basal plus biotin, 10 × (10 mg./kilo)	4	10	10	22.8	19.3	10	0	10	1	0	0	1	10
7	1157	Fox chow, bread, milk	4	9	10	24.0	20.3	2	0	10	6	1	3	10	100
8	191	Synthetic, basal	8	10‡	10‡	24.2	19.0	8§	0	10	0	1	0	1	10
9	1157	Fox chow, bread, milk	8	10‡	10‡	25.4	21.1	0	0	10	1	2	7	10	100

* All males in this group by technical error. The 2 survivors failed to show ADE.
‡ 5 of each 10 treated with penicillin (see text).
§ Composed of 5 untreated and 3 treated.

178 weanling BSVS mice were divided, by litter and sex, into nine groups as indicated in Table III. Each mouse was caged individually and the following diets were supplied: Group 1, diet 191, synthetic basal diet; Group 2, diet 1000, basal plus, per kilogram, 1 mg. biotin, 10 mg. folic acid, and 50 μg. crystalline vitamin B_{12}; Group 3, diet 1153, basal plus, per kilogram, 10 mg. biotin, 100 mg. folic acid, and 500 μg. crystalline vitamin B_{12}; Group 4, diet 1154, basal plus 100 mg. folic acid per kilogram; Group 5, diet 1155, basal plus 500 μg. crystalline vitamin B_{12} per kilogram; Group 6, diet 1156, basal plus 10 mg. biotin per kilogram; Group

7, diet 1157, fox chow plus bread and milk; Group 8, diet 191, synthetic basal; and Group 9, diet 1157, fox chow, bread, and milk. Groups 1 to 7 were fed the diets for a period of 4 weeks, groups 8 and 9 for a period of 8 weeks. All animals were weighed weekly. At the end of the preparative feeding period, with the experimental diets being continued, the mice were tested for susceptibility to ADE by the IC_2 method. The experiment was terminated 21 days after the second intracutaneous injection of proteolipide and histopathological examination of brains followed with lesions being scored as previously. Results are presented in Table III.

A Digression: The Incitement of Fatal Pasteurellosis from Latency by the Manipulations of the IC_2 Method, with Diet, Sex, Latent Infection, and the Inoculation Event as Determinants.

In performing the last experiment an unanticipated event occurred which, when analyzed, illuminated a hazard latent in the dietary analysis when pursued by the IC_2 method of testing for susceptibility to ADE, and since this unexpected experience involved the incitement of an infectious disease in somewhat novel circumstances, it seems worthwhile to report it here. Beginning on the 2nd day after the first intracutaneous injection of proteolipide emulsion a wave of deaths occurred which was confined exclusively to male mice on the simplified synthetic diets. These deaths reached their peak during the 3rd to 7th postinjection day and by the 10th day, when the second injection was scheduled, of 70 males on the synthetic diets 65, or 93 per cent, died. In sharp contrast with this, females remained unaffected. Male mice on the stock diet were markedly less affected with only 2 dead out of 8. That these events were directly connected with the injections in some manner was indicated by the remaining 40 uninjected mice being fed for an additional 4 weeks in this experiment, housed in identical and adjacent cages, and which remained completely unaffected during this period. Several hundred other mice in the same room also remained unaffected.

When death occurred it was preceded by only a short period, of perhaps a day, during which the animal showed lassitude, anorexia, and a ruffled coat. Conjunctival exudate which glued the eyelids together was frequently seen. At autopsy the only noteworthy finding was a mottling of the lungs with frequently frank areas of reddish consolidation. From lungs, spleen, and occasionally from heart's blood a short coccoid bipolar Gram-negative rod-like organism was cultivated which was tentatively identified as a species of *Pasteurella*. This encounter with *Pasteurella* was a repetition of previous episodes (17) in other investigations in this laboratory in which deaths due to apparently latent *Pasteurella* infection have been brought about in mice by the simple operation of an intraperitoneal injection. These previous episodes have had an irregular periodicity, occurring usually in the winter months during a period of several weeks and disappearing spontaneously. Severity of the incited disease has varied from year to year, and appeared only by elicitation by means of some experimental procedure. No outbreaks among the stock animals or among untreated mice on a great variety of diets have ever been observed. On balance it would appear that the outbreak of respiratory disease observed in the present instance, as well as previous episodes, was an instance of infection with *Pasteurella pneumotropica* as described by Jawetz (18). (See also footnote 2.) Interestingly this author has implicated "sensitivity" in some of the infection phenomena observed by him (19). In the present experiment it would seem that the well known sensitizing effects of *H. pertussis* vaccine were implicated in the explosive outbreak of pneumonic disease.

When Groups 8 and 9 in the present experiment, after a feeding period of an additional 4 weeks, were tested for susceptibility to ADE by the same IC_2 method an attempt was made to test the practicability of controlling the incidence of *Pasteurella* infection by intramuscular injection of 2 doses of 0.05 ml. of bicillin (Wyeth) penicillin = 30,000 units, at the time the *H. pertussis* vaccine was introduced intraperitoneally, and 11 days later. Half the number of

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the mice were thus treated, the remainder serving as a control. The results (Table III) indicated that latent *Pasteurella* was still present. All 5 of the untreated males on the synthetic diet of Group 8 died of pasteurellosis following the first injection of proteolipide. Again, the 5 untreated females on the same diet escaped the infection. Of the 5 penicillin-treated males, 3 died and 2 survived, indicating the unsatisfactory nature of the attempted prophylaxis. On the stock diet all mice escaped pasteurellosis. Whether other antibiotics would be more successful has remained untested. From the work of Jawetz and Baker (19) streptomycin would appear a more hopeful choice.

In summing up this digression it would appear that latent *Pasteurella* can be incited to frank and fatal pneumonic disease in BSVS mice subjected to the manipulations of the IC_2 method of testing for susceptibility to ADE. The provocation of the latent infection by this means is restricted to mice which (*a*) are on a synthetic diet, (*b*) are males, (*c*) are carriers of *Pasteurella*, and (*d*) come under the stress of inoculation. Stated in another way it can be said that the natural resistance of mice to a pasteurellosis incited from the latent state is markedly conditioned by (*a*) sex, with males more susceptible, and (*b*) by diet, with mice on a synthetic diet being more susceptible.

Completion of ADE Susceptibility Tests

In view of the outbreak of pasteurellosis among the male mice of the experiment the completion of the susceptibility test was confined to the females, as indicated in Table III. These results clearly reveal that on diet 191 susceptibility to ADE fell to very low levels (0 to 10 per cent) compared to the 100 per cent susceptibility supported by the stock diet of fox chow, bread, and milk. This dietary effect was elicited by the IC_2 method thereby confirming the previous observation (Table II) in which the SC_3 method was used. The shift to the IC_2 method as a technique in pursuing the nutritional analysis of the dietary effect on susceptibility was thus supported. Extension of the preparatory feeding period from 4 to 8 weeks (Groups 1 and 7 *vs.* 8 and 9) offered no advantage.

Again in confirmation of previous experience (*cf.* Table II) supplementation of the basal synthetic diet (diet 191) with biotin, folic acid, and vitamin B_{12} resulted in a partial (60 per cent) restoration of susceptibility to ADE. Multiplying the supplement tenfold had no additional effect on this partial restoration of susceptibility (Group 2 *vs.* Group 3, $P > 0.4$). The nutritional gap between diet 191 and the stock diet is thus only partially explained by the effect of the three vitamin supplement. The suggestion consequently offered itself that still other qualitative nutritional entities remain to be identified as supplements to diet 191 before susceptibility can be restored to the 100 per cent level.

The analysis of the effects of the individual members of the triple vitamin supplement revealed that folic acid alone was as effective in restoring susceptibility as the combination (Group 4 *vs.* Group 3). The single supplementation

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with biotin seemed not to support this restoration of susceptibility to the same degree (Group 6 *vs.* Group 3) but the small size of the samples left this issue in doubt ($P > 0.3$). The vitamin B_{12} comparison was lost, of course, because of the initial error of failing to provide females in this group, a blunder which proved catastrophic upon the appearance of the "male-pasteurellosis" phenomenon.

In order to cope with the statistical necessities indicated by the preceding experience and to test the feasibility of circumventing the "male-pasteurellosis" problem inherent in these experiments, a second and similar experiment was performed.

In this new experiment, only female mice were used and the size of the group receiving vitamin B_{12} as a supplement was increased to compensate for its loss in the previous trial. As a test of the continued endemicity of *Pasteurella* in the mice, 2 months after the previous experiment, December, 1955, males were included in the groups receiving the synthetic and the stock diet.

Specifically, 80 female weanling BSVS mice were litter-divided into 6 groups of 10 each and one group of 20. To 2 of these 7 groups a further addition was made of 9 and 10 males respectively. All mice were individually caged and fed the experimental diets for a preparatory period of 4 weeks as follows: (The number identifying each diet and the composition of each was the same as in the preceding experiment.) Group 1, 9 males, 10 females, diet 191; Group 2, 10 females, diet 1000; Group 3, 10 females, diet 1153; Group 4, 10 females, diet 1154; Group 5, 20 females, diet 1155; Group 6, 10 females, diet 1156; Group 7, 10 males, 10 females, diet 1157. All animals were weighed weekly. At the end of the 4 week preparative feeding period, with the experimental diets being continued, the mice were tested for susceptibility to ADE by the IC_3 method. The experiment was terminated 21 days after the second intracutaneous injection of proteolipide and histopathological examination of brains followed with lesions being scored as previously. Results are presented in Table IV.

As the results presented in Table IV indicate, the use of female BSVS mice in the susceptibility test was successful in providing test animals which were not lost from the experiment by pasteurellosis. As the control males showed, latent *Pasteurella* infection was still present at the time of the experiment, and once again male mice on a synthetic diet had a high death rate attributable to the incitement of the latent infection to frank and fatal disease. The effect of diet in this infectious disease was again evident for no deaths occurred among the males on the stock diet. Until other, tested methods are available to eliminate this "male-pasteurellosis" problem as encountered in these mice, it is evident that the use of females in the susceptibility test permits a by-pass and furnishes animals adequate for the continuation of the analysis begun here.

To confine comparisons to the females at test: Diet 191 again failed to support the phenotypic susceptibility of BSVS mice (30 per cent) compared to the 100 per cent susceptibility exhibited by mice receiving the stock diet of fox chow, bread and milk ($P < 0.005$). Supplementation of diet 191 with biotin, folic acid, and vitamin B_{12} resulted again in a partial restoration of susceptibility

TABLE IV

Repeat Test of the Effect of Single and Combined Supplements of Biotin, Folic Acid and Vitamin B_{12} on the Diminished Susceptibility of BSVS Mice Fed a Simplified Synthetic Diet

Group	Diet		No. of Mice		4 wks. Av. weight		Pasteurellosis deaths		Females for test	Lesion score			Total susceptibility	Susceptibility
	No.	Description	♂	♀	♂	♀	♂	♀		Mild	Moderate	Marked		
					gms.	gms.								per cent
1	191	Synthetic, basal	9	10	23.0	18.9	3	0	10	0	3	0	3	30
2	1000	Basal plus B_{12} biotin, folic acid, (low level)	—	10	—	18.6	—	0	10	3	2	3	8	80
3	1153	Basal plus B_{12}, biotin, folic acid, (10 × low)	—	10	—	18.4	—	0	10	3	2	5	10	100
4	1154	Basal plus folic acid, (10× low) (100 mg./kilo)	—	10	—	19.1	—	0	10	2	3	3	8	80
5	1155	Basal plus B_{12} (10 × low) (500 γ/kilo)	—	20	—	18.1	—	0	20	7	4	5	16	80
6	1156	Basal plus biotin (10 × low) (10 mg./kilo)	—	10	—	19.1	—	0	10	2	2	4	8	80
7	1157	Fox chow, bread, milk	10	10	22.1	20.1	0	0	10	2	4	4	10	100

TABLE V

Dietary Effects on Susceptibility to ADE of Female BSVS Mice (Tables III and IV, combined)

Diet		Susceptible / Total tested	Susceptible	Probability that difference from diet 191 group arose by chance	
No.	Supplements to synthetic basal			χ^2	P
			per cent		
191	None	3/20	15	—	—
1000	Folic acid, 10 mg./kilo B_{12}, 50γ/kilo Biotin, 1 mg./kilo	14/20	70	10.2	<0.002
1153	Above, × 10	14/20	70	10.2	<0.002
1154	Folic acid, 100 mg./kilo	12/19	63	7.62	<0.006
1155	B_{12}, 500 γ/kilo	16/20	80	14.4	<0.001
1156	Biotin, 10 mg./kilo	9/20	45	2.98	>0.08
1157	Fox chow, bread, milk	20/20	100	26.2	<0.001

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to the 80 per cent level, and multiplying the supplements tenfold resulted in 100 per cent susceptibility. Considering the sample sizes this achievement of complete susceptibility is probably more attributable to chance than to any real effect of increasing supplement concentration. Indeed, in the previous experiment (Table III) the trend was reversed.

When the three vitamins were fed as single supplements it was found that each resulted in a partial restoration of susceptibility and these rises were all equally to the 80 per cent level. Whether this ostensible equivalence is real or is only a chance effect cannot be determined by samples of this size. It can be concluded, however, that each of these vitamin supplements tended to restore the diminished susceptibility arranged by diet 191. That these effects of single vitamin supplements were not of equal magnitude was suggested, but not proved, by combining the results obtained in these last two experiments, as presented in Table V.

The statistical analysis of this total experience indicates that the partial restoration of susceptibility by the three vitamin supplement is a real effect and that increasing the concentration of these supplements tenfold does not result in any further increase in susceptibility. Folic acid and vitamin B_{12} are apparently equal to each other and to the combined supplement in their effect, while the suggestion arises that biotin has a slighter effect than either of these two vitamins.

DISCUSSION

It will be recalled that the experiences recorded here had their beginning in an attempt to round out a genetic analysis of susceptibility to ADE in the mouse. The hypothetical case of a nutritional influence on the degree of penetrance of the dominant allele, resistance to ADE, remains unexamined, and must be deferred until certain primary phenomena have been analyzed and brought under control. These antecedent matters have to do with the nature of susceptibility to ADE, for, as the experiments above have demonstrated, if the phenomenon of susceptibility is itself nutritionally dependent in homozygous BSVS mice then this nutritional dependency must first be analyzed and brought under control before other nutritional manipulations can be entered upon in a study of the consequences of the introduction of genetic factors for resistance. All this is to say that what is first needed is a nutritional benchmark, a definition of the nutritional environment in which the BSVS mouse genotype achieves, as a phenotype, a susceptibility of 100 per cent. That this is an attainable goal is certified by the 100 per cent susceptibility invariably observed when BSVS mice are on the stock regimen of commercial fox chow, bread, and milk. In the present experiments this goal of definition has not been completely achieved for the defined simplified diet with its three additional vitamin supplements reaches a susceptibility of only 70 per cent.

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This would suggest that there remain other unidentified nutrients, supplied by the stock regimen, which are needed as further supplements. What their nature might be is not known, but must remain as objects for further investigation.

What is to be made of the role of folic acid, vitamin B_{12}, and, to a lesser extent, biotin in the present experiments? In the first place the basal synthetic diet is fully capable of supporting the growth of the mice and mice thus fed seem to have no overt need of these three vitamins, either because of innate, small but adequate biosynthesis, or by supply from imperfectly purified materials such as the "vitamin-free" casein, or by intestinal synthesis due to the bacterial flora. But whatever may be the case it is clear that the pathological events set in train by the attempt to produce ADE pose an additional and crucial requirement in order that the lesion which we seek to incite can be brought into being. For this end the BSVS mouse needs dietary supplements, of which we have been able to identify three. The identification of these three is, in a sense, an historical accident, for when, in 1940, the basal synthetic diet was formulated for use in this laboratory (14) these three items were not available for inclusion. Their inclusion here as pure chemical entities to be tested was a direct consequence of the historical development of the science of nutrition in the intervening years. Indeed, folic acid, biotin, and vitamin B_{12} may have no unique connection with susceptibility to ADE but, as may well turn out, other vitamins and other nutrients now included in the basal synthetic diet may be similarly necessary for the susceptibility of BSVS mice to ADE. This possibility is now under examination.

Returning to the information now directly in hand it is interesting that folic acid and vitamin B_{12} are found once again in close association, so close, indeed, as to be mutually replaceable. It is tempting to speculate that the dependency of susceptibility to ADE on folic acid or vitamin B_{12} rests, at the metabolic level, on the well known participation of these vitamins in the formation and transfer of one-carbon intermediates, and in the synthesis of nucleic acids. This may, however, be somewhat extravagant; rather more justifiable, seemingly, is the more direct connection which is indicated in the recent nutritional investigations on the problem of antibody formation. This connection can now be elaborated as follows:

There are significant indications (20) that acute disseminated encephalomyelitis can perhaps be regarded as a specific manifestation of hypersensitivity. In this view brain tissue or proteolipide as incitants of ADE are regarded as antigens in the susceptible host as an antibody-forming system. The pathological lesions of ADE in the CNS are hence the sequelae of the antigen-antibody reaction. It follows that to be susceptible the host must be an antibody producer. Now, mainly through the work of Axelrod and Pruzansky (21), it has been shown that the fabrication of antibodies is quantitatively dependent on

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certain vitamins in the nutritional environment. Specific deficiency results in impaired antibody production, and it has been shown that the various vitamins range from trivial to pronounced effects in this connection. Further, any single vitamin, which may have a pronounced effect in the antibody response to one antigen may have a relatively trivial effect in the response to a second antigen. It should occasion no surprise, therefore, that in the present experiments two vitamins have emerged as important supporters of susceptibility to ADE and a third vitamin be less effective in this connection. The harmony of these results with the more generalized analysis of Axelrod and Pruzansky on antibody production as influenced by nutrition can be regarded as one more piece of evidence in support of antigen-antibody, or hypersensitivity, as the mechanism of the pathological process in ADE.

SUMMARY

The susceptibility of homozygous BSVS mice to acute disseminated encephalomyelitis (ADE) has been found to be nutritionally dependent. On a laboratory stock regimen of commercial fox chow pellets, whole wheat bread, and milk this genotype is 100 per cent susceptible to the disease. On a "synthetic" diet, containing a minimal list of vitamins adequate for growth and maintenance, susceptibility was found to be reduced to 15 per cent. Supplementation of the "synthetic" diet with biotin, folic acid, and vitamin B_{12} restored susceptibility to a frequency of 70 per cent. Increasing the supplements tenfold had no further effect in restoring susceptibility frequencies to the 100 per cent level.

In the restoration of susceptibility, folic acid and vitamin B_{12} were equally effective as single supplements and equivalent to the triple vitamin supplement. The effect of single biotin supplementation was less.

An outbreak of fatal pasteurellosis among BSVS mice latently infected with *Pasteurella* and used in an ADE susceptibility test has been described. The fatal pasteurellosis has been ascribed to a constellation of determinants including (*a*) diet, (*b*) sex, (*c*) inoculation events, and (*d*) latent infection with *Pasteurella*. With males the susceptible sex it was possible to avert the fatal pasteurellosis and continue the nutritional experiments by using females exclusively.

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EXPLANATION OF PLATE 28

Brains deriving from 2 BSVS mice reared on natural diet 100, and exposed intracutaneously to proteolipide A + B. Stained with hematoxylin-eosin:

FIG. 1. Cerebellum, showing chiefly the infiltrated vascular lesions; vessel partly in granular layer exhibits a thrombus. × 105.

FIG. 2. Subependymal area of lateral ventricle, showing the massed infiltrations apart from vascular lesions. × 315.

Litter mates similarly exposed to proteolipide A + B, but reared on synthetic diet 191, revealed no changes in the CNS.

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Biosynthesis of Natural Products

Problems of substrate nonpermeability and location of isotopic label are described.

A. Ian Scott

Natural products, for many years the touchstones of structural and synthetic [illegible] chemists, continue to be dis[illegible]d from various sources; and 1000 structures are added annually to a storehouse of some 12,000 described molecules whose molecular weights are between 20 and 2000. These compounds are usually referred to as "secondary" metabolites because their biochemical function in the host organism is in most cases obscure. Yet in the few cell-free systems that have been developed to study the biosynthesis of natural products, the enzymology appears to follow the operation of mechanisms identical or closely allied to "primary" metabolism. In view of the considerable practical difficulties in the preparation of the enzymes of secondary metabolism, knowledge of this field has mainly been derived from the results of administration of ^{14}C-, ^{3}H-, and ^{3}H-labeled substrates to the intact plant or organism, with subsequent appropriate degradative techniques to locate the label, and, more recently, from the use of isotope ratio techniques to ensure nonrandomization. Although [illegible] progress in experimental tech[illegible] has been made, it seems to be an intrinsic property of many higher plant systems that specific incorporations of between 0.01 and 0.1 percent represent an average level of success. The purpose of this article is to suggest that several techniques can be used to improve a given situation which at first sight might be indicative of negative incorporation of a suspected precursor. In addition, in systems where incorporation of 5 percent or more can be achieved (usually in fungi or bacteria) the use of ^{14}C as biosynthetic label offers unique advantages and can be combined very successfully with the results of other kinds of radioactive labeling experiments.

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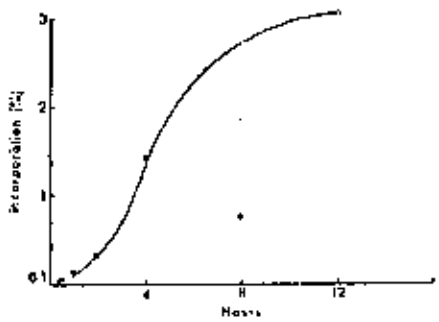
Identification of Intermediates by Short-Term Incubation in Vinca rosea

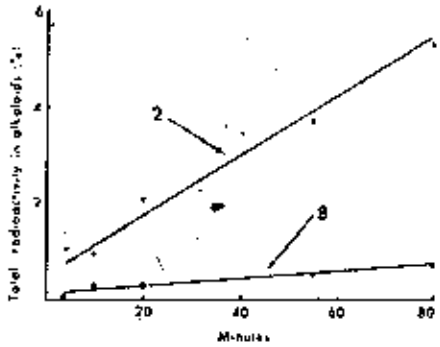
Of the plants in which indole alkaloids occur, perhaps none holds more interest for the chemist or the biochemist than *Vinca rosea* in that the periwinkle produces a profuse array of indole alkaloids including some 100 representatives of all the important structural classes (*1–3*). It is also an ideal plant with which to begin cell-free studies, for the viable seeds are readily available, easy to cultivate, and the seedlings possess a remarkable capacity for synthesizing alkaloids with a vigor that is perhaps matched only by the chemotaxonomists who have bestowed such complex names on these compounds.

Earlier biosynthetic studies (*2*) with *V. rosea* utilized either intact shoots or seeds. After some experimentation it was decided to use young seedlings (9 to 17 days from germination) grown from a mixed strain (Burpee) at 33°C in an environmental chamber with full light. Intact seedlings were removed and placed in groups of four (the average weight was 16 milligrams) in ¼-dram vials (1 dram = 1.8 grams). The seedlings remained healthy and continued to develop root growth for about 9 to 10 days in water at 33°C. The range of alkaloids present in the 9-day seedlings approximated to that of mature *V. rosea*, and the more predominant compounds were easily detectable by thin-layer chromatography (TLC) (*4*). These were vindoline (**13**), coronaridine (**12**), catharanthine (**11**), akuammicine (**5**), and vinervine (**6**) (see chart 1). One of the main difficulties in carrying out cell-free studies with *V. rosea* is the absence (or virtual absence) in the mature plant of many alkaloids which correspond to the intermediates of the various pathways. In practical terms this will probably require preparative scale cell-free incubations to isolate and characterize these dynamic compounds. However, with the use of short-term incubation techniques with the above seedlings, some progress can be made toward the solution of this problem.

The main events of indole alkaloid biosynthesis based on earlier incorporation data (*2*) are summarized in chart 1. Here the structures marked with an asterisk (*) correspond to alkaloids identified below. The remaining alkaloids have already been described from mature plants and germinating seeds of *V. rosea* (*2*).

Incorporation of tryptophan into the alkaloids takes place in two distinct phases. For the first 2 hours of incubation it is linear (Fig. 1), but after this interval a rapid increase in the rate of incorporation is observed. Between 12 and 48 hours, a maximum of 3 percent is reached and this level is maintained during the full time of the experiment





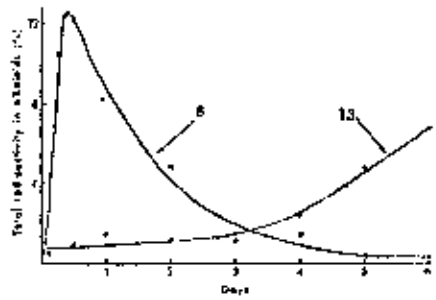


Fig. 1 (left). Incorporation (percent) of DL[^{14}C]tryptophan into alkaloids in *V. rosea* seedlings during 12 hours. Fig. 2 (right). Relative radioactivities of geissoschizine (2) (triangles) and tabersonine (8) (circles) during the first 80 minutes of DL[^{14}C]tryptophan feeding.

Fig. 3. Comparison of radioactivity in tabersonine (8) (circles) and vindoline (13) (triangles) in *V. rosea* seedlings after administration of DL[^{14}C]tryptophan during 6 days.

(up to 8 days). It is concluded from these results that no large pool of tryptophan is present and also that 8 days is insufficient time for the distribution of radioactivity to approximate to the "normal" percentage of alkaloid distribution in the seedlings since after 8 days vindoline (13) (a major alkaloid of *Vinca*) contains only 7 percent of all the activity. Shoots of mature *Vinca* which have been used for most of the available feeding data incorporate tryptophan but at a reduced initial rate, and the final (constant) percentage is about 1.0. The latter observation is of particular interest for comparative studies with diversely aged biological material.

As can be seen from Fig. 2, activity is being incorporated into geissoschizine (2) at eight times the rate of incorporation into tabersonine (8). This is consistent with geissoschizine being a precursor of tabersonine, and, although the experiment does not demonstrate this relationship, the necessary evidence for the sequence has been secured in another way (2). Further, the geissoschizine and tabersonine pools have not in 80 minutes reached their maximum value of radioactivity. In fact the tabersonine pool attains maximum radioactivity after 9 hours (Fig. 3). At this time [2-^{14}C]tryptophan with a specific activity of 52 millicuries per millimole gives rise to tabersonine of specific activity of 15.6 mc/mmole (30 percent specific incorporation), indicating the extremely small size of the tryptophan pool available for alkaloid synthesis and of tabersonine. Autoradiograms show that tabersonine is metabolized almost as rapidly as it is formed, and in fact its activity falls (Fig. 3) from 12 to 2 percent in 3 days. Refeeding ^{14}C-labeled tabersonine isolated after tryptophan had been fed for 9 hours gives a number of labeled metabolites. After 1 day when there is still a large amount of unchanged tabersonine (8), it was found that epoxytabersonine (9), methoxytabersonine (10), and coronaridine (12) are all labeled. After 6 days there is almost no tabersonine remaining while vindoline (13) has gained activity (Fig. 3).

The role of epoxytabersonine [(−)-lochnericine] (9) in the overall biosynthetic map is at present obscure. It is a minor constituent of the normal alkaloid pattern of *Vinca*, but after 9 hours it has 4.3 percent of all the activity and a specific activity of 10 mc/mmole (from tryptophan of specific activity 52 mc/mmole). It is formed from tabersonine (8) as shown from refeeding [^{14}C]tabersonine. It is not converted to tabersonine, nor is it transformed to vindoline.

Although vindoline can be detected on autoradiograms after 1 day, its activity does not begin to increase rapidly until after 3 days (Fig. 3). At this stage the amount of radioactivity in the alkaloids has reached a maximum, and the activity in vindoline will presumably increase rapidly to a maximum value that will not alter unless vindoline is broken down. In contrast, the amount of radioactivity in the *Strychnos* alkaloid akuammicine (5) (approximately 8 percent of the total) and its phenolic derivative vinervine (6) (14 percent!), remains unchanged from 9 hours to 6 days, a fact which suggests that these alkaloids are shunt

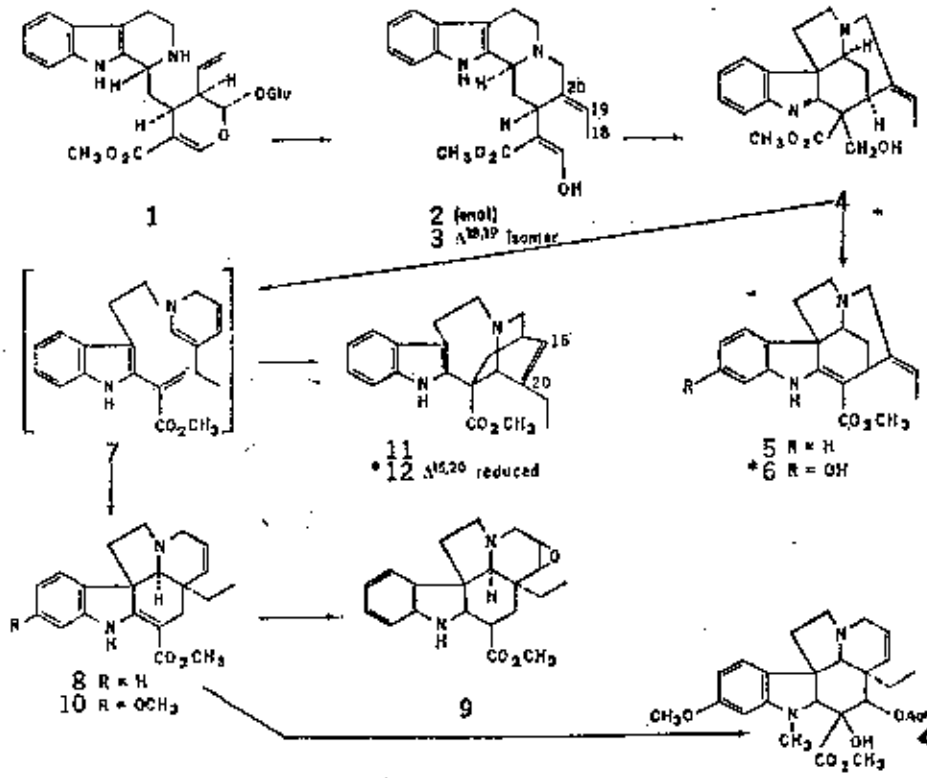


Chart 1. Structures of the monomeric alkaloids of *V. rosea*

products formed from a rapidly metab-
zing key intermediate postulated (2)
ne preakuammicine (4) at a con-
tant rate during the early part of the biosynthesis, that is, when the incorporation of label from tryptophan is still increasing.

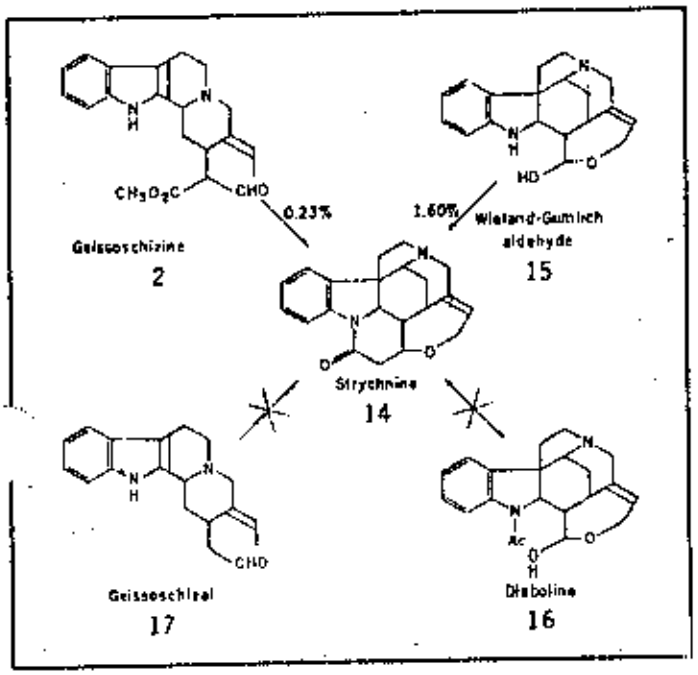
The precursor activity of tabersonine (8) for both vindoline (13) (*Aspidosperma*) and coronaridine (12) (*Ibogа*) emerges from the above experiments and confirms the earlier findings with seeds and shoots. The pathway from tabersonine to coronaridine (12) has until recently been presumably via catharanthine (11) and presents an intriguing problem of sequence, for the postulated acrylic ester (7) has been invoked at two stages in the biosynthesis (2), that is, after geissoschizine (2) and again after tabersonine (8). The obligatory role of latter alkaloid *Ibogа* synthesis may require reassessment when the relative incorporation data is available at the enzyme level. However, the special place of tabersonine in the pattern of alkaloid synthesis in *Vinca* is demonstrated by the
ngoing results because after 9 hours
alkaloid is the dominantly labeled species—yet it is almost undetectable on the sixth day

In summary the technique of short-term incubation has led to the discovery of both static and dynamic constituents of the maturing seedling. The specific incorporation of tryptophan into the alkaloids is approaching a more satisfactory value (25 to 30 percent), and the profiles revealed for tabersonine and geissoschizine are suggestive that true intermediates can be identified and subsequently confirmed by extension of this easily applied technique to other species.

Biosynthesis of Strychnine in Strychnos nux-vomica

In contrast to the relative ease of incorporation of the building blocks and indeed the larger intermediate substrates in *V. rosea*, species such as *Rauwolfia serpentina* and *Strychnos nux-vomica* have, from the outset of the biosynthetic studies, displayed most disappointing properties as far as precursor uptake has been concerned. Indeed if these had been the only plants available for study it might be suggested that our knowledge of the intricate mechanisms whereby mevalonate and tryptophan are cometabolized to the complex indole alkaloids of the major structural types (for example, the *Strychnos* alkaloids) would still be in its infancy. Thus the administration of geissoschizine (2) [a known precursor of the "*Strychnos*" alkaloid akuammicine (5) in *V. rosea*] to *S. nux-vomica* under "normal" feeding conditions (5 to 7 days) led to insignificant ($<2 \times 10^{-3}$ percent) incorporations into strychnine (14). More surprisingly the Wieland-Gumlich aldehyde (15) and diaboline (16), which seem almost certain candidates for intermediacy (near the end of the pathway), failed to incorporate over the same time scale. Reinvestigation of the problem involved the technique of replanting young seedlings after 5 days incubation with 2, 15, 16, and 17 labeled with ^3H in the aryl group. After a further 100 days strychnine was isolated from each feeding, crystallized to constant radioactivity, and (in the case of positive incorporation) degraded to locate the position of the tritium label. Reference to Fig. 4 shows that indeed geissoschizine (2) fulfills the same pivotal role in *S. nux-vomica* as it does in *V. rosea*, but that the result would have been in doubt without resource to the "prolonged contact" technique. Similarly the anticipated but hitherto unrealized conversion of Wieland-Gumlich aldehyde (15) to strychnine (14) (1.6 percent) could be demonstrated (5). Finally the nonincorporation of diaboline (16) after 70 days (6) suggests strongly that the seventh ring of the complex strychnine molecule is built in a clockwise carbon-to-carbon addition of the acetic acid unit, rather than from the nitrogen-carbon condensation which would have required diaboline as an intermediate. The nonincorporation of geissoschizal (17) reveals that, by a hitherto undisclosed mechanism, a carbon atom is lost somewhere between geissoschizine and Wieland-Gumlich aldehyde but not by a simple decarbomethoxylation reaction (5).

These experiments do not begin to answer the question of genetic control and evolution in a plant such as *S. nux-vomica*, which, although producing quite massive concentrations of strychnine and brucine by a process now shown to emanate from geissoschizine, does not elaborate the variety of rearranged structures of the *Aspidosperma* and *Ibogа* families engendered by *V. rosea* from the latter precursor.



Application of ^{13}C-Labeling in the Biosynthesis of Natural Products

Although ^{13}C was used in the early studies of steroid biosynthesis and its distribution was analyzed by mass spectrometry, it is only recently that the first reports of the application of continuous wave and Fourier transform ^{13}C nuclear magnetic resonance (NMR) spectroscopy to the solution (without recourse to degradation of the intact metabolite) of a precursor relationship have appeared. Several descriptions of the techniques are now available (7) and, provided that successful assignment of the natural abundance spectrum is made, enrichments above the natural abundance (1.1 percent) of ^{13}C can be used to delineate the pathway with 60 to 90 percent enriched substrates. A recent example from our own laboratory (8, 9) may be used to illustrate the power of this tool (along with ^{14}C-labeling) in the study of the intermediary metabolism

of one of the most complex of natural products, vitamin B_{12} (19; cyanocobalamin). Several other recent biosynthetic studies with ^{14}C have been made (7).

Biosynthesis of Vitamin B_{12}

Early experiments with ^{14}C in *Propionibacterium shermanii* had revealed that the pathway succinate-glycine to δ-aminolevulinic acid (20) to cobyrinic acid (21) obtains in corrin biosynthesis. Thereafter by successive amidation and addition of the aminoisopropanol-phosphate-ribose-dimethylbenzimidazole side chain and finally the cobalt atom the full structure of the vitamin and coenzyme are reached. First, Fourier transform ^{13}C NMR was used to confirm that eight molecules of aminolevulinic acid were utilized, and this was borne out by feeding [2-^{13}C]aminolevulinic acid (240 mg) to *P. shermanii* (500 grams of wet cells) for 70 hours. The resultant cyanocobalamin showed enrichment of seven methylene and one methyl signal (Fig. 5a), a result in full accord with this concept (9). Similarly the intact incorporation of 4 moles of [8-^{13}C]porphobilinogen (22) is revealed in Fig. 5b, where dicyanocobalamin was used to separate the otherwise convergent signals in cyanocobalamin (the *mono*cyano form of the vitamin). It has always seemed an attractive possibility by analogy with the magnesium (chlorophyll) and iron (heme) pathways that the cobalt pathway forms B_{12} from uroporphyrinogen III (23) by a methylative ring-contraction sequence. This has now been shown (8) to be correct by obtaining the same pattern (Fig. 5) of labeling as in the [8-^{13}C]porphobilinogen experiment by feeding 23 prepared in turn from the [8-^{13}C]porphobilinogen specimen used to obtain the data in Fig. 5b. All that remains is to account for the so-called "extra" methyl groups in B_{12}, of which there seemed to be either six or seven. The latter number is shown to be correct by feeding experiments with [5-^{13}C]aminolevulinic acid in which only seven enrichments were obtained at sp^2 carbons but none in the methyl region of corrin; and ^{13}C-enriched methionine which, in the derived dicyanocobalamin (Fig. 5d), shows the insertion of seven methionine-derived methyl groups (9). Furthermore, comparison of the Fourier transform ^{13}C NMR spectra of cobinamide and neocobinamide enriched with [^{13}C]methionine reveals (10) a downfield shift of its enriched methyl group in ring C corresponding to a deshielding of 11.7 parts per million (Fig. 6). Hence the stereochemistry of methylation in ring C must be in the α-configuration (10). The second methyl group of the gem-dimethyl array in ring C comes from decarboxylation of an acetate side chain of porphobilinogen; that is, it is derived from carbon 2 of aminolevulinic acid (see Fig. 5a). Thus the pathway porphobilinogen to uroporphyrinogen III, and the gain of seven methionine-derived methyl groups can be used to construct a mechanism for the process.

Such a mechanism embodying these results has been suggested (10) (Fig. 7). The stage is now set for the approach to the enzymology of the various steps in corrin formation between uroporphyrinogen III and cobyrinic acid. To this end a cell-free system has been developed (11) which converts aminolevulinic acid to cobyrinic acid.

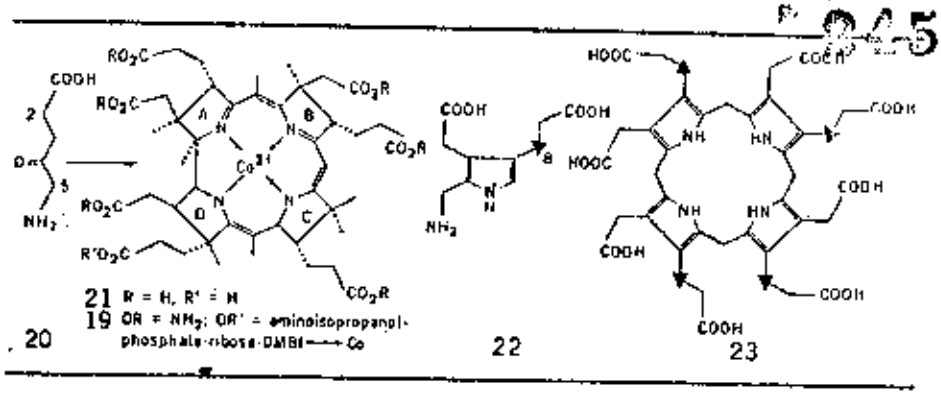


Chart 2. Biosynthesis of B_{12}.

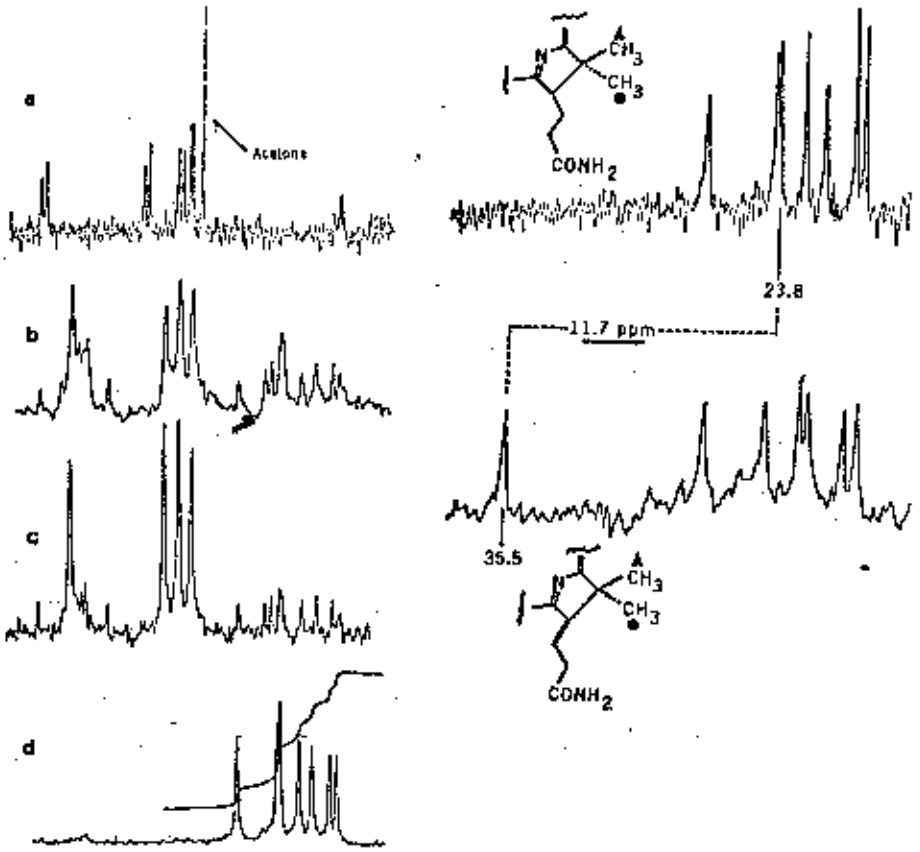


Fig. 5 (left). Proton-noise decoupled Fourier transform ^{13}C NMR spectra of vitamin B_{12} as (a) cyanocobalamin enriched with [2-^{13}C]aminolevulinic acid; (b) and as dicyanocobalamin enriched with [8-^{13}C]porphobilinogen; (c) uroporphyrinogen III; and (d) [methyl-^{13}C]methionine. Details of chemical shift data and experimental conditions are described in (9). Fig. 6 (right). Comparison of the proton-noise decoupled Fourier transform ^{13}C NMR spectra of dicyanocobinamide (top) and dicyanoneocobinamide (bottom). Chemical shifts are downfield from hexamethyldisilazine.

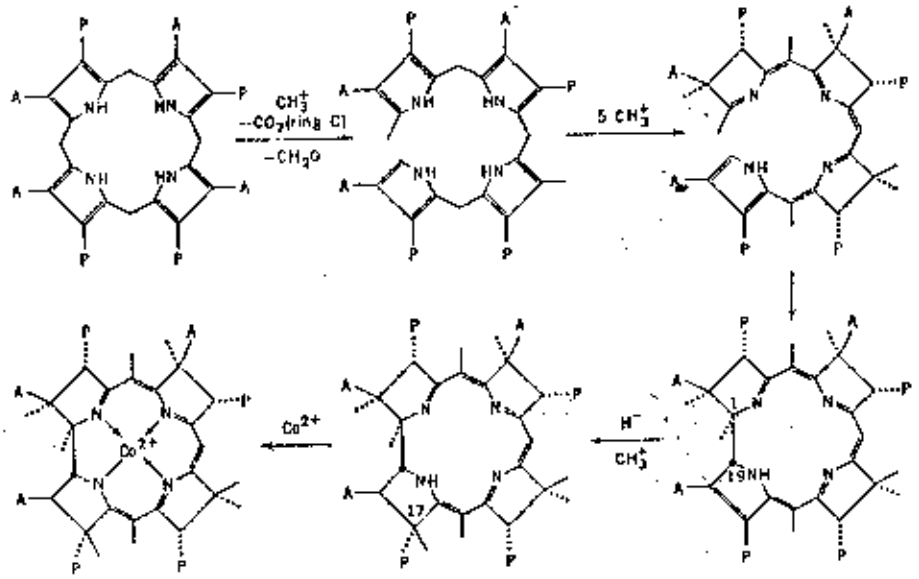


Fig. 7. Proposed mechanism for the biosynthesis of cobyrinic acid from uroporphyrinogen III.

and in addition utilizes uroporphyrinogen III as a precursor, thereby removing the permeability problems associated with larger precursors. Fractionation of the crude homogenate on a large scale to permit ^{13}C analysis of the interactions between enzyme and substrate is in progress.

Summary and Conclusions

The experimental verification of a proposed biosynthetic pathway for a given natural product is often difficult to obtain with the use of the whole organism (permeability factors) or, in the case of higher plants, a cell-free system. Until the purified enzyme for each step of biosynthesis is available, biosynthetic studies can, however, be carried out, albeit with modest incorporation values, by means of either hydroponic or injection methodology. Where viable seed sources are available it is suggested that improvements of several orders of magnitude in incorporation can be achieved by a short-term incubation (small pool size of precursors; trapping of reactive intermediates) or a long-term feeding (equilibration of precursor with the compartmentalized or induced synthetases). In bacterial, fungal, mammalian, and plant systems, where incorporation efficiencies provide the opportunity to study ^{13}C enrichment (at least equal to natural abundance of the isotope), we can expect a rapid expansion since the method removes the tedium of carbon-by-carbon degradation. For the next few years, however, the prognosis would seem to favor parallel studies of ^{13}C and ^{2}H, and of $^{14}C/^{3}H$ ratio techniques since the last-mentioned method provides more information concerning the stereoselectivity of labeling processes on the microgram scale.

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Growth and Development of Central American Children

II. The Effect of Oral Administration of Vitamin B_{12} to Rural Children of Preschool and School Age

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THE DISCOVERY of vitamin B_{12} and its stimulatory effect on the growth of experimental animals under certain conditions soon led to studies of its influence on the growth of children, particularly on those whose development appeared to be retarded. Unfortunately, these studies have frequently yielded quite conflicting or inconclusive results, as recently reviewed by Howe.[1]

The first report[2] on the administration of vitamin B_{12} introduced an experimental design and statistical procedures which are still controversial. In this study the authors concluded that 5 of 11 children responded in a dramatic and highly significant manner. This has been followed by the inconclusive results of Chow[3,4] and reports of suggestive positive effects on at least some children by O'Neil and Lombardo,[5] Bidault,[6] Wetzel *et al.*,[7] Wilde,[8] Jolliffe and his associates,[9] Larcomb and collaborators,[10] Crump and Tully,[11] and Grüninger and coworkers.[12] Negative reports also appeared beginning with Benjamin and Pirrie[13] and Spies and his associates.[14] The papers of Scrimshaw and Guzmán,[15] Someswara Rao *et al.*,[16] and Mackay *et al.*,[17] deal with poorly nourished children showing a considerable degree of retardation in growth and development, and all failed to find a significant stimulatory effect of orally administered vitamin B_{12} despite the relatively large numbers and careful balancing of control and placebo groups. Moreover, Downing,[18] Mitchell *et al.*,[19] and Rascoff *et al.*,[20] administering vitamin B_{12} to premature infants, were unable to demonstrate any significant effect on growth. The second paper by Wetzel[7] gave additional data to support his original hypothesis but as before did not make use of placebo controls. More recently, Montoye *et al.*[21] found no positive effect of vitamin B_{12} administration to school children, and Howe[1] cites a dissertation by Wittich[22] in which this vitamin stimulated the growth of school children.

The present study is an attempt to determine with reasonable certainty whether or not 20 μg of vitamin B_{12} administered daily by mouth has any tendency to improve the growth of rural Guatemalan children of either preschool or school age. The growth and development of these children were, on the average, markedly retarded, and their intake of protein of animal origin extremely low. The trials were run concurrently with those of Mackay *et al.*[17] in Jamaica and have yielded similar results.

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TABLE I

Average Monthly Height and Weight Gains of Preschool Children Given Orally 20 μg Vitamin B_{12} Daily

Period	Height (cm) Control	Height (cm) B_{12}	Weight (kg) Control	Weight (kg) B_{12}
	N = 26	N = 24	N = 26	N = 24
I	0.70	0.65	0.10	0.17
	N = 15	N = 20	N = 15	N = 20
II	0.48	0.43	0.18	0.17
	Approx. L.S.$D_{0.05}$ = 0.08 cm		Approx. L.S.$D_{0.05}$ = 0.05 kg	

MATERIAL AND METHODS

Fifty preschool children in the small rural highland village of San Lorenzo el Cubo (SLC) and 228 school children in four other villages—Magdalena Milpas Altas (MMA), Santa María Cauqué (SMC), San Antonio las Flores (SAF), and Chinautla (CHI)—in the same area were ranked by age and sex and randomly assigned to placebo or experimental groups. Tablets containing 20 μg of vitamin B_{12} or placebo identical in appearance were administered daily by mouth six days a week. In physical appearance and development, social and economic status, as well as dietary habits, the children included in this study were similar to those described for previous field experiments.[15,23] Their diets provided protein of animal origin in the range of only 8 to 14 g daily and their height, weight, and bone maturation were approximately two to four years behind those of well-nourished children of comparable age in the United States and Guatemala. All children were eating ad libitum in their own homes throughout the study.

Height and weight measurements were obtained monthly and a careful record of attendance kept. Personnel administering the tablets had no knowledge as to which children were receiving placebos. It was possible to administer the treatment without substantial interruption to preschool children, but treatment of the school children was discontinued during the school vacation, from October through December. The study of preschool children was begun in October, 1954, and terminated in October, 1955; that of school children was started in March, 1954, and finished in October, 1955, during 12 weeks of which time they were on vacation and did not receive treatment.

Both height and weight gains were adjusted by multiple regression methods for initial differences in age, height, and weight. No effect of frequency of treatment was encountered, so that no correction for this variable was made in the data presented. The approximate least significant differences (α = 0.05) were calculated using pooled estimates of error.

RESULTS

The results of the administration of vitamin B_{12} to the preschool children are shown in Table I. There is no suggestion in these data of a positive effect of vitamin B_{12} at this dose on rate of gain in either height or weight.

Similarly, the data for the school children (Table II) make possible twelve sets of com-

TABLE II

Average Monthly Gains of School Children Given Orally 20 μg Vitamin B_{12} Daily

Village	First 6 mo Control	First 6 mo B_{12}	Second 6 mo Control	Second 6 mo B_{12}	Third 6 mo Control	Third 6 mo B_{12}
Height (cm)						
MMA	0.36	0.37	0.41	0.36	0.42	0.36*
SMC	0.41	0.37	0.42	0.53*	0.43	0.42
SAF	0.42	0.45	0.44	0.45	0.47	0.41
CHI	0.37	0.34	0.51	0.46	0.27	0.28
Weight (kg)						
MMA	0.18	0.20	0.11	0.08	0.22	0.20
SMC	0.14	0.12	0.18	0.26*	0.20	0.16
SAF	0.10	0.22	0.18	0.14	0.20	0.19
CHI	0.18	0.21	0.08	0.08	0.19	0.21

Approx. L.S.$D_{0.05}$ = 0.06 cm in height; 0.06 kg in weight. * Indicates significant differences.
Approx. Number: MMA-83, SMC-53, SAF-58, CHI-34.

parisons, one for each of three 6-month periods in four villages. Of the twelve comparisons for the effect of vitamin B_{12} on height, one is significantly positive, one significantly negative, and ten show no effect. Of those for weight, eleven show no significant difference. The single period in which a positive effect on height and weight was observed in one village had the lowest frequency of treatment due to the 3-month school vacation.

DISCUSSION

The data presented provide no basis for assuming that vitamin B_{12} has any effect, positive or negative, on growth as estimated by the rate of gain in either height or weight. Although subjective benefits from vitamin B_{12} administration have often been mentioned in the literature, such alterations as were noted by teachers and field workers in our experiment seemed to occur with equal frequency in the experimental and control groups. The data available did not permit any further attempt to appraise subjective changes.

It was felt that such a study in a population known to be greatly retarded in growth and development and subsisting on a diet containing sufficient calories and total protein but only a small amount of animal protein would give the maximum opportunity for demonstrating a vitamin B_{12} effect. Even if the effect of vitamin B_{12} were only to stimulate appetite, the children should have been able to show a response, since they were on an ad libitum diet which was limited more by quality than quantity, and in most cases a child could have had greater quantities of food if he had desired it. It is noteworthy that our results are identical with those of a parallel field trial by Mackay *et al.*[17] and also those of Someswara Rao *et al.*,[18] whose studies failed to show a positive effect on undernourished Jamaican and Indian children, respectively.

In view of the earlier concept that vitamin B_{12} might improve the growth of children on diets low in animal protein by supplying a missing "animal protein factor" or by improving the utilization of protein of vegetable origin, the studies by Someswara Rao *et al.*[18] in undernourished Indian children, Kaye *et al.*,[24] and Chow *et al.*[8] in infants 3–6 and 3–12 months of age are significant. All these studies failed to demonstrate a significant effect of vitamin B_{12} on nitrogen retention, although the Indian workers tested it as an addition to both restricted and adequate vegetarian diets and the other investigators studied it with intakes of high and low protein content. Closely related to the problem is the failure of Aguirre and Scrimshaw[25] to find any effect of vitamin B_{12} on hematologic values in the same or similar children.

It is possible in theory, as Howe[1] suggests, that these children would have responded to vitamin B_{12} administration if some other factor had not been more limiting. Although the design of the present study does not specifically exclude such a possibility, previous trials in both Guatemala and El Salvador[15] failed to demonstrate a statistically significant difference between vitamin B_{12} and placebo when included in dietary supplements designed to make up existing deficiencies. Although the intake of vitamin A in these trials was below the recommended dietary allowance, subsequent experiences indicate that this vitamin was not a limiting factor.

Many of the reported studies suffer from deficiencies in experimental design and interpretation of the data which make them inconclusive. One is the danger of using children of one village as a control and those of another as an experimental group. The difference in growth rates among villages shown in Table II is such that any conclusion could have been obtained by chance, had this practice been followed in the present study. Equally unreliable is the use of a group as "its own control." The variations from one 6-month period to the next are so great that had the previous or subsequent growth experience of any of these groups been used for comparison with a period of vitamin B_{12} administration, again any conclusion could have been obtained fortuitously. Another procedure has been to pick out those children in an experimental group showing growth spurts during the experimental period and call attention to them as examples of significant and unexpected response. Analysis of our own data has shown that individual spurts

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of growth during an experimental period are equaled by individual spurts in the control groups, and our groups are of sufficient size to validate this observation.

We also feel that undue significance has been attached in some studies to the month-to-month fluctuations in the height and weight measurements recorded for individual children. Although the curves for child growth which have been developed from the analysis of the data from large numbers of individuals give the impression of a smooth progression, many individuals do not follow such a pattern in a regular manner. The fine studies of the growth of individual children (Stuart *et al.*[26,27] and Reynolds and Sontag[28]) show that many children grow with types of variation similar to those characterized by some authors as "growth failure" and "growth response."

It is noteworthy that vitamin B_{12} has been without effect in studies conducted in technically underdeveloped areas with children showing some degree of malnutrition and growth retardation. On the basis of these reports and our own experience there would appear to be no indication for the use of vitamin B_{12} in public health or school programs designed to improve the nutritional status of children whose growth and development are retarded.

The present data do not preclude the possibility that occasional apparently well-nourished children whose growth and maturation are chronologically retarded will respond to vitamin B_{12} administration. This question will have to be resolved by additional critical studies among well-nourished groups of children.

SUMMARY

The daily oral administration of 20 μg of vitamin B_{12} in controlled trials involving 50 preschool children in one rural village and 228 school children in four rural schools in Guatemala did not produce any effect on their rates of gain in either height or weight. These data illustrate some of the errors in interpretation which can result from the considerable variation and apparently fortuitous changes commonly observed in the rates of growth and maturation in children. The present findings are in agreement with the results of trials conducted in other areas involving children showing some degree of malnutrition and growth retardation and subsisting on relatively inadequate diets.

On the basis of the foregoing it appears that the growth retardation commonly observed in technically underdeveloped areas will not be affected by the administration of vitamin B_{12}.

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Combinations of Chemical Compounds in Experimental Cancer Therapy*

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The ability of microbial cells to adapt themselves to toxic agents leads to the development of "drug fastness" in anti-bacterial chemotherapy. Experimental evidence indicates that two mechanisms may be important in the resistance of bacteria to chemical agents. Bacterial mutants may arise which are no longer dependent upon the metabolic pathway which was blocked in the parent strain by the anti-bacterial agent. Adaptations of metabolism may occur so that an alternative pathway is developed which is not affected by the drug. In the chemotherapy of infectious diseases, significant advances have been made by the discovery of more potent anti-bacterial chemical compounds and by the judicious use of combinations of effective drugs. Combination therapy is effective against selected microbial diseases, presumably because the drugs used may attack the metabolic function of the microbial cell simultaneously at different points.

In experimental and clinical cancer chemotherapy no chemical compound has yet been developed which destroys neoplastic cells effectively without serious toxicity to normal cells. In view of the well recognized qualitative similarities between normal and malignant cells, it seems unlikely that a single chemical agent will be found which will selectively kill tumor cells. It is for this reason that the use of combinations of chemical compounds in the treatment of tumors in experimental animals has been explored in our laboratory. Limited trial of this approach has been made. Mitchell (13) has reported that the combination of parenterally administered tetra-sodium 2-methyl-1:4-naphthohydroquinone diphosphate (synkavit) and irradiation treatment has slightly prolonged the mean survival time of patients with inoperable carcinoma of the bronchus (13), but this has yet to be substantiated and has even been questioned (6). Skipper (16) has observed a possible anti-leukemic synergism between urethan and nitrogen mustard (HN_2) against a strain of mouse leukemia. Emerson, Wurtz, and Zanetti (5) have reported the rapid regression of lymphosarcoma transplants in mice receiving cortisone in conjunction with a riboflavin-deficient diet, and methyl testosterone or cortisone administered to pyridoxine-deficient rats increases the tumor inhibition of a rat lymphosarcoma produced by either steroid alone (2).

Reports of the effect of 5-amino-7-hydroxy-1*H*-*s*-triazolo (*d*) pyrimidine (guanazolo, 8-azaguanine) on a variety of neoplasms in experimental animals (3, 7, 8, 11, 12, 17, 18, 20) and of its mechanism of action (15, 25) have appeared with increasing frequency during the past year. In the present report, preliminary experiments will be summarized which indicate that the administration of certain chemical agents together with 8-azaguanine can lead to a greater inhibition of tumor growth than is obtained with 8-azaguanine alone.

MATERIALS AND METHODS

The carcinostatic action of guanazolo upon the 755 tumor, a transplantable mammary adenocarcinoma in C57 black mice, has been previously established and recorded in this laboratory (7). In the experiments to be reported, combinations of chemical compounds were assayed for their inhibitory effect upon the growth of this mouse tumor, and 8-azaguanine always constituted one component of the combination under trial. The effectiveness of combinations has been measured by the comparison of results obtained following the intraperitoneal administration of 8-azaguanine alone with those observed following combination therapy.

The tumor was transplanted unilaterally in the axillary region by the usual trocar method. The mice were allowed a stock diet of Rockland pellets and water *ad libitum*. Therapy was initiated at

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various intervals after transplantation as indicated in the tabular summaries, the experiments were terminated by killing the animals, and the tumors were dissected out and weighed to the nearest milligram.

The chemical compounds[1] were dissolved as follows: 8-azaguanine in 0.1 N NaOH as previously described (7); desoxypyridoxine and vitamin B_{12} in isotonic saline; pteroylglutamic acid and 7-methyl folic acid in 2 per cent sodium bicarbonate. Control animals received injections of comparable volumes of the appropriate vehicle.

RESULTS

Desoxypyridoxine.—The results of the administration of the pyridoxine analog, desoxypyridoxine, are summarized in Table 1. In this table and in all subsequent ones, the pertinent information on the number of animals, their mortality, the number of tumors, the duration of the experiment, the time of administration of the drugs, dosage schedules, mean tumor weights, and the standard deviation of the mean is presented so that the protocol of each experiment can be duplicated. In comparing the difference between two mean tumor weights (m_1 and m_2), the results have been considered beyond chance variation when

$$\frac{m_1 - m_2}{\sqrt{\sigma m_1^2 + \sigma m_2^2}} = 2.5$$

or greater. The average body weight of all groups of animals is recorded at the beginning and the end of each experiment in order to assess the possible nonspecific contribution of weight loss on tumor inhibition.

As can be seen from Table 1, desoxypyridoxine, given alone, significantly inhibited tumor growth in two experiments (Exp. Nos. 74 and 79), as compared with untreated controls, and significantly failed to modify tumor growth in two other experiments (Exp. Nos. 67 and 92). It is to be noted that in no instance was the desoxypyridoxine-induced tumor inhibition as great as that consistently observed with 8-azaguanine. In five out of seven experiments the combination of 8-azaguanine and desoxypyridoxine inhibited tumor growth to a significantly greater extent than the purine analog alone. In the two experiments (Exp. Nos. 74 and 106) in which the difference in mean tumor weights was not significant, the trend was in the same direction as in the other experiments.

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The dosage levels employed ranged from 200 to 175 mg/kg initially, dropping during the course of treatment to 175–150 mg/kg of body weight. When 150 to 135 mg desoxypyridoxine/kg body weight were employed initially in combination with 8-azaguanine, the results of such experiments, although showing a definite trend in the direction of augmented inhibition of tumor growth, were statistically insignificant. It would appear that dosage levels in the neighborhood of 150 mg/kg would be in the threshold range of a desoxypyridoxine-azaguanine combination effect.

With the exception of Experiment 85, the weight loss produced by this combination falls into a range which, in our experience, has not affected tumor growth. Experiment 85 is nevertheless considered a valid experiment, because the weight loss is of the same degree in both the azaguanine-treated and in the desoxypyridoxine-azaguanine-treated animals.

Folic acid.—Tables 2 and 3 illustrate the effects of the administration of pteroylglutamic acid 1 hour before that of 8-azaguanine on tumor growth in male and female mice. It can be noted that in neither series of experiments did pteroylglutamic acid have an inhibitory effect on the tumor when compared with untreated controls. As can be seen from the results, a difference in the effect of the combination of 8-azaguanine and folic acid on tumor inhibition was detectable, depending upon the sex of the host. In four out of five experiments on male mice, significant inhibition of tumor growth followed the administration of the combination, whereas in none of the three experiments on female mice was the difference between the mean tumor weights of the combination-treated and 8-azaguanine-treated animals significant. The observed variation in response in the two sexes is not understood.

7-Methyl folic acid.—The effects on tumor growth of the simultaneous administration of 7-methyl folic acid and 8-azaguanine, compared with 8-azaguanine therapy alone, are summarized in Table 4. It is to be noted that 7-methyl folic acid alone failed to inhibit tumor growth, whereas the combination of the folic acid derivative and the purine analog decreased the size of tumors to a significantly greater extent than did 8-azaguanine alone in all experiments except No. 118. It is also of interest that, unlike the observations with folic acid previously described, the inhibitory effect of 7-methyl folic acid and 8-azaguanine was noted whether the tumor-bearing hosts were male or female.

Vitamin B_{12}.—The results of combination therapy with Vitamin B_{12} and 8-azaguanine are sum-

marized in Table 5. Significant tumor inhibition by the combination is far less consistent than in the previously recorded experiments, and B_{12} alone has no effect.

In the nine experiments conducted, the combination of B_{12} and 8-azaguanine significantly depressed tumor growth in three instances (Exp. Nos. 67, 91, and 95). In one experiment (Exp. No. 74) the tumors of combination-treated animals were significantly larger than those in the 8-azaguanine controls, and in the remaining observations no significant difference between 8-azaguanine and 8-azaguanine plus B_{12} treatment could be determined. Table 5 indicates that the dosage of vitamin B_{12} varied from 25 μg to 2,000 μg/kg body weight and that the B_{12} was injected at different time-intervals in relation to the 8-azaguanine. The results of experiments 91, 95, and 108 suggest the possibility that the dosage and the time of administration of the B_{12} may be important in obtaining consistent tumor inhibition when combined with 8-azaguanine.

Histology.—In all the experiments, histological sections of tumors from the experimental and control animals failed to show distinctive morphological changes which distinguished the groups.

DISCUSSION

The basis for the selection of the chemical compounds used in combination with 8-azaguanine in these experiments rested on two hypotheses.

Qualitative differences in the biochemical patterns of normal and neoplastic cells have not yet been described; however, many quantitative dif-

TABLE 1

EFFECT OF DESOXYPYRIDOXINE AND 8-AZAGUANINE ON THE 755 TUMOR IN C57 MICE

Exp. No.	Group	No. Animals Dead/Alive	No. Tumors	Duration Tumor Growth (Days)	Day Therapy Begun After Transplantation	Daily Dosage (mg/kg) DP*	Daily Dosage (mg/kg) 8-AG†	Mean Tumor Weight ± Standard Deviation of Mean (mg.)	Av. Body Weight (gm.) Beginning	Av. Body Weight (gm.) End
74	Controls	1/10	9	18	8			888± 51	22.3	23.3
	Desoxypyridoxine	3/10	6	18	8	175		486±151	22.4	22.0
♂	8-Azaguanine	0/10	10	18	8		50	51± 7	21.4	21.1
	8-Azaguanine +Desoxypyridoxine	5/10	5	18	8	175	50	86± 10	22.4	20.0
79	Controls	0/30	26	18	1			284± 23	18.8	18.2
♀	Desoxypyridoxine	9/30	16	18	1	‡		155± 43	19.5	19.0
92	Controls	4/20	16	23	13			1110±172	24.0	25.8
♂	Desoxypyridoxine	1/20	19	23	13	§		1135±117	23.4	22.5
	8-Azaguanine	0/30	28	23	13		50	344± 32	24.1	22.9
	8-Azaguanine +Desoxypyridoxine	5/30	22	23	13	‖	50	209± 25	23.0	20.8
	Sacrificed controls	0/20	20	18				181± 24	23.5	
67	Controls	0/20	19	16	5			587± 61	21.5	22.1
♂	Desoxypyridoxine	0/10	10	16	5	175		459± 76	22.0	21.6
	8-Azaguanine	0/16	16	16	5		50	82± 11	22.2	22.5
	8-Azaguanine +Desoxypyridoxine	1/10	7	16	5	175	50	29± 4	21.7	20.3
95	Controls	0/10	10	20	8			1032±147	22.9	25.8
♂	8-Azaguanine	0/10	10	20	8		50	200± 28	20.6	19.9
	8-Azaguanine +Desoxypyridoxine	0/10	10	20	8	‖	50	103± 18	20.1	19.2
85	8-Azaguanine	6/30	24	25	14		50	959± 57	23.1	19.5
♂	8-Azaguanine +Desoxypyridoxine	5/30	25	25	14	175	50	512± 58	22.2	18.8
100	Controls	1/10	9	20	7			1420±251	21.1	20.9
♂	8-Azaguanine	1/10	9	20	7		50	220± 45	20.5	19.9
	8-Azaguanine +Desoxypyridoxine	0/10	10	20	7	175	50	105± 16	20.5	17.9
114	8-Azaguanine	0/10	10	24	7		50	398± 66	18.7	18.3
♂	8-Azaguanine +Desoxypyridoxine	0/10	10	24	7	175	50	195± 27	18.1	17.0

* DP = Desoxypyridoxine.
† 8-AG = 8-Azaguanine.
‡ 800 (×4), then 175 (×11).
§ 800 (×8), then 175 (×7).
‖ 175 (×4), then 150 (×8).

ferences in the two types of cells have been recorded (10). Of particular pertinence to the present discussion are the quantitative assays of the concentrations of members of the Vitamin B complex in normal and tumor tissue (2, 23). Since, for example, it has been demonstrated that pyridoxine is in lower concentration in certain tumors than in the analogous normal tissue, it was considered possible to inhibit the function of this vitamin completely, by means of an antagonist in cancer tissue, while minimally depressing the metabolic activities of the vitamin in normal tissues. This is a reasonable hypothesis, since metabolite inhibition by an anti-metabolite depends to a large degree upon the concentration of the metabolite. Ackermann and Potter have recently presented evidence which indicates that the same holds true for enzymes (1).

On the basis of the above hypothesis, a series of vitamin analogs were selected for chemotherapeutic trial in combination with 8-azaguanine on the 755 tumor. At this time, desoxypyridoxine is the only analog with which preliminary observations have been completed. The mechanism of action, whereby desoxypyridoxine in combination with 8-azaguanine inhibits tumor growth to a greater extent than is observed following treatment with either chemical compound alone, is not known. In support of the concept presented is the fact that desoxypyridoxine has been shown to be a pyridoxine antagonist (4, 21); however, a number

TABLE 2

EFFECT OF 8-AZAGUANINE AND PTEROYLGLUTAMIC ACID ON THE 755 TUMOR IN ♂ C57 MICE

Exp. no.	Group	No. animals dead/alive	No. tumors	Duration tumor growth (days)	Day therapy begun after transplantation PGA*	Day therapy begun after transplantation 8-AG†	Daily dosage (mg/kg) PGA	Daily dosage (mg/kg) 8-AG	Mean tumor weight ± standard deviation of mean (mg.)	Av. body weight (gm.) Beginning	Av. body weight (gm.) End
84	8-Azaguanine	1/20	28	34		14		50	830 ± 69	23.0	22.5
	PGA+8-AG	1/20	29	34	15	14	50	50	441 ± 81	23.8	22.0
105	8-Azaguanine	0/20	20	26		12		50	1159 ± 182	20.2	20.4
	PGA+8-AG	0/20	19	26	11	12	50	50	820 ± 69	20.3	20.1
76A	Controls	1/10	9	25					1770 ± 320	23.7	23.8
	PGA	4/10	6	25	7		50		2140 ± 431	21.6	24.7
	8-Azaguanine	0/10	10	32		8		50	522 ± 72	21.9	21.5
	PGA+8-AG	0/10	10	32	7	8	50	50	521 ± 84	23.9	22.2
76B	Controls	1/10	9	25					1770 ± 320	23.7	23.8
	PGA	5/10	5	25	7		37.5		1786 ± 358	22.1	24.0
	8-Azaguanine	0/10	10	32		8		50	522 ± 72	21.9	21.6
	PGA+8-AG	0/10	10	32	7	8	37.5	50	262 ± 37	23.7	23.1
	Sacrificed controls	0/10	10	8					36 ± 11	24.0	
72	8-Azaguanine	1/10	9	22		18		50	129 ± 11.1	17.7	18.7
	PGA+8-AG	2/10	8	22	12	18	37.5	50	49 ± 9.4	15.5	18.6

* PGA = pteroylglutamic acid.
† 8-AG = 8-azaguanine.

TABLE 3

EFFECT OF 8-AZAGUANINE AND PTEROYLGLUTAMIC ACID ON THE 755 TUMOR IN ♀ C57 MICE

Exp. no.	Group	No. animals dead/alive	No. tumors	Duration tumor growth (days)	Day therapy begun after transplantation PGA	Day therapy begun after transplantation 8-AG†	Daily dosage (mg/kg) PGA*	Daily dosage (mg/kg) 8-AG	Mean tumor weight ± standard deviation of mean (mg.)	Av. body weight (gm.) Beginning	Av. body weight (gm.) End
88	8-Azaguanine	1/29	28	34		14		50	905 ± 96.5	20.6	20.0
	PGA+8-AG	1/30	29	34	19	14	50	50	790 ± 61.6	20.9	21.5
104	8-Azaguanine	2/20	18	26		12		50	790 ± 84	18.2	18.5
	PGA+8-AG	0/20	20	26	11	12	50	50	590 ± 64	18.7	19.1
76	Controls	0/10	10	18					508 ± 122	19.6	19.8
	PGA	0/10	10	18	7		37.5		847 ± 78	19.7	20.5
	8-Azaguanine	0/10	10	18		8		50	67 ± 14.1	18.9	18.3
	PGA+8-AG	1/10	9	18	7	8	37.5	50	80 ± 6.7	18.2	19.7

* PGA = pteroylglutamic acid.
† 8-AG = 8-azaguanine.

TABLE 4

Effect of 7-Methyl Folic Acid and 8-Azaguanine on the 755 Tumor in C57 Mice

Exp. no.	Group	No. animals dead/alive	No. tumors	Duration tumor growth (days)	Day therapy begun after transplantation: 7-Methyl PGA	8-AG	Daily dosage (mg/kg): 7-Methyl PGA	8-AG	Mean tumor weight ± standard deviation of mean (mg.)	Av. body weight (gm.): Beginning	End
91 ♀	Controls	1/10	9	19					788 ± 111	17.7	18.4
	8-Azaguanine	0/10	10	19		7		50	278 ± 58	17.9	17.6
	7-Methyl PGA	0/10	10	19	7		*		693 ± 147	18.2	17.3
	7-Methyl PGA +8-Azaguanine	0/10	10	19	7	7	*	50	43 ± 4	18.5	16.8
95 ♂	Controls	0/10	10	20					1125 ± 147	22.9	23.8
	8-Azaguanine	0/10	10	20		8		50	200 ± 28	20.6	19.9
	7-Methyl PGA	0/10	10	20	8		†		1400 ± 18	22.6	23.1
	7-Methyl PGA +8-Azaguanine	1/10	9	20	8	8	†	50	67 ± 9	19.3	18.9
107 ♀	8-Azaguanine	1/10	8	22		8		50	446 ± 89	18.2	18.7
	7-Methyl PGA +8-Azaguanine	1/10	8	22	8	8	‡	50	169 ± 48	17.2	17.0
109 ♂	8-Azaguanine	0/10	10	22	8	8		50	386 ± 47	19.2	19.3
	7-Methyl PGA +8-Azaguanine	0/10	10	22	8	8	‡	50	220 ± 39	19.4	19.3
118 ♀	8-Azaguanine	2/10	8	33		7		50	785 ± 113	17.1	18.3
	7-Methyl PGA +8-Azaguanine	0/15	15	33	7	7	25	50	1117 ± 121	17.3	18.6

* 50 (×4), then 25 (×4).
† 25 (×4), then 25 (×4).
‡ 50 (×5), then 25 (×3).

TABLE 5

Effect of Vitamin B_{12} and 8-Azaguanine on the 755 Tumor in C57 Mice

Exp. no.	Group	No. animals dead/alive	No. tumors	Duration tumor growth (days)	Day therapy begun after transplantation: B_{12}	8-AG	Daily dosage: μgm/kg B_{12}	mg/kg 8-AG	Mean tumor weight ± standard deviation of mean (mg.)	Av. body weight (gm.): Beginning	End
67 ♂	Controls	0/20	10	16					587 ± 61	22.4	22.3
	B_{12}	0/10	10	16	5		*		618 ± 116	23.3	22.3
	8-Azaguanine	0/18	18	16		5		50	82 ± 11	22.2	22.5
	B_{12}+8-AG	1/10	9	16	5	5	*	50	37 ± 7	23.3	22.6
80 ♂	8-Azaguanine	6/30	24	28		15		50	1055 ± 99	21.6	20.6
	B_{12}+8-AG	0/30	28	28	14	15	200	50	1208 ± 78	21.9	19.9
74 ♂	Controls	1/10	9	18					888 ± 51	22.3	23.3
	B_{12}	0/10	10	18	8		1,250		630 ± 95	21.2	22.1
	8-Azaguanine	0/10	10	18		8		50	51 ± 7	21.4	21.1
	B_{12}+8-AG	0/10	10	18	8	8	1,250	50	96 ± 9	20.7	20.1
87 ♂	8-Azaguanine	5/30	25	28		15		50	1404 ± 98	21.4	20.4
	B_{12}+8-AG	8/30	22	28	14	15	2,000	50	1149 ± 93	21.5	20.0
91 ♀	Controls	1/10	9	19					788 ± 111	17.7	18.4
	8-Azaguanine	0/10	10	19		7		50	278 ± 58	17.9	17.6
	B_{12}+8-AG	0/10	10	19	4	7	1,250*	50	85 ± 14	18.5	19.2
95 ♀	Controls	0/10	10	20					1125 ± 147	22.9	23.8
	8-Azaguanine	0/10	10	20		8		50	200 ± 28	20.6	19.9
	B_{12}+8-AG	0/10	10	20	5	8	† ‡	50	93 ± 15	20.3	20.4
106A ♂	8-Azaguanine	0/10	10	22		8		50	386 ± 47	19.2	19.3
	B_{12}+8-AG	0/10	10	22	6	8	1,000*	50	240 ± 42	14.1	19.3
109B ♂	8-Azaguanine	0/10	10	22		8		50	386 ± 47	19.2	19.3
	B_{12}+8-AG	0/10	10	22	6	8	500*	50	285 ± 30	19.7	19.3
116 ♀	8-Azaguanine	2/10	8	33		7		50	785 ± 113	17.1	18.3
	B_{12}+8-AG	1/15	14	33	7	7	1,000*	50	983 ± 112	17.9	19.5

* 25 (×6), then 250 (×8).
† = B_{12} dose given ½ hour before 8-azaguanine dose.
‡ 1250 (×9), then 900 (×7).

of other possibilities must be considered and studied before accepting the mechanism of action as due to competitive inhibition of pyridoxine function.

Microbiological studies with pteroylglutamic acid and vitamin B_{12} suggest their importance in nucleic acid metabolism (14, 24). Since other studies from this laboratory have shown an effect of 8-azaguanine on mitotic activity in tumor cells (15), it was considered possible that folic acid and vitamin B_{12} might prevent the carcinostatic effect of the guanine analog. As was shown in the "Results," these vitamins not only failed to reverse the 8-azaguanine action but, rather, led to a greater inhibition of tumor growth. 7-Methyl folic acid, a relatively weak antagonist of pteroylglutamic acid, was selected primarily because of its structural similarity to folic acid.

The mechanism by which pteroylglutamic acid, its 7-methyl analog, or vitamin B_{12}, when combined with 8-azaguanine, lead to greater tumor inhibition than that observed following 8-azaguanine alone is not known. A possible explanation for the folic acid effect is that one of its degradation products, 2-amino-4-hydroxy-6-formylpteridine, inhibits a liver enzyme system *in vitro* which deaminates 8-azaguanine.[1] The resulting 2,4-dihydroxytriazolopyrimidine is inactive as a carcinostatic agent (8). This finding may be comparable to that of Williams and Elvehjem that "the metabolism of guanine (presumably via guanase to xanthine . . .)" is decreased by folic acid (22). Whether this possible explanation of the mechanism involved in the folic acid observations is pertinent to the studies with 7-methyl folic acid or vitamin B_{12} is not known.

The mechanism of action of the combinations utilized in these experiments is of particular interest, as it may provide some insight into the metabolism of the tumor cells. The most obvious explanation for the observed enhancement of carcinostasis would be that the administration of two chemical compounds caused greater toxicity to the host than either drug alone and that the tumor inhibition was merely a reflection of over-all toxicity. The evidence does not support this mechanism, however. It can be seen from the results that in the great majority of instances there was either no weight loss or a minimal loss in the animals, and, therefore, on the reasonable assumption that loss of body weight constitutes a measure of general toxicity, there was no significant deleterious effect of the combinations on the host. Furthermore, initial trials with a number of other vitamin analogs in combination with 8-azaguanine have failed to show an enhancement of tumor inhibition, thus lending circumstantial support to our conviction that the reported results are specific rather than nonspecific.

[1] A. Graff, J. Kream, and A. Gellhorn, unpublished results.

SUMMARY AND CONCLUSIONS

The effect of combinations of chemical compounds in cancer chemotherapy has been investigated. By the use of the guanine analog, 5-amino-7-hydroxy-1*H*-*s*-triazolo (*d*) pyrimidine, together with either desoxypyridoxine, pteroylglutamic acid, 7-methyl pteroylglutamic acid, or vitamin B_{12}, it has been shown that the growth of a transplantable carcinoma of the breast in mice can be inhibited to a greater extent by a combination than by any one of the drugs alone. Evidence has been presented to show that the combinations are not acting by causing host toxicity, for the observed effects are associated with minimal to no weight loss. The possible mechanisms of action are discussed.

It is recognized that in no instance did a combination eradicate the tumor being treated; however, the possibility of utilizing more effective agents in combination or of increasing the number of active chemical compounds in combination offers an attractive approach to achieve this end.

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THE EFFECT OF VITAMIN B_{12} ADMINISTRATION ON JAPANESE CHILDREN[1]

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Since the isolation of crystalline vitamin B_{12} (B_{12}) from liver many reports have appeared during the past several years on the supplemental effect of the vitamin on the growth of children in U.S.A. and in some other countries (1). The duration of the studies was from 4 weeks to 2 years, and the number of children subjected to individual study was from 9 to 418. The status of the subjects in each investigation was not the same, and positive results were obtained in some of the studies but not in all of them. The studies have been carried out in different parts of the world, but never in Japan.

Observations on the effect of supplementing B_{12} to the diet of a large number of Japanese children aged 3 to 14 years were conducted by the authors representing the Vitamin B Research Committee of Japan from 1959 to 1961 with the aid of Merck, Sharpe and Dohme Co., Inc. The children were chosen from four different areas: Tokyo and Osaka as large cities, Tokushima as a mountain area, and Hirosaki as a rural area. The children in both the large cities were residing either in orphanages or nurseries. The children in Tokushima and Hirosaki were attending elementary schools from their individual homes.

Before and after the period of administration of B_{12}, the height and weight of all the children were carefully measured; B_{12} content in whole blood, and hemoglobin value were also determined. The children were divided into the test (A) and the control groups (B), and 25 μg of cyanocobalamin per day was given orally in a tablet form to the former and a placebo to the latter.

The method of determination of B_{12} in whole blood was first carefully studied for nearly a year by a study group consisting of the members from four different areas together with the members of the Vitamin B Research Committee who had been working on the microbiological assay of B_{12} in blood. They set up the standard method (2), which was used for this work.

[1] Cooperative study of the Vitamin B_{12} Study Group of the Vitamin B Research Committee, N. Shimazono being a chairman
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After the end of the observation period, the difference between the means of the data of group A and those of group B was statistically analysed for each regional group, and also for the age groups as mentioned below (Table II). This paper deals with the experimental procedure for the study and the results of the observations.

MATERIALS AND METHODS

1. *Experimental Subjects*

The numbers of boys and girls included in this experiment in different parts of Japan are listed in Table I together with the names of the institutions where they resided. They were all clinically normal children and their weight and height were measured at the beginning of the experimental period. The children were divided into two equivalent group A and B on the basis of age, sex and size.

TABLE I

Number of the Experimental Subjects Chosen from Different Areas

Area	Age	Subjects A ♂	Subjects A ♀	Subjects B ♂	Subjects B ♀	Name of Institute
Hirosaki	6–8	17	0	18	0	
	9–11	35	0	35	0	
Tokushima	10–11	24	26	24	26	
Osaka	12–13	21	17	25	25	Hakuaisha
Tokyo	3–5	8	5	4	3	Ninomiya
	6–8	12	9	8	7	Ichinomiya
	9–11	21	17	21	13	Futatsubashi
	12–14	4	5	5	3	Futatsubashi
	9–11	15	13	17	11	Kurume
	12–14	3	5	5	3	Kurume
Total		160	97	162	91	
		257		253		
		510				

The total number of children were classified into four groups according to age: 3–5, 6–8, 9–11, and 12–14 years, as summarized in Table II. Group A (test

TABLE II

Total Number of the Experimental Subjects in Age Group

Age	Group A ♂	Group A ♀	Group A Total	Group B ♂	Group B ♀	Group B Total	Total
3–5	8	5	13	4	3	7	20
6–8	29	9	38	26	7	33	71
9–11	95	59	154	97	50	147	294
12–14	26	27	53	35	31	66	119
Summation	160	97	257	162	91	253	510

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group) consisted of 160 boys and 97 girls, and group B (control group) 162 boys and 91 girls. The total number was 510 at the beginning of the experiment; some of them dropped out of the experiment, and at the end of the whole experimental period of six months and one year, respectively, the data of only 431 and 400 children could be used for the statistical analysis.

The children received the regular institutional diet or their home diet. The nutritional values are shown in Table III.

Table III

Average Nutritional Intake of Separate Groups in Different Areas

Area	Sex	Calories	Protein	Fat	Animal protein	Ca	Fe	Vitamin A	Vitamin B_1	Vitamin B_2	Vitamin B_{12}	Vitamin C
		Cal	*g*	*g*	%	*g*	*mg*	I.U.	*mg*	*mg*	*μg*	*mg*
Hirosaki	♂	1915	63.5	18	37.1	0.28	13.1	677	0.8	0.54	1.16	39
Tokushima (10–11)	♂	(2100) 2159	(70) 59	13	20.0	(0.6) 0.33	(10) 11.7	(2000) 341	(1.0) 1.5	(1.0) 0.58	(1.0) 1.08	(65) 42
	♀	(2000) 2038	(75) 59	14.7	20.5	(0.7) 0.29	(10) 12.0	(2000) 357	(0.9) 1.5	(0.9) 0.59	1.02	(65) 46
Osaka (12–13)	♂	(2500) 2358	(95) 76	25.2	25.2	(0.9) 0.61	(12) 12.9	(2000) 1855	(1.2) 1.1	(1.2) 0.85	1.60	(80) 65
	♀	(2400) 2171	(85) 73	26.1	28.0	(0.8) 0.57	(12) 11.9	(2000) 1826	(1.1) 1.1	(1.1) 0.86	1.64	(75) 72
Tokyo: Ninomiya (3–5)	♂	(1550)	(50)			(0.4)	(8)	(1200)	(0.7)	(0.7)		(40)
	♀	(1400) 1656	(45) 57	34	47.0	0.56	6.9	3542	1.8	1.7	1.36	60
Tokyo: Ichinomiya (6–8)	♂	(1850)	(55)			(0.5)	(9)	(1500)	(0.9)	(0.9)		(50)
	♀	(1700) 2347	(50) 78	28	33.0	0.49	12.0	1876	1.0	1.0	1.11	89
Tokyo: Futatsubashi (9–14)		2035	73	28	38.0	0.47	12.6	1722	1.3	1.0	1.45	77
Kurume (9–14)		2473	88	47	40.3	0.68	20.0	2926	1.4	1.3	2.16	98

Nutritional allowances are shown in ( ).

2. *Administration of B_{12}*

B_{12} tablets and placebos were furnished by Merck, Sharpe and Dohme and Co., Inc. One tablet containing 25 μg of cyanocobalamin was given daily to the children of group A, and a placebo to those of group B for one year.

3. *Determination of B_{12} Content in Blood*

The blood samples were withdrawn by venipuncture in tubes with heparin and shaken thoroughly. They were then treated according to the standard method (2), as shown in Diagram, to extract B_{12}. The extracts were stored in a refrigerator and microbiologically analysed within two weeks.

The commercially available Nissan medium was used for the microbiological assay of B_{12}. The assays were carried out using *Lactobacillus leichmannii* ATCC 4797 or 7830.

DIAGRAM

The Procedure of B_{12} Extraction from Whole Blood

Two ml of whole blood with heparin
↓ 0.2 *M* acetate buffer (pH 4.5), 2 ml; 50 mg% KCN, 0.1 ml; and water, 5 ml
Heat for 30 min in a boiling water bath
↓ Cool off, add 10% metaphosphoric acid, 0.2 ml; and make up to 25 ml with water
Centrifuge (3,000 rpm for 15 min)
↓
Supernatant, 10 ml
↓ Adjust to pH 7 with NaOH, and make up to 20 ml with water
Use for microbiological assay

4. Nutritional Intake of the Children

The nutritional intake of the separate groups of the children in different areas is shown in Table III. Although protein intake was lower than its nutritional allowance, the amount of calories taken was just within the value. The calculated amount of daily intake of B_{12} from their diets was more than 1 μg, the value which is assumed to be the requirement for an adult. The main sources of B_{12} were dried fish with viscera and shell fish, and the particularly high figures in Kurume special school were attributed to the daily intake of 180 ml of milk as an extra nutriment.

RESULTS

The B_{12} content of the whole blood of the children was determined at the beginning of the experimental period (before the start of the vitamin administration), and then at 2 to 2.5 month intervals. The data of six such observations from I to VI are shown in Table IV.

TABLE IV

Dates of the Observations

	Observation time	Dates of observations for all groups
I	Before the start of B_{12} or placebo administration	Feb. 4, 1959–Aug. 20, 1959
II	After 2.5 month administration	April 17, 1959–Oct. 30, 1959
III	After 4.6 month administration	June 3, 1959–Jan. 19, 1959
IV	After 7.5 month administration	Sept. 25, 1959–March 21, 1960
V	After 9.5 month administration	Nov. 25, 1959–June, 1960
VI	After 12 month administration	Feb. 4, 1960–Aug. 20, 1960

All the children were also weighed and measured at regular intervals at I, III, and VI of Table IV. The means of the initial weight and height of the children of each group in different areas and those of the increase after one year are shown in Tables V and VI. The means of the B_{12} values of the whole blood of the children aged 3 to 8 years and 9 to 14 years are shown in Tables VII and VIII respectively.

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TABLE V

Means of Initial Weight of Children and Weight Increase after One Year

Sex	Age	Group A: Initial weight (I)	Group A: Weight gain $\bar{x}$ (VI − I)	Group A: N_1	Group B: Initial weight (I)	Group B: Weight gain $\bar{x}$ (VI − I)	Group B: N_2
		kg	kg		kg	kg	
♂	3-5	14.0	1.6	5	17.9	1.5	2
	6-8	22.8	1.9	24	22.6	1.6	21
	9-11	27.7	2.6	77	27.7	2.8	79
	12-14	35.0	5.1	23	33.9	5.1	30
♀	3-5	15.8	3.1	4	15.2	2.3	3
	6-8	22.6	1.7	5	22.1	2.1	4
	9-11	25.7	3.5	44	25.5	3.4	36
	12-14	38.6	5.4	22	39.4	4.7	32

TABLE VI

Means of Initial Height of Children and Height Increase after One Year

Sex	Age	Group A: Initial height (I)	Group A: Height increase $\bar{x}$ (VI − I)	Group A: N_1	Group B: Initial height (I)	Group B: Height increase $\bar{x}$ (VI − I)	Group B: N_2
		cm	cm		cm	cm	
♂	3-5	92.6	6.3	5	100.6	5.3	2
	6-8	118.7	5.1	24	118.7	5.0	21
	9-11	129.7	5.3	77	129.5	5.1	79
	12-14	140.7	6.6	23	139.3	7.1	30
♀	3-5	98.7	8.0	4	98.2	6.3	3
	6-8	120.0	4.4	5	117.8	4.4	4
	9-11	126.9	6.0	44	127.8	6.1	36
	12-14	142.1	4.7	22	143.3	3.7	32

TABLE VII

Means of B_{12} Values of Whole Blood of Subjects Aged 3 to 8 Years

Group	Sex	Age		B_{12} (mμg/ml) in whole blood: I	II	III	IV	V	VI
A	♂ ♀	3-5	*m*	0.36	—	0.65	—	—	0.42
			σ	0.16	—	0.22	—	—	0.07
			N	13	—	13	—	—	8
	♂ ♀	6-8	*m*	0.44	—	0.62	—	—	0.46
			σ	0.13	—	0.37	—	—	0.39
			N	21	—	21	—	—	11
	♂	6-8	*m*	0.35	0.27	0.29	0.42	0.34	0.50
			σ	0.07	0.13	0.50	0.16	0.12	0.52
			N	17	16	8	16	17	16
B	♂ ♀	3-5	*m*	0.26	—	0.56	—	—	0.36
			σ	0.09	—	0.30	—	—	0.09
			N	7	—	7	—	—	3
	♂ ♀	6-8	*m*	0.52	—	0.47	—	—	0.46
			σ	0.34	—	0.33	—	—	0.39
			N	16	—	16	—	—	7
	♀	6-8	*m*	0.27	0.19	0.28	0.25	0.35	0.23
			σ	0.05	0.08	0.12	0.07	0.09	0.07
			N	14	17	17	18	17	18

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TABLE VIII

Means of B_{12} Values of Whole Blood from All Subjects Aged 9 to 14 Years

Group	Sex	Age		B_{12} (mcg/ml) in whole blood I	II	III	IV	V	VI
A	♂	9–11	*m*	0.337	0.395	0.448	0.247	0.383	0.376
			σ	0.121	0.142	0.200	0.186	0.158	0.190
			N	84	60	84	60	60	60
		12–14	*m*	0.393	0.456	0.374	0.342	0.420	0.491
			σ	0.154	0.153	0.211	0.136	0.202	0.183
			N	27	21	27	21	21	21
	♀	9–11	*m*	0.357	0.494	0.414	0.464	0.468	0.553
			σ	0.137	0.161	0.193	0.132	0.112	0.262
			N	51	33	52	33	33	33
		12–14	*m*	0.386	0.521	0.550	0.489	0.469	0.558
			σ	0.138	0.132	0.182	0.205	0.237	0.398
			N	22	19	22	19	19	19
B	♂	9–11	*m*	0.337	0.295	0.339	0.300	0.318	0.315
			σ	0.127	0.118	0.133	0.115	0.097	0.219
			N	84	60	84	60	60	60
		12–14	*m*	0.409	0.362	0.450	0.342	0.277	0.296
			σ	0.173	0.125	0.158	0.123	0.103	0.290
			N	32	26	32	26	26	25
	♀	9–11	*m*	0.375	0.365	0.393	0.308	0.348	0.377
			σ	0.141	0.320	0.089	0.092	0.081	0.290
			N	47	31	47	31	31	31
		12–14	*m*	0.400	0.433	0.509	0.422	0.322	0.396
			σ	0.120	0.088	0.168	0.172	0.148	0.174
			N	33	27	33	27	27	27
Summation			*m*	0.361					
			σ	0.135					
			N	380					

The differences between the means of the B_{12} values of the whole blood, the body weight gain and the height increase of the children in groups A and B from I to III and from I to VI were statistically analysed. As shown in Table IX, most of the groups studied did not show any statistically significant differences in body weight gain and height increase after the six month or one year administration of B_{12}, but some groups showed a statistically significant difference in the increase of the B_{12} value of the whole blood.

The distribution of the numbers of the blood samples in which the B_{12} value was increased, decreased or remained unchanged after the administration of the vitamin or placebo is shown in Table X. The number of blood samples with an increase of more than 20 % in the B_{12} value of the whole blood after 6 or 12 month administration was greater in group A than in group B. The χ^2 test showed a highly significant difference between the two groups.

The means of the B_{12} values of the whole blood at regular intervals from I to VI of the boys and girls aged 9 to 14 years as shown in Table VIII were plotted in Fig. 1. As shown in the figure, the mean value of B_{12} in the whole blood of group A was higher than that of group B after 3 month administration, and this difference was maintained throughout the year.

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TABLE IX

Results of the Statistical Analyses for the Data of Individual Groups

Area	Age	Sex	I–III (after 6 months) 3A–3B: Number of children	Bre value of whole blood	Height increase	Weight gain	I–VI (after 12 months) 3A–3B: Number of children	Bre value of whole blood	Height increase	Weight gain
Hirosaki	6-8	♂	A 10 B 14	[illegible]	[illegible]	—◎	A 17 B 14	□	□	×
	9-11	♂	A 33 B 34	[illegible]	□	—×	A 33 B 34	◎	×	□
Tokushima	9-11	♂	A 24 B 24	◎	×	×	A 24 B 26	□	□	×
	9-11	♀	A 24 B 26	□	□	×	A 26 B 26	◎	□	□
Osaka	12-14	♂	A 21 B 25	◎	—◎	—◎	A 21 B 25	◎	—×	—×
	12-14	♀	A 17 B 25	×	×	×	A 17 B 25	×	×	×
Tokyo	3-5	♂	A 8 B 4	×	□	×	A 4 B 2	×	×	□
	3-5	♀	A 5 B 3	×	×	×	A 4 B 1	◎	×	□
	6-8	♂	A 12 B 8	□	□	×	A 6 B 3	—◎	×	×
	6-8	♀	A 9 B 7	□	×	×	A 4 B 4	□	—×	—×
	9-11	♂	A 19 B 18	◎	—×	□	A 13 B 13	◎	□	□
	9-11	♀	A 14 B 10	◎	—×	—×	A 9 B 4	□	□	□
	12-14	♂	A 2 B 5	—×	—×	×	A 2 B 5	×	×	□
	12-14	♀	A 3 B 5	□	—×	×	A 3 B 5	◎	—×	×
Tokyo (Kurume)	9-11	♂	A 15 B 17	×	×	—×	A 6 B 8	—×	×	×
	9-11	♀	A 13 B 11	□	□		A 9 B 5	◎	×	□
	12-14	♂	A 3 B 5	—×	×	×	—	—	—	—
	12-14	♀	A 5 B 3	×	×	□	A 2 B 2	×	×	□
Total			**481**				**400**			

◎, highly significant; ○, significant; ×, insignificant; □, population variance of random difference between Group A and B is significant; Δ, III–I or VI–I of each item.

The statistical analyses for B_{12} values of the whole blood between the two groups were made on the children aged 9 to 14 years, 380 boys and girls at the observation time I (before starting the vitamin administration), and at III (after 6 month administration), 276 boys and girls at VI (after one year administration). As shown in Table XI, the difference between the two groups A and B was not

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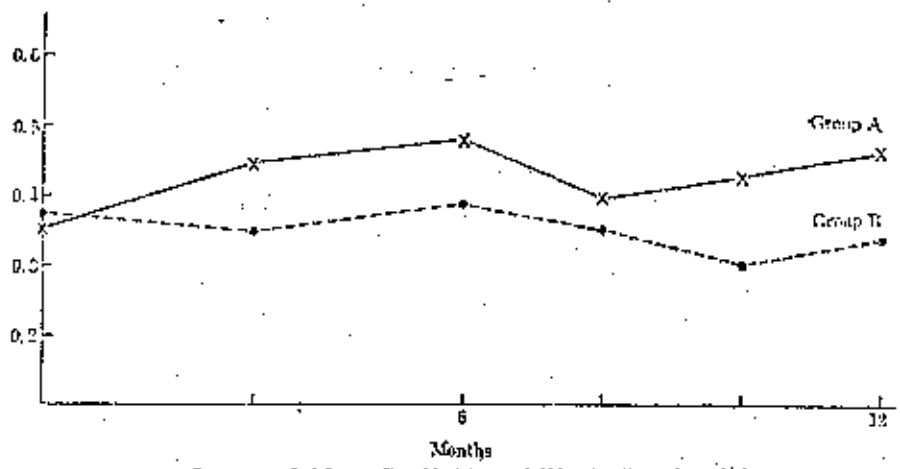

FIG. 1 *Curves of Mean B_{12} Values of Whole Bloods of Children Aged 9 to 14 Years During the Experimental Period*

TABLE X

Number of Cases in which B_{12} Value Increased, Decreased or Remained in the Blood after the Vitamin or Placebo Administration

Area	Age	Sex	I–III After 6-month administration						I–VI After 12-month administration					
			A			B			A			B		
			+	0	−	+	0	−	+	0	−	+	0	−
Hirosaki	6–8	♂	6	1	2	4	7	4	10	4	2	1	7	6
	9–11	♂	16	13	5	14	11	7	22	12	19	5	11	19
Tokushima	9–11	♂	19	4	10	10	10	4	15	8	0	9	12	3
	9–11	♀	13	13	0	4	18	4	15	11	0	7	14	5
Osaka	12–14	♂	19	1	1	13	5	7	14	5	2	1	12	12
	12–14	♀	13	4	0	15	8	2	9	7	1	6	11	8
Tokyo	3–5	♂♀	12	1	0	6	1	0	7	2	0	0	3	0
	6–8	♂♀	13	7	1	2	5	9	1	3	6	0	1	6
	9–11	♂	11	8	0	5	8	5	1	7	4	0	4	8
	9–11	♀	12	2	0	6	3	2	4	5	0	0	2	2
	12–14	♂	2	0	0	1	3	1	1	1	0	2	1	2
	12–14	♀	2	1	0	1	4	0	1	1	1	1	0	1
Tokyo (Kurume)	6–8	♂♀	2	1	1	0	1	2	3	0	0	0	6	1
	9–11	♂	9	5	5	8	9	2	3	1	2	8	1	0
	9–11	♀	6	1	6	4	6	5	8	1	0	2	2	1
	12–14	♂	1	1	1	3	1	1	2	0	0	1	1	0
	12–14	♀	1	3	1	2	0	1	0	0	0	0	0	1

$>\sqrt{20\%}$, +; 20% to −20%, 0; $<$ −20%, −.

χ^2 Test

	I–III			
	+	0	−	Total
A	157	66	23	246
B	98	130	56	254
Total	255	196	79	500

$\chi^2_0 = 34.3 > \chi^2$ (p = 0.001)

	I–VI			
	+	0	−	Total
A	116	68	37	221
B	43	82	78	203
Total	159	150	115	424

$\chi^2_0 = 48.4 > \chi^2$ (p = 0.001)

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significant at the observation time I, but highly significant at III and VI. It was also found that the average value of B_{12} in the whole blood of normal children aged 8 to 14 years was 0.261 mμg/ml (σ: 0.175 mμg).

TABLE XI

Mean Value of B_{12} in Whole Blood of Children Group A and B (aged 9 to 14 years)

		I	III	VI
		Before starting administration	After 6-month administration	After 12-month administration
		μg/ml	μg/ml	μg/ml
Group A	m	0.252	0.523	0.465
	σ	0.133 (N: 184)	0.205 (N: 184)	0.241 (N: 130)
Group B	m	0.268	0.387	0.340
	σ	0.141 (N: 196)	0.153 (N: 196)	~~0.340~~ (N: 149)
Significant difference between Group A and B		No F_0 1.28<F 3.87 (α 0.05)	Yes F_0 52.0>F 6.72 (α 0.01)	Yes F_0 21.5>F 6.74 (α 0.01)

TABLE XII

Means of Hemoglobin Values of Blood from All the Subjects Aged 9 to 14 Years

Group	Sex	Age		Hemoglobin in blood I	VI	VI-I	Increased or decreased
					g/100 ml		%
A	♂	9–14	m	12.05	11.97	0.09	0.45
			σ	0.88	0.80	4.63	7.03
			N	58	58	58	58
		12–14	m	12.07	12.95	0.84	7.65
			σ	1.04	0.62	1.09	10.03
			N	21	21	21	21
	♀	9–11	m	12.39	12.38	−0.01	−0.27
			σ	1.04	0.85	0.67	10.20
			N	26	26	26	26
		12–14	m	12.33	12.80	0.53	3.71
			σ	1.57	0.86	1.33	10.86
			N	17	17	17	17
B	♂	9–11	m	12.19	12.16	−0.05	−0.10
			σ	0.95	0.78	0.78	5.99
			N	59	59	59	59
		12–14	m	12.90	13.14	1.11	9.92
			σ	0.88	0.93	0.87	7.74
			N	25	25	25	25
	♀	9–11	m	12.6	12.38	−0.22	−1.50
			σ	1.10	0.27	0.95	6.90
			N	26	26	26	26
		12–14	m	12.35	12.94	0.59	6.08
			σ	1.10	0.96	1.37	11.55
			N	25	25	25	25
Summation			m	12.22			
			σ	1.25			
			N	257			

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Since the number of children aged 3 to 8 years was small, the means of the B_{12} values of the whole blood as shown in Table VII were not statistically analysed. The means of hemoglobin values of the boys and girls aged 9 to 14 years did not show any significant difference between group A and B at the time I and VI respectively as shown in Table XII.

SUMMARY

The studies of the effect of vitamin B_{12} administration to a large number of Japanese children in different areas were carried out from 1959 to 1961 by the B_{12} Study Group of the Vitamin B Research Committee of Japan.

It was observed that oral administration of 25 μg of B_{12} daily to the children aged 3 to 14 years over a period of one year did not show any definite promoting effect on the growth of the children. However, in the statistical analyses for B_{12} values of the whole blood, 276—330 children aged 9 to 14 years showed a significant increase in the B_{12} level when B_{12} was supplemented for 6 months to one year.

The average normal value of B_{12} in the whole blood of Japanese children aged 9 to 14 years was found to be 0.361 $m\mu g$ ($\delta = 0.135$ $m\mu g$) ml.

As the children in this study were taking daily around 1 μg or more of B_{12} from their diet, the findings mentioned above do not exclude the possibility of growth-promoting effects on children when the intake of the vitamin is not sufficient.

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Effect of *p*H changes on the binding of vitamin B_{12} by intrinsic factor

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SYNOPSIS The binding of vitamin B_{12} by human gastric juice has been found to be *p*H dependent. Maximum binding occurs between *p*H 6·5 and 10. Outside this *p*H range the vitamin B_{12}-binding ability of human gastric juice decreases and at *p*H below 2 or above 12·2 this drops sharply to about 10 to 15% of the maximum. Three commercial hog intrinsic factors have been found to give a similar response to *p*H changes. The *p*H-dependent binder in human gastric juice has been shown to be intrinsic factor by the addition of intrinsic factor-blocking antibody. About 10% of vitamin B_{12} bound by human gastric juice is not bound by intrinsic factor and is not *p*H dependent. The reduction in the vitamin B_{12}-binding capacity of human gastric juice induced by an adverse *p*H is reversed by neutralization. The physiological and clinical significance of these observations is discussed and their relevance to various procedures *in vitro* noted.

Intrinsic factor is now generally recognized as a substance which is necessary for the normal absorption of vitamin B_{12} in man. It has been shown to be secreted by the parietal cells of the stomach but its chemical identity is still not certain although it has been reported to be a glycoprotein with a molecular weight equal to some multiple of 50,000 (Grasbeck, 1969). The exact mode of action of intrinsic factor in the normal absorption of vitamin B_{12} is not known, but it is known to be a very potent binder of vitamin B_{12}. The mechanism by which intrinsic factor binds vitamin B_{12} and what part of the intrinsic factor molecule is involved are not known.

In this paper we report the results of our studies on the effect of *p*H on the ability of human gastric juice intrinsic factor to bind vitamin B_{12}. This problem was studied because intrinsic factor has been used in a number of unbuffered assay procedures *in vitro* for serum vitamin B_{12} (Lau, Gottlieb, Wasserman, and Herbert, 1965; Raven, Robson, Walker, and Barkhan, 1969), serum intrinsic factor blocking antibody (Gottlieb, Lau, Wasserman, and Herbert, 1965; Ungar, 1967), and in the estimations of intrinsic factor content of human gastric juice (Ardeman and Chanarin, 1963; Gottlieb *et al.* 1965). The *p*H of the various parts of the alimentary tract may also be important in the absorption of B_{12} *in vivo* in normal and abnormal states.

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Materials

^{57}CO-VITAMIN B_{12} (^{57}CO-B_{12})

This was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. Preparations of specific activities from 10 μCi/μg to 20 μCi/μg were used. Each batch was diluted to a concentration of 1,000 pg/ml and stored at −20°C in 10 ml aliquots.

HUMAN GASTRIC JUICE

Human gastric juice was obtained from a large number of non-achlorhydric patients undergoing histamine test meals. Mucus and cellular debris was removed by centrifugation and the supernatant was then depepsinized by adjusting it to *p*H 10 with NaOH, incubated at room temperature for 20 minutes, neutralized with HCl, and stored at −20°C until required. The pooled human gastric juice was found to have a vitamin B_{12}-binding capacity of about 20 ng/ml at *p*H 7·4. Its intrinsic factor activity was demonstrated *in vivo* by its ability to correct vitamin B_{12} malabsorption in two patients with proven pernicious anaemia. As a working solution, human gastric juice was diluted so that the amount used would maximally bind about 80% of the total ^{57}Co-B_{12} added in the various test procedures.

INTRINSIC FACTOR BLOCKING ANTIBODY

Intrinsic factor blocking antibody serum was obtained

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from a pernicious anaemia patient. The intrinsic factor blocking antibody activity in this patient's serum was detected and assayed by a previously described method (Ungar, 1967). It was found to have a titre of 57 ng units/ml.

HUMAN GASTRIC JUICE TREATED WITH INTRINSIC FACTOR BLOCKING ANTIBODY

One ml of human gastric juice was mixed with 1 ml of intrinsic factor blocking antibody serum, then incubated at room temperature for 10 minutes so that all its intrinsic factor activity was blocked. It was then diluted with distilled water to the required concentration for each experiment.

COMMERCIAL HOG INTRINSIC FACTOR

The following three different commercial hog intrinsic factor preparations were tested.

Nutritional biochemical intrinsic factor 10 x

This was obtained from the Nutritional Biochemicals Corp, Cleveland, Ohio. A stock aqueous solution of 0·075 g% was made up and stored at −20°C. The vitamin B_{12}-binding capacity of this solution was 200 ng/ml at *p*H 7·4.

National Formulary intrinsic factor

This was obtained from the National Formulary, American Pharmaceutical Association, Washington, D.C. A stock aqueous solution of 0·15g% was made up and stored at −20°C. The vitamin B_{12}-binding capacity of this solution was 100 ng/ml at *p*H 7·4.

Lederle intrinsic factor

This was obtained from the Lederle Laboratories Division, American Cyanamid Comp., Pearl River, N.Y. A saturated aqueous stock solution was made up and stored at −20°C. The vitamin B_{12}-binding capacity of this solution was found to be 57 ng/ml at *p*H 7·4.

ALBUMIN-COATED CHARCOAL

This was prepared by mixing equal volumes of a 5% aqueous suspension of Norit A neutral pharmaceutical grade decolorizing carbon (Amend Drug and Chemical Co., Inc., N.Y.), and a 1% aqueous solution of bovine albumin (Armour fraction V, Armour Pharmaceutical Company Ltd., England). Albumin-coated charcoal was prepared freshly for each assay since we have previously noted an appreciable deterioration on storage (O'Neill, Streeter, and Shum, 1968).

BUFFER SOLUTION

Sorensen's phosphate buffer was used. *p*H values outside the range of this buffering system were obtained using different concentrations of NaOH and HCl.

Methods

EFFECT OF *p*H ON VITAMIN B_{12} BINDING BY HUMAN GASTRIC JUICE AND HOG INTRINSIC FACTOR

Experiments were set up according to the protocol shown in Table I to cover a wide *p*H range. The radioactive ^{57}Co-B_{12} solution was brought to the required *p*H and then the intrinsic factor source was added. This was allowed to incubate at room temperature for 30 minutes and then the albumin-coated charcoal was added. This reagent acts as a 'molecular sieve' and adsorbs free vitamin B_{12} but not the bound form (Lau *et al*, 1965). The coated charcoal was removed by centrifugation and the radioactivity in the supernatant determined. The counts from the control series were counts due to unbound ^{57}Co-B_{12} not taken down by the coated charcoal at the various *p*H levels (usually about 1-2% of the total counts added, irrespective of the *p*H). The difference between the test count and the control count was a direct measure of the amount of ^{57}Co-B_{12} bound to intrinsic factor at each *p*H. In each experiment the highest 'net count', ie, test count minus control count, was taken as 100% binding capacity; the other net counts from the same experiment were then expressed as a percentage of this highest net count.

EFFECT OF *p*H ON VITAMIN B_{12} BINDING BY HUMAN GASTRIC JUICE TREATED WITH INTRINSIC FACTOR BLOCKING ANTIBODY

This was carried out as in Table I with two additional series. One series had human gastric juice treated

	Test Series	*Control Series*
^{57}Co-B_{12} (1,000 pg/ml)	1·0 ml	1·0 ml
Buffer solution/N NaOH/N HCl	8·5 ml	8·5 ml
Intrinsic factor source (human gastric juice or hog intrinsic factors)	0·5 ml	—
Distilled water	—	0·5 ml
	Mix, stand at room temperature for 30 minutes; take 5·0 ml aliquot for *p*H determination	
Coated charcoal	3·0 ml	3·0 ml
	Mix, centrifuge at 4,000 rpm (3,500g) for 20 minutes; take 5·0 ml of clear supernatant for radioactivity determination[1]	
Counting result	Test count	Control count

Table I *Protocol for experiments*

[1]This was carried out in an automatic Nuclear-Chicago, well-type scintillation counter.

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with intrinsic factor blocking antibody in place of the intrinsic factor source; the other series had intrinsic factor blocking antibody serum instead of the intrinsic factor source. Subtracting the supernatant radioactivity of the intrinsic factor series from the supernatant radioactivity of the human gastric juice treated with intrinsic factor blocking antibody series gave a measure of the ^{57}Co-B_{12} bound to the non-intrinsic factor component of human gastric juice at each *p*H.

REVERSIBILITY OF *p*H EFFECT ON VITAMIN B_{12} BINDING BY HUMAN GASTRIC JUICE

This was performed as in Table I with the following modifications. After the human gastric juice had been incubated with the ^{57}Co-B_{12} mixture for 30 minutes at the various *p*Hs, each solution of the test series was divided into two equal portions. Portion 1 was neutralized and an equal volume of distilled water added to portion 2. Further distilled water was then added so that all tubes of both portions had the same final volume. The *p*H of all tubes was then determined. After 30 minutes' incubation at room temperature coated charcoal was added. The net counts of each tube from portion 1 were taken as 100% binding capacity and the net counts of portion 2 from the corresponding tubes were expressed as a percentage of this.

Results

EFFECT OF *p*H ON VITAMIN B_{12} BINDING BY HUMAN GASTRIC JUICE AND HOG INTRINSIC FACTOR PREPARATIONS

The results of four separate experiments of the effect of *p*H on vitamin B_{12} binding by human gastric juice appear in Fig. 1 (curve A). Agreement between these experiments was satisfactory. It can be seen that maximal binding of vitamin B_{12} by human gastric juice occurs in the *p*H range 6·5-10. Outside this optimal *p*H range the binding capacity of human gastric juice drops and at *p*H values below 1·6 and above 12, the binding of vitamin B_{12} by human gastric juice drops below 10% of its maximum binding capacity. We have used acetate buffer as well as NaOH and HCl solutions to control the *p*H changes and have observed similar results. This therefore rules out the possibility that our observations may be due to the effect of Na^+, K^+, or phosphate ions.

The results of similar experiments using hog intrinsic factor are shown in Figure 2. There were no apparent differences in the *p*H sensitivity of three brands of hog intrinsic factor tested. The pattern of *p*H dependence shown by hog intrinsic factor resembles the effect of *p*H on human gastric juice except that at *p*H values between 1·4 and 3·5 the

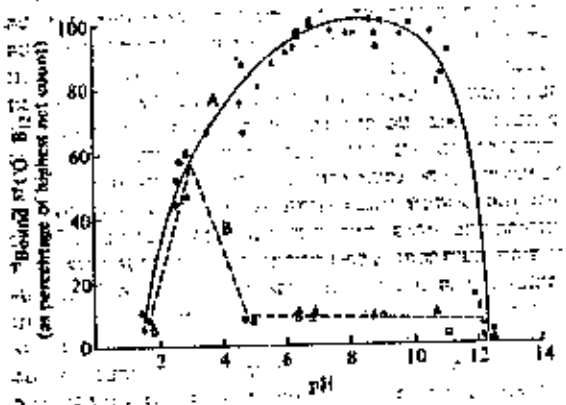

Fig. 1. *Effect of pH on the binding of vitamin B_{12} by human gastric juice (curve A) and by human gastric juice treated by intrinsic factor blocking antibody) (curve B). ○, ●, ■, ♦ = results of four separate experiments for curve A. □, ▲ = results of two separate experiments for curve B.*

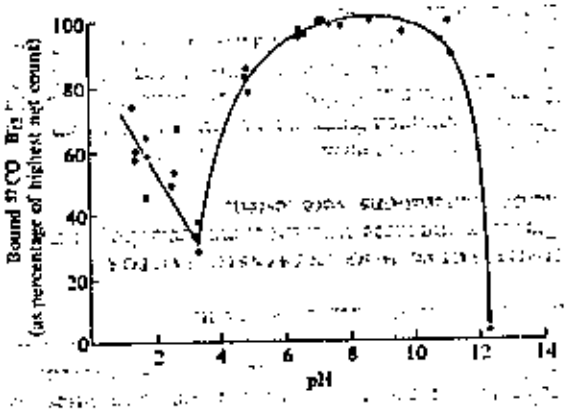

Fig. 2. *Effect of pH on the binding of vitamin B_{12} by hog intrinsic factor preparations. ○ = Nutritional biochemical intrinsic factor; ♦ = National Formulary intrinsic factor, and ● = Lederle intrinsic factor.*

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vitamin B_{12}-binding pattern of the hog intrinsic factors was rather irregular.

EFFECT OF *pH* ON VITAMIN B_{12} BINDING BY HUMAN GASTRIC JUICE TREATED WITH INTRINSIC FACTOR BLOCKING ANTIBODY

The results of two experiments are shown in Figure 1 (curve B). When added to human gastric juice in excess the intrinsic factor blocking antibody would block all the intrinsic factor activity and any vitamin B_{12} bound may be attributed to the non-intrinsic factor vitamin B_{12} binders. It can be seen that, at least for *pH* levels above 5, the amount of ^{57}Co-B_{12} bound by the non-intrinsic factor binders is not significantly altered by changes in *pH*. The fact that the serum had no effect on human gastric juice vitamin B_{12} binding at *pH* levels below 4 probably indicates that the antibody is not active at these low levels (McGuigan, 1967). This implies that the pattern of vitamin B_{12} binding by human gastric juice in response to *pH* changes is due to the effect of *pH* on human intrinsic factor.

REVERSIBILITY OF *pH* EFFECT ON VITAMIN B_{12} BINDING BY HUMAN GASTRIC JUICE

This experiment was designed to determine whether, *in vitro*, the binding ability of human gastric juice is irreversibly destroyed by subjecting it to extremes of *pH*. The results (Table II) suggest that exposure of human gastric juice to non-optimal *pH* values for 30 minutes results in a loss of vitamin B_{12} binding capacity which is almost completely restored by neutralization.

pH	*B_{12}-binding Capacity of Human Gastric Juice (%)*[1]	
	Before Neutralization	*After Neutralization*
2·1	0·6	100
2·7	24·2	100
3·2	45·8	100
10·1	98·3	100
10·5	85·7	100
11·8	10·2	100

Table II *Reversibility of pH effect on binding of B_{12} by human gastric juice*

[1]These are arbitrary figures calculated by taking the amount of ^{57}Co-B_{12} bound after neutralization as 100%.

Discussion

The present studies show that human gastric juice binding of vitamin B_{12} is *pH* dependent. Between *pH* 6·5 and 10 the binding capacity is maximal and is fairly constant; outside this *pH* range, however, the binding capacity decreases. When the *pH* is below 1·6 or above 12·2, less than 10% of the maximal binding occurs. In the *pH* range of 5 to 12, the presence of intrinsic factor blocking antibody removed 85-90% of the total binding ability, indicating that the predominant vitamin B_{12} binder in human gastric juice is intrinsic factor. Below *pH* 4 the presence of intrinsic factor blocking antibody did not significantly reduce the vitamin B_{12} binding ability of human gastric juice. This is not unexpected since a number of antibody-antigen complexes dissociate at such low *pH*s and the intrinsic factor blocking antibody was therefore probably not active. The vitamin B_{12} binding ability of the non-intrinsic factor fraction of human gastric juice did not appear to be affected by *pH*. Apparently it is the intrinsic factor itself, rather than the non-intrinsic factor fraction of human gastric juice, which is affected by *pH* changes.

It was shown that, in the *pH* range 3·5-12·2, three commercial hog intrinsic factor preparations responded in a similar manner to human gastric juice. Below *pH* 3·5, however, they respond differently to human gastric juice. This unexpected phenomenon has not been investigated further. It might be due to the presence of other non-intrinsic factor proteins which may bind vitamin B_{12} at such low *pH*s or the freeing of intrinsic factor binding sites which are not usually available at other *pH* levels in these manufactured reagents.

Goldberg and Fudenberg (1969) supported our suggestion that the vitamin B_{12} binding ability of intrinsic factor is *pH* dependent (O'Neill *et al*, 1968). They found that this increased when the *pH* was increased from 2 to 9. They, however, did not extend their studies to higher *pH* values and consequently did not report any inhibitory effects at *pH*s above 12·2. However, using equilibrium dialysis, McGuigan (1967) reported that there was no significant difference in the vitamin B_{12} binding by human gastric intrinsic factor at *pH* 4·5, 7·4, and 11. This appears to be inconsistent with our results, but due to the differences in techniques and in the assessment of results, it is not apparent whether a genuine inconsistency exists.

These effects of *pH* on intrinsic factor might explain some of the abnormalities of vitamin B_{12} absorption previously reported in man. Veeger, Abels, Hellemans, and Neiweg (1962) and Le Bauer, Smith, and Greenberger (1968) reported that in some patients with pancreatic insufficiency and a low intestinal *pH*, vitamin B_{12} malabsorption was not due to a lack of intrinsic factor but could be corrected by the administration of sodium bicarbonate. Shimoda, Saunders, and Rubin (1967) also reported that in a case of Zollinger-Ellison syndrome malabsorption of vitamin B_{12} was corrected when

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acidity of the upper small intestine was neutralized. The gastric juice of this patient was shown to exhibit normal intrinsic factor activity. Furthermore, an adverse effect *in vitro* of acid *pH* on vitamin B_{12} uptake by everted sacs of rat small intestine has been reported (Herbert, 1959).

It appears that a neutral or slightly alkaline *pH* condition in the intestine is probably necessary for normal vitamin B_{12} absorption. Whether this *pH* effect is concerned solely with the optimal binding of vitamin B_{12} by intrinsic factor is still not clear. It is possible that intraluminal neutrality may also be necessary for the normal attachment of the intrinsic factor-vitamin B_{12} complex to the intestinal mucosal cells.

The reversibility experiments (Table I) indicate that intrinsic factor is not destroyed when subjected to extreme *pH* values for 30 minutes. This implies that the intrinsic factor molecule, or at least its binding site or sites, remains intact under those conditions. This is not unexpected as, under normal physiological conditions, intrinsic factor is subjected to a wide spectrum of *pH* changes through the gastro-intestinal tract.

Our results suggest that a certain ionic conformation of the binding site or sites of the intrinsic factor molecule facilitates maximum binding of vitamin B_{12}. It is further suggested that this ionic conformation is acquired at neutral or slightly alkaline *pH*. The exact mode of the binding mechanism, however, still remains to be elucidated.

The effect of *pH* on the binding of vitamin B_{12} by intrinsic factor appears to have been neglected in a number of assay procedures *in vitro* for serum vitamin B_{12} (Lau *et al*, 1965; Raven *et al*, 1969), serum intrinsic factor blocking antibody (Gottlieb *et al*, 1965; Ungar, 1967), and intrinsic factor in human gastric juice (Ardeman and Chanarin, 1963; Gottlieb *et al*, 1965). These assay methods do not use a buffering system to control the *pH* and it is therefore not surprising that inconsistent results have sometimes been reported (Lau *et al*, 1965; Raven, Walker, and Barkhan, 1966). We have recently reported on the reproducibility of a modified isotope dilution vitamin B_{12} assay using a buffering system to maintain the *pH* within a narrow range (Shum, Streeter, and O'Neill, 1970).

We wish to thank Mr K. A. Steele of the Division of Biochemistry for the supply of gastric juice.

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The Biochemistry of B_{12}-Mediated Reactions in Man

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The report by Minot and Murphy [1], in 1926, that pernicious anemia could be controlled by the ingestion of large quantities of liver stimulated many studies aimed at identification of the factor or factors responsible for this therapeutic effect. These investigations led in 1948 to the crystallization of vitamin B_{12} (cyanocobalamin) from liver [2,3]. This substance in microgram amounts produced hematologic responses in patients with pernicious anemia.

The very low concentration of vitamin B_{12}-active derivatives in liver may explain why twenty-two years elapsed until this compound was isolated, and another two decades were required to establish the present, still incomplete, concepts of its function. We will attempt to summarize current knowledge of the biochemistry of vitamin B_{12}, with special emphasis on the reactions described in man. Several detailed reviews on the chemistry and biochemistry of vitamin B_{12} have recently appeared [4-7].

PROPERTIES AND STRUCTURE

Vitamin B_{12}, a dark red compound containing a cobalt atom, has a molecular weight of 1,355 and decomposes when autoclaved at 115°C for thirty minutes. Optical rotatory dispersion studies suggest that the cobalt constitutes an asymmetric center for the molecule. The absorption spectrum of vitamin B_{12} in aqueous solutions shows three maximums, at 278, 361 and 550 mμ. The vitamin is stable in the pH range of 4 to 7 at room temperature. Photolysis with sunlight results in a loss of activity of the vitamin and its coenzyme forms [4].

For this discussion, the molecule (Fig. 1) may be considered to consist of three regions [4,8]:

(1) The Corrin Nucleus. This planar structure is composed of four pyrrole rings, three of which are linked together through bridged carbon atoms as in the porphyrin nucleus. In contrast to porphyrins, a direct bond links the alpha position of rings A and D. The central trivalent cobalt atom links the three regions of the molecule. The coordination number of cobalt is 6. The ligands satisfying these requirements are the four nitrogens of the corrin nucleus, a coordinate linkage to the N-3 of the benzimidazole lying "below" the planar ring and an ionic Co-C bond by which the cobalt is linked to the cyano group lying "above" the corrin ring. Upon chemical reduction, this group is removed as hydrogen cyanide and the vitamin is reduced to a brown compound, vitamin B_{12r}, which contains cobalt in a divalent state. Further reduction yields vitamin B_{12s} containing Co in a monovalent state [9].

(2) The Benzimidazole Nucleotide. This unusual nucleotide is situated "below" and nearly at right angles to the corrin moiety. It consists of a 5,6-dimethyl-benzimidazole base attached to ribose-3-phosphate by an alpha glycoside linkage. The nucleotide is connected to the planar portion of

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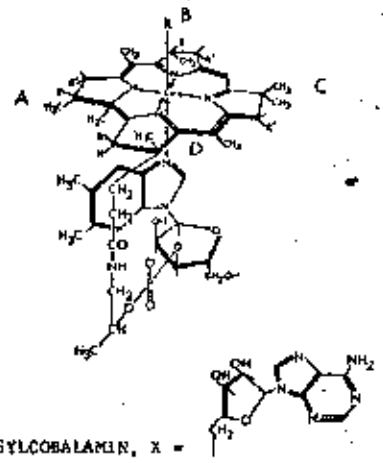


FOR 5'-DEOXYADENOSYLCOBALAMIN, X =

FOR METHYLCOBALAMIN, X = CH_3

FOR VITAMIN B_{12} (CYANOCOBALAMIN), X = CN

Fig. 1. Structure of vitamin B_{12} and B_{12} coenzymes.

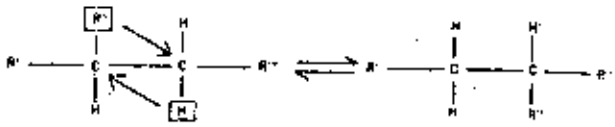
the molecule by two linkages: the Co-N bond and by its phosphate group, which is linked through aminoisopropanol to a propionic acid side group of the D pyrrole.

(3) The Cyano Group. This group is probably not a part of the molecule in vivo but represents an artifact of isolation, stemming from the presence of cyanide in the charcoal used during the purification process. In the physiologically active coenzyme forms of the vitamin, either a deoxyadenosyl group or a methyl group is bound to the cobalt instead of the cyano ligand. In the deoxyadenosyl B_{12} coenzyme, which is involved in hydrogen transfer reactions, the 5'-deoxyadenosyl group is connected via a Co-C linkage to the C-5 of deoxyribose. A methyl group occupies this position in the methyl B_{12} coenzyme. This coenzyme, the only other natural compound known to contain a Co-C bond, participates in methyl transfer reactions. An intact Co-C bond is of paramount importance to the catalytic properties of the B_{12} coenzymes.

The vitamin B_{12} coenzymes were first discovered in bacteria, but soon thereafter 5'-deoxyadenosyl B_{12} was isolated in moderate purity from sheep, rabbit, chicken and human livers [10]. The human preparation had molar activities very similar to those of the bacterial coenzyme in a specific enzymatic assay and yielded 1 mole of 5,6-dimethyl-benzimidazole on acid hydrolysis. Approximately two thirds of the total liver B_{12} is in the 5'-deoxyadenosyl form. At least some, and perhaps most of the remainder, consists of the methyl B_{12} form of B_{12} coenzyme [11].

REACTIONS DEPENDENT ON B_{12} COENZYMES

The enzyme reactions in which vitamin B_{12} derivatives participate as cofactor can be divided into two major categories [6]. The reactions in the first group are catalyzed by enzymes dependent on 5'-deoxyadenosyl B_{12} as a prosthetic group [5-7]. These include glutamate [12] and methylmalonyl-CoA mutase [13], diol dehydrase [14], ribonucleotide reductase [15], ethanolamine deaminase [16] and beta-lysine isomerase [17]. Table I lists these systems in the order of their discovery. The over-all reaction mechanism for this category involves the transfer of hydrogen in one direction with formation of a new carbon-hydrogen bond and migration of a second group in the opposite direction as depicted in equation 1 [7].



(1)

Ribonucleotide reductase also belongs to this category, except that the exchange is intermolecular, that is, the hydrogen donor and acceptor are different compounds [18].

The methyl B_{12}-dependent enzymes which constitute the second group catalyze a methyl group transfer in the methylation of homocysteine, the formation of methane and the synthesis of acetate (Table II) [7]. To date only two B_{12}-dependent enzymes, methylmalonyl-CoA mutase and N^5-methyl tetrahydrofolate-homocysteine methyl transferase, have been demonstrated in man [6]. It has been postulated that the ribonucleotide reductase reaction in man would show dependence on a B_{12} enzyme [19]. These

TABLE I Reactions Requiring 5'-Deoxyadenosyl B_{12}

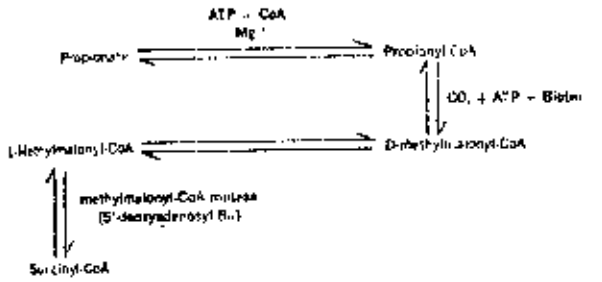
Reaction	Pathway	Organism
Glutamate mutase	Glutamic acid fermentation	Clostridium tetanomorphum
Methylmalonyl-CoA mutase	Propionate oxidation	Propionibacterium shermanii; animals, man
Diol dehydrase	Glycol metabolism	Aerobacter aerogenes
	Glycerol metabolism	Lactobacillus (unidentified)
Ribonucleotide reductase	Deoxyribonucleotide formation	Lactobacillus leichmenii
Ethanolamine deaminase	Ethanolamine fermentation	Choline-fermenting Clostridium
β-Lysine isomerase	Lysine fermentation	Clostridium sticklandii

TABLE II Reactions Requiring Methyl-B_{12}

Reaction	Pathway	Organism
N^5-methyl tetrahydrofolate: homocysteine methyl transferase	Methionine synthesis	Esch. coli, pig, man
Methane synthesis	Methanol fermentation	Methanosarcina barkeri
Acetate synthesis	Carbon dioxide metabolism	Clostridium thermoaceticum

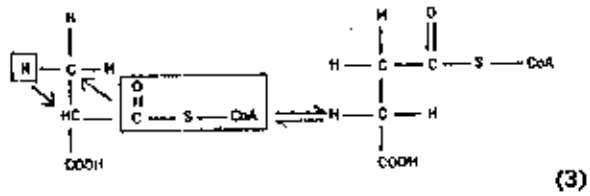
three reactions will therefore be considered in some detail.

Deoxyadenosyl-B_{12} Mediated Reactions. Methylmalonyl-CoA mutase: This enzyme, which catalyzes the interconversion of L-methylmalonyl-CoA and succinyl-CoA, two intermediates of the propionate oxidation pathway, has been obtained in high purity from sheep liver [20] and Propionibacterium shermanii [21] (equation 2).



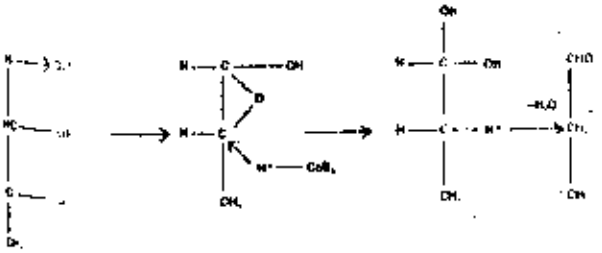
(2)

The over-all sequence consists of the carboxylation of propionate to D-methylmalonyl-CoA, isomerization of the D-methylmalonyl-CoA to L-methylmalonyl-CoA and isomerization of this product to succinyl-CoA [5]. The methylmalonyl-CoA mutase reaction involves a hydrogen transfer and cleavage of a C-C bond with migration of the thioester group [22] (equation 3).



(3)

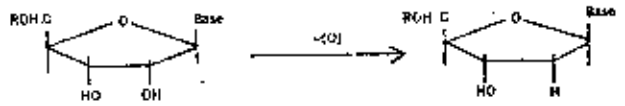
The hydrogen migrates from the methyl group of methylmalonyl-CoA to C-3 of succinyl-CoA with concomitant transfer of a carbonyl thioester group from the C-2 to the C-3 position [23]. This transfer takes place without incorporation of solvent hydrogen, and free intermediates have not been detected. Experiments with tritium-labeled 5'-deoxyadenosyl-B_{12} have indicated that hydrogen transfer occurs via the C-5 position of the deoxyadenosyl coenzyme [24,25]. The reaction mechanism for this transfer may be analogous to the diol dehydrase reaction [6] shown.



When this enzyme is incubated with unlabeled coenzyme and tritiated substrate, tritium appears in the deoxyadenosyl-B_{12} [26]. The tritium is subsequently transferred from the coenzyme to the unlabeled substrate. These experiments indicate that the coenzyme functions as a hydrogen donor [27]. The C-Co bond must be modified in such a way that either of the two hydrogens attached to C-5 in the deoxyadenosyl coenzyme becomes equivalent in the transfer process [7]. The clinical aspects of methylmalonic aciduria in the diagnosis of vitamin B_{12} deficiency and the significance of the recently described inborn errors involving methylmalonyl-CoA mutase are discussed elsewhere in this symposium.

Ribonucleotide reductase: Ribonucleotide reductase [28] catalyzes the conversion of ribonucleotides to deoxyribonucleotides, which are the substrates necessary for DNA synthesis. It thus constitutes a likely site for the operation of physiologic control mechanisms or for the action of pharmacologic agents designed to inhibit cell division.

Although isotope experiments with intact cells had suggested, as early as 1950 [29], that ribose bound in a ribonucleoside or ribonucleotide was reduced to the corresponding deoxyribosyl compound, efforts to demonstrate the appropriate biosynthetic enzymes in cell-free extracts were unsuccessful for almost a decade. It has now been shown [28] that the reaction involves the substitution of a hydroxyl group by a hydrogen ion in the 2' position of the ribosyl moiety of a nucleoside diphosphate or triphosphate, according to equation 4.



(4)

In this equation, R stands for either a pyrophosphoryl group or a triphosphoryl group, depending on the source of the ribonucleotide reductase.

Two different but related enzyme systems have been defined in Escherichia coli [30,31] and Lactobacillus leichmanii [19]. Some properties of the two enzymes are compared in Table III. Perhaps the most important difference between the two enzymes lies in the requirement for deoxyadenosyl-B_{12} by the L. leichmanii ribonucleotide reductase [15]. The involvement of vitamin B_{12} was first suggested by the growth requirements of this organism, since vitamin B_{12} could be substituted for deoxyribosyl

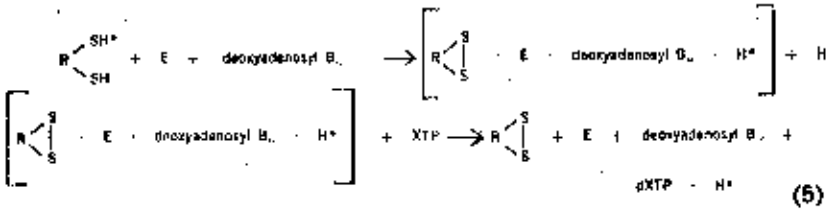
TABLE III Comparison of Ribonucleotide Reductases

Enzyme	L. leichmanii	Esch. coli	Mammalian
Nucleotide substrate	Triphosphate	Diphosphate	Diphosphate
Hydrogen donor	Thioredoxin like protein	Thioredoxin	Thioredoxin
Cation requirement	None	Magnesium	Magnesium
Stimulation by metal	None	Iron	Iron
Allosteric regulation	Yes, dATP less important	Yes, dATP negative effector	Yes, dATP negative effector
Deoxyadenosyl B_{12} requirement	Yes	None	Probably none

compounds in the growth medium. The mechanism of enzyme action differs from that of the other deoxyadenosyl-B_{12}-catalyzed reactions. In the dioldehydrase and methylmalonyl-CoA mutase reactions, an over-all intramolecular transfer of hydrogen occurs [5-7]. By contrast, in the ribonucleotide reductase reaction an intermolecular transfer of hydrogen takes place [32,33]: a hydrogen is transferred from a dithiol $R\begin{smallmatrix}SH\\SH\end{smallmatrix}$ to the 2' carbon of the deoxyribosyl moiety of the nucleotide (XTP). The reaction mediated by the enzyme (E) involves the formation of a transient enzyme–hydrogen donor–coenzyme complex. The H^+ is transferred from a thiol group to the 5' carbon of the coenzyme deoxyadenosyl moiety, from whence it is exchanged for the -OH of the C-2 in ribose. This sequence is summarized in equation 5.

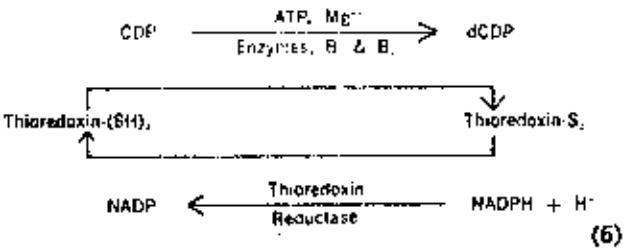
The mammalian ribonucleotide reductase seems to share most of the properties of the Esch. coli enzyme [37, 42]. It appears that reduction occurs at the diphosphate level, Mg^{++} is required, positive as well as negative effectors are operative. No stimulation by a B_{12} coenzyme has been shown.

Since it had been demonstrated that impaired synthesis of DNA is a constant feature in the marrow of patients with pernicious anemia, an attractive scheme had been postulated suggesting that the human bone marrow reductase was cobamide-dependent [19] and therefore not functioning properly in these B_{12}-depleted patients. The lack of deoxyribonucleotides would in turn have explained the decrease in DNA synthesis. Recent studies in this laboratory, using B_{12}-depleted bone marrow from patients with pernicious anemia, showed no stimulation of ribonucleotide reductase activity by vitamin B_{12} or deoxyadenosyl B_{12} [39]. These observations fail to support the hypothesis that this B_{12} coenzyme is involved in ribonucleotide reduction in man. Of significance, perhaps, is the finding that the level of ribonucleotide reductase in B_{12} deficient bone marrow is about twenty-fold higher than that found in normal marrow [40].



The physiologic source of reducing power is a low molecular weight protein, shown as $R\begin{smallmatrix}SH\\SH\end{smallmatrix}$, which donates the hydrogen. This protein closely resembles Esch. coli thioredoxin, to be discussed later.

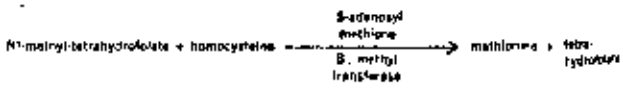
The reaction sequence for the Esch. coli ribonucleotide reductase is shown in equation 6.



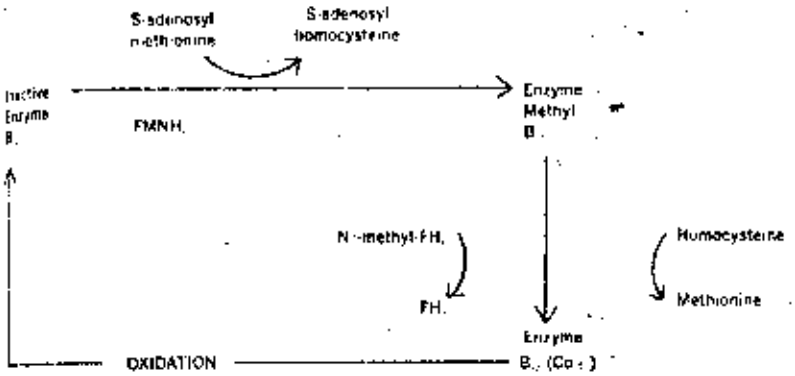
The principal features of this system are as follows: The reduction occurs at the nucleoside diphosphate level and is catalyzed by two enzymes. The physiologic dithiol hydrogen donor has been shown to be a protein, thioredoxin [34]. When isolated in its oxidized form, thioredoxin-S_2, this protein contains a disulfide bridge formed by two half-cystine molecules. An NADPH-dependent enzyme, thioredoxin reductase, catalyzes the reduction of the -S-S-bridge in thioredoxin-S_2 to the dithiol found in the reduced form, thioredoxin-$(SH)_2$ [35]. Studies on the mechanism of the ribonucleotide diphosphate reduction performed in ^3H_2O and ^2H_2O showed that the sulfhydryl groups of reduced thioredoxin are labeled with tritium or deuterium coming from the labeled water. The hydrogen is then introduced into position 2' of the deoxyribonucleotide during the enzymatic reduction of the ribonucleotide [36]. The participation of cobamides appears to have been firmly excluded from this reaction [28].

Methyl Transfer Reactions. Evidence that vitamin B_{12} is involved in the biosynthesis of methionine arose out of the demonstration that rats were able to grow on a diet lacking methionine but containing homocysteine [43]. Rats that failed to gain weight on a diet devoid of methyl groups but rich in folic acid and homocysteine did so when vitamin B_{12} was added [44]. Additional support for the involvement of a B_{12} derivative in this pathway was provided by studies with a mutant of Esch. coli which required vitamin B_{12} or methionine for growth [45]. This organism was unable to synthesize methionine from homocysteine in the absence of vitamin B_{12}.

Further investigations employing cell-free systems [46, 47] reveal that the transfer of a methyl group from N^5-methyl tetrahydrofolate (N^5-methyl FH_4) to homocysteine (equation 3) is catalyzed by a B_{12} requiring methyl transferase enzyme:



Methyl-B_{12}, the coenzyme form of the vitamin involved in this reaction, contains a methyl group attached to a cobalt as well as several methyl groups attached to carbon atoms. Only the methyl group attached to the cobalt is transferred during the reaction. The following mechanism has been suggested:



The methyl group is transferred from N^5-methyl tetrahydrofolate to a reduced cobamide species on the enzyme to form methyl-B_{12} and tetrahydrofolate. Subsequent transfer of this methyl group to a homocysteine yields methionine and regenerates a reduced B_{12}-enzyme [6]. The role of S-adenosyl-methionine in the alkylation of the enzyme by N^5-methyl tetrahydrofolate has recently been clarified [48,49]. It appears that S-adenosyl methionine has a "priming action" when, in the presence of a reducing agent, it will methylate the initial molecules of the reduced B_{12} enzyme. Methyl groups from the folate donor will then replace the methyl group on the cobamide donated by S-adenosyl methione and serve as the actual methyl donor for methionine synthesis. Decreased activity of N^5-methyl-FH, methyl transferase in vitamin B_{12} deficiency has been described in animals [50,51].

SUMMARY AND COMMENTS

The usual sequence of events in the discovery of a vitamin's function is as follows: (1) A nutritional deficiency leads to the discovery of a vitamin. (2) A variety of biochemical reactions are found which are stimulated by this substance. (3) The vitamin as such is not the active principle but has to be converted to a coenzyme form. (4) Further knowledge of the specific reactions involving the coenzyme forms is acquired. (5) Attempts are made to explain the biologic effects of the nutritional deficiency at the molecular level. It may be said that we are approaching this last stage in our understanding of Vitamin B_{12}. The coenzyme forms have been identified, and several abnormalities involving these pathways have been described in man.

Despite the significant progress made in the enzymology of vitamin B_{12} derivatives many questions remain unanswered. The following comments emphasize some problems yet to be fully clarified.

(1) The pathogenesis of the neurologic lesions in vitamin B_{12} deficiency remains obscure. Neither of the reactions known to occur in man has been shown to bear a direct relationship to the observed pathology. It is possible that other B_{12}-dependent reactions will be discovered which will shed light on this matter.

(2) Considerable evidence discussed elsewhere in this symposium implicates the "folate trap" as a cause of the megaloblastic changes. This hypothesis states that methionine biosynthesis is impaired in B_{12} deficiency and N^5-methyl FH, cannot be converted to N^5,N^{10}-methylene FH_4. Since the latter folate derivative is essential for formation of the DNA precursor thymidylate, a failure in folate utilization becomes the ultimate cause for the decreased DNA synthesis in megaloblastic anemia. However, positive evidence for decreased methyl transferase activity in vitamin B_{12} deficient patients is still lacking and the levels of N^5,N^{10}-methylene FH_4 have not been determined. Further direct enzymologic studies are therefore desirable as definitive proof of this hypothesis.

(3) The profound differences between the manifestations of vitamin B_{12} deficiency in man and experimental animals are unexplained. Conceivably, species differences in host metabolism or intestinal flora may account for some aspects of these discrepancies.

(4) The regulatory mechanisms responsible for the control of vitamin B_{12} dependent reactions have not been fully explored.

(5) The nature of the active sites in the B_{12}-dependent enzymes has not been elucidated.

In a sense, the molecular biology of the megaloblastic anemias is just beginning to be understood. The next decade should bring some answers to these fundamental problems.

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Vitamin B_{12} levels in the serum and cerebrospinal fluid in multiple sclerosis

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Vitamin B_{12} has been widely used in the treatment of neurological disease, if only as a placebo. Whilst the results of a series of uncontrolled trials of its use in multiple sclerosis have not been impressive (Lereboullet, Pluvinage, and Coty, 1950; Schumacher, 1950; Simson, Herfort, Krim, and Meyer, 1950; Booth, Lawyer, and von Storch, 1951; Lereboullet and Pluvinage, 1952; Levin, 1955), there have been some reports of abnormalities both in the serum and cerebrospinal fluid levels of vitamin B_{12} in multiple sclerosis. These, however, are not consistent amongst themselves. Thus low serum values (O'Connor, Davis, Langworthy, and Chow, 1960), elevated serum and cerebrospinal fluid values (Sobotka, Christoff, and Baker, 1958), and a low ratio of cerebrospinal fluid to serum vitamin B_{12} (Gjertsen and Schrumpf, 1962) have all been reported. Grann and Glass (1961) from their study of vitamin B_{12} metabolism in 21 patients with multiple sclerosis concluded that it was probably normal (although they did not study cerebrospinal fluid). It was accordingly felt that a further investigation of vitamin B_{12} in cerebrospinal fluid and serum was necessary, particularly as this department was carrying out a controlled trial of massive doses of hydroxocobalamin in the treatment of multiple sclerosis. Since commencing this investigation the work of Worm-Petersen (1962) has appeared and records no difference in the serum and cerebrospinal fluid vitamin B_{12} levels between multiple sclerosis and other neurological disease. These discordant findings encouraged continuance of the present study.

METHODS

Specimens of blood (collected with disposable Mediplast syringes) and lumbar cerebrospinal fluid were collected at the same time into specially cleaned, sterile containers (Hutner, Bach, and Ross, 1956). Serum was separated aseptically and transferred to similarly treated containers. All samples were stored at −20°C. There is no appreciable loss of activity in specimens stored under these conditions for six to 24 months (Killander, 1957).

The specimens were assayed by the method of Hutner, Bach, and Ross (1956) with *Euglena gracilis, Z strain*, with the modification that hydroxocobalamin replaced cyanocobalamin. Total vitamin B_{12} content was estimated after heating the assay mixture at 100°C. for 15 minutes before inoculation. In addition free vitamin B_{12} was estimated in some specimens by the method of Russ (1950, 1952). Readings were made using a turbidimetric method in a spectrophotometer (Unicam SP 500) at 625 mμ.

RESULTS

Specimens were obtained from 46 patients (average age 39 years, range 22-55) in whom the diagnosis of multiple sclerosis was beyond all reasonable doubt. From these 46, five were taken during acute exacerbations. The remaining cases were all chronic; four chairbound, the rest ambulant and only mildly or moderately afflicted. The control group consisted of 23 patients (average age 48 years, range 30-77) with miscellaneous neurological and mental disorders, namely, cerebral tumour, 4; cervical spondylosis, 4; prolapsed lumbar disc, 3; motor neurone disease, 2; polyneuropathy, 2; cranial arteritis, 2; and one each of, pituitary tumour, headache, epilepsy, spinal artery thrombosis, obsessional neurosis, and hysteria. A truly 'normal' control group was considered to be unobtainable as there is little ethical justification for carrying out lumbar puncture on normal people. No patient had received previous treatment with vitamin B_{12}, and haemoglobin estimation, mean corpuscular haemoglobin concentration, and blood film examination were normal in all cases.

Since Worm-Petersen (1962) has shown that the distribution of vitamin B_{12} levels fits a logarithmic distribution better than a normal one and the present results support this, the logarithms of the serum or cerebrospinal fluid levels of vitamin B_{12} have been used for the calculations.

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TABLE I

VITAMIN B_{12} LEVELS IN SERUM AND CEREBROSPINAL FLUID ($\mu\mu$g./ml.)

		Control	Multiple Sclerosis	Significance
(a) Mean log. serum vitamin B_{12} and standard error (s.e.)		2·526 ± 0·046	2·427 ± 0·042	Difference of means P = 0·05
(b) Mean log. cerebrospinal fluid vitamin B_{12} and standard error (s.e.)		1·045 ± 0·011	0·947 ± 0·56	Difference of means P = 0·1
Antilog. (a)	(a) + 2 × s.e.	415	324	
	(a)	336	267	
	(a) − 2 × s.e.	271	220	
Antilog. (b)	(b) + 2 × s.e.	11·7	11·5	
	(b)	11·1	8·9	
	(b) − 2 × s.e.	10·5	6·8	
r		0·53	0·79	Control and multiple sclerosis P<0·005
Slope of regression line		0·72	0·97	Difference between slopes P = 0·4 (Difference between intercepts P = 0·4)

Means of the log. vitamin B_{12} values in serum and cerebrospinal fluid are given in Table I. The log. cerebrospinal fluid levels of vitamin B_{12} have been plotted against the log. serum levels for both control and multiple sclerosis groups and the regression lines calculated (Figs. 1 and 2). The coefficients of correlation (r) and slopes are given in Table I.

It should be noted (Table II) that in six patients with multiple sclerosis the serum vitamin B_{12} was less than 135 $\mu\mu$g./ml. (the lowest control value) but this is not significant (P>0·7). The serum and cerebrospinal fluid vitamin B_{12} values for the five acute cases ranged from 428 (59) to 182 (10) $\mu\mu$g./ml. which is comparable with results for chronic cases.

Free (uncombined) vitamin B_{12} was measured in five control and 14 multiple sclerosis sera. A small quantity of free vitamin B_{12} was detected in one control and one multiple sclerosis serum. No difference between free and total vitamin B_{12} levels occurred in 13 samples of cerebrospinal fluid but free vitamin B_{12} was not detected in one control and five multiple sclerosis samples. Log. cerebrospinal fluid vitamin B_{12} was plotted against log. cerebrospinal fluid protein (measured in the routine laboratory) and there was no correlation between the two.

DISCUSSION

Good correlation between cerebrospinal fluid and serum vitamin B_{12} levels exists in both controls and patients with multiple sclerosis. This is contrary to the findings of Gjersten and Schrumpf (1962) on nine cases of multiple sclerosis. There is no signifi-

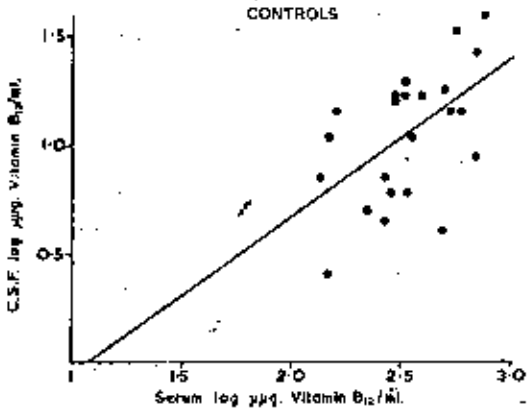

FIG. 1. *In control patients, log. cerebrospinal fluid vitamin B_{12} against log. serum vitamin B_{12}. The regression line is shown.*

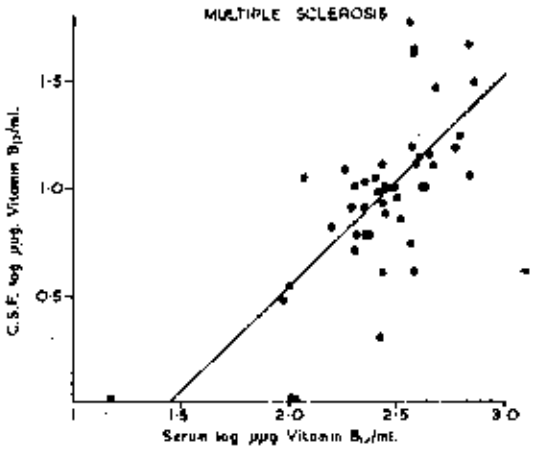

FIG. 2. *In multiple sclerosis patients, log. cerebrospinal fluid vitamin B_{12} against log. serum vitamin B_{12}. The regression line is shown.*

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TABLE II

DETAILS OF PATIENTS WITH LOW SERUM VITAMIN B_{12} LEVELS

Serum Vitamin B_{12} Level (μμg./ml.)	*Diagnosis*	*Length of History (yr.)*	*Severity of Disease*	*Course of Disease*	*Physique*
64	Multiple sclerosis	6	Severe	Progressive	Thin
94	Multiple sclerosis	6	Mild	Fluctuating	Thin
101	Multiple sclerosis	3	Mild	Fluctuating	Normal
103	Multiple sclerosis	2	Moderate	Progressive	Normal
108	Multiple sclerosis	2	Mild	Fluctuating	Thin
118	Multiple sclerosis	12	Mild	Static	Normal
135	Obsessional neurosis	--	--	--	Normal

cant difference in the cerebrospinal fluid levels of vitamin B_{12} between multiple sclerosis and control groups, which is also contrary to the findings of Gjertsen and Schrumpf (1962), and to the raised values found by Sobotka *et al.* (1958). The latter, using *Ochromonas malhamensis*, studied 20 cases of multiple sclerosis, of which 11 fell within the accepted normal range of cerebrospinal fluid vitamin B_{12} (0-30 μμg./ml.), six had high serum and cerebrospinal fluid levels, of which two were very high; and one had an abnormally low serum level.

Although the difference is not significant there is a suggestion that serum vitamin B_{12} levels may be abnormally low in some patients with multiple sclerosis. Six of these patients had serum vitamin B_{12} levels which lay below the lowest control value and only one of these could be considered severely afflicted and possibly malnourished (Table II). Grann and Glass (1961) found one of 21 cases of multiple sclerosis with a subnormal serum vitamin B_{12} level. Intestinal absorption of Co^{60}-labelled vitamin B_{12} in all their cases was normal. One control, two patients with migraine, and five of the nine cases of multiple sclerosis of Gjertsen and Schrumpf (1962) had serum vitamin B_{12} values of 100 μμg./ml. or below (by *Euglena gracilis* method). O'Connor *et al.* (1960) found that where the mean serum vitamin B_{12} in 360 control subjects was expressed at 100%, the mean in 21 cases of probable, long-standing multiple sclerosis was only 50%. Whilst they do not quote the mean levels, they claim that 10 of the 21 patients with multiple sclerosis (only three of whom were severe and not ambulant) had readings comparable with those for pernicious anaemia (50 μμg./ml., assayed using *L. leichmanii*). They further found that absorption of Co^{60}-labelled vitamin B_{12} was normal but that serum-binding capacity for vitamin B_{12} was lower in multiple sclerosis, and urinary excretion higher, than in the controls. From this they deduced that tissue retention of vitamin B_{12} was impaired in some cases of multiple sclerosis.

The results of the present investigation are in agreement with those of Worm-Petersen (1962) who also found that Ross's (1950, 1952) method of estimating free vitamin B_{12} showed no free vitamin B_{12} in the serum whereas the cerebrospinal fluid contained roughly equal quantities of free and total vitamin B_{12}. He pointed out, however, that using dialysis there was no free vitamin B_{12} in the serum or cerebrospinal fluid, and that *Euglena gracilis, Z strain* can utilize non-dialysable vitamin B_{12} (Hoff-Jørgensen and Worm-Petersen, 1962). This factor may account for the observations in cerebrospinal fluid but does not appear to operate in the serum. The ratio of serum protein to cerebrospinal fluid protein (200 : 1) is very different from the ratio serum vitamin B_{12} to cerebrospinal fluid vitamin B_{12} (25 : 1). Both these differences suggest that vitamin B_{12} binding in serum may differ from that in cerebrospinal fluid.

Meyer, Bertcher, and Mulzac (1959) found in dialysis experiments with cerebrospinal fluid and radioactive vitamin B_{12} that the ratio of radioactive vitamin binding capacity to protein concentration was higher for cerebrospinal fluid than for serum. They concluded that in cerebrospinal fluid vitamin B_{12} was not bound to protein or that this protein was different from that in serum.

Normally vitamin B_{12} is bound in both serum and cerebrospinal fluid to an immunologically indistinguishable alpha-1-globulin and it is suggested that the greater binding capacity of cerebrospinal fluid protein may be associated with some physicochemical difference in this protein (Clausen and Munkner, 1962).

There is probably a difference between vitamin B_{12} binding in serum and in cerebrospinal fluid, but there is not yet any convincing proof of abnormal vitamin B_{12} metabolism in multiple sclerosis. Although the present work offers no rational basis for the use of vitamin B_{12} in the treatment of multiple sclerosis, there is still a place for placebo therapy and the psychosomatic effect of an injection is greater than that of a tablet. As some patients with multiple sclerosis have low serum vitamin B_{12} levels, there may be something to be said for the administration of vitamin B_{12} in this disease but the results of a controlled trial are awaited.

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SUMMARY

Serum and cerebrospinal fluid vitamin B_{12} levels were estimated in 46 patients with multiple sclerosis and 23 patients with miscellaneous disorders were used as a control group.

No significant difference between multiple sclerosis and control groups could be found either in the absolute values of serum or cerebrospinal fluid vitamin B_{12} or in the serum: cerebrospinal fluid ratio. There was a significant correlation between serum and cerebrospinal fluid vitamin B_{12} levels but not between vitamin B_{12} and protein concentrations in cerebrospinal fluid. Vitamin B_{12} binding in serum and cerebrospinal fluid is discussed. It was noted that a few patients with multiple sclerosis in this and previous reports have abnormally low serum vitamin B_{12} levels although this is not statistically significant.

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The February 1964 Issue

THE FEBRUARY 1964 ISSUE CONTAINS THE FOLLOWING PAPERS

Serum enzyme studies in muscle disease Part I *Variations in serum creatine kinase activity in normal individuals* JOHN M. S. PEARCE, R. J. PENNINGTON, and JOHN N. WALTON

Renal tubular acidosis presenting with muscle weakness DESMOND CARROLL and PAGET DAVIES

The behaviour of attenuated strains of poliovirus in monkeys T. S. L. BESWICK, C. R. COID, E. HARTLEY, MOIRA HENDERSON, and MAUREEN WINTER

Antibody studies in multiple sclerosis and experimental 'allergic' encephalomyelitis E. A. CASPARY, E. J. FIELD, and E. JANET BALL

A clinical, pathological, and genetic study of an unusual form of Tay-Sachs disease with macular degeneration in the family PAUL R. DYKEN and WOLFGANG ZEMAN

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J. Pediatr.

Vitamin B_{12} studies in myelogenous leukemia of childhood

Chronic myelogenous leukemia (CML), in early childhood frequently presents a diagnostic problem. Plasma vitamin B_{12} levels and radioactive B_{12} clearance studies have been beneficial in diagnosing CML in adults. Plasma radioactive B_{12} clearances and/or plasma B_{12} concentrations were determined in 39 infants and children. In this group the radioactive B_{12} clearance was abnormally slow in 6 patients; these patients were the only patients diagnosed as having myelogenous leukemia. These data would indicate the usefulness of this simple and rapidly performed test for the age group in which myelogenous leukemia is a difficult diagnostic problem. The data are compared to those reported in adults, and the physiologic significance of this altered vitamin metabolism is reviewed.

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MINNEAPOLIS, MINN.

CHRONIC MYELOGENOUS leukemia (CML) is at times difficult to diagnose in infants and young children. The usual diagnostic procedures including bone marrow, liver, and lymph node biopsies with histochemical staining may fail to confirm the diagnosis at the onset of the disease.

Elevated plasma vitamin B_{12} concentration and the prolonged rate of clearance of radioactive B_{12} have been reported as helpful in the diagnosis of this disease in adult patients.[1-3] The clearance of intravenously injected radioactive vitamin B_{12} has been performed in 31 pediatric patients. The six prolonged clearance rates observed were in patients in whom the diagnosis of myelogenous leukemia was confirmed at some stage of their disease. In the remaining group of 25 patients with normal B_{12} clearance values there were no patients with myelogenous leukemia. The data presented demonstrate the value of the radioactive B_{12} clearance for patients in the age group in which a diagnosis of myelogenous leukemia is most difficult. The pathophysiology of altered B_{12} metabolism in these patients is considered in the discussion.

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MATERIALS AND METHODS

A standard dose of 0.5 microcurie of Co^{60} cyanocobalamine was injected intravenously. Samples were taken at 1 minute, 10 minutes, 30 minutes, 1½ hours, 2½ hours, 6

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hours, and 24 hours after injection. The radioactivity was measured in a plasma aliquot of each specimen in a scintillation counter. The plasma volume of the patient was estimated from the hematocrit and weight. The total plasma radioactivity was then expressed as a percentage of the injected dose. In two of the patients with myelogenous leukemia the studies were performed twice.

The vitamin B_{12} in the serum was measured by the microbiologic assay method of Hutner and co-workers[6] using the green algae, *Euglena* var. *gracilis*.

The leukocyte alkaline phosphatase was determined by a modification of the histochemical method of Hayhoe and Quaglino[7] using the diazonium salt of Brentamine garnet red as the dye. This is precipitated by the alkaline phosphatase contained in the granules.

Sudan Black B granules were stained by a method devised in the University of Minnesota Hospitals Special Hematology Section by Grave.[8]

The diagnosis of CML was established by examination of peripheral blood and bone marrow, by leukocyte alkaline phosphatase determinations, or by postmortem examination.

Acute myelogenous leukemia was separated from other acute leukemias by the demonstration of Auer rods, Sudan Black B positive granules, or peroxidase positive granules in the myeloblasts of the marrow and blood smears.

RESULTS

All six of the patients with myelogenous leukemia had a prolonged clearance of radioactive B_{12} from the plasma (Fig. 1). In contrast, all of the patients with acute lymphatic leukemia (ALL) had a normal clearance (Fig. 2).

Four of the children with ALL were newly diagnosed and untreated, two had been in a drug-induced remission but at the time of the test were in exacerbation. One ALL patient had lymphosarcoma with a leukemic transformation, and was in remission at the time of the examination. In the "other leukemia" group, there was one example each of erythroleukemia, myelomonocytic leukemia, and leukemic reticuloendotheliosis.

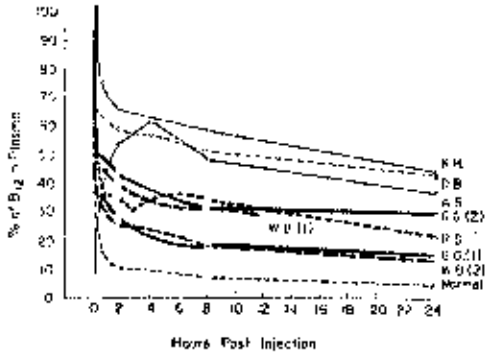

Fig. 1. Plasma radioactive vitamin B_{12} clearance of six patients with myelogenous leukemia.

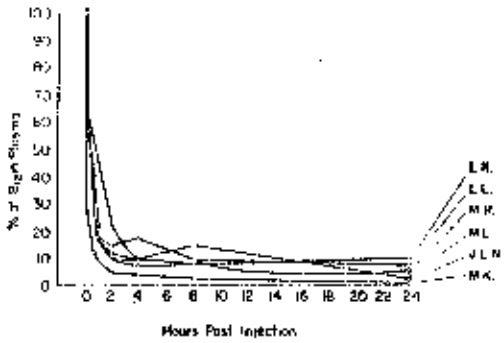

Fig. 2. Plasma radioactive vitamin B_{12} clearance of six patients with acute lymphatic leukemia.

In the remaining 15 subjects, there are patients with various disorders, including diseases of the liver, reticuloendothelial system, and solid tumors, also a few that were considered to be hematologically normal. The plasma clearance of B_{12} in each of these was normal.

The serum B_{12} concentrations are listed in Table 1. Of the 8 patients with CML from whom serum B_{12} concentrations were obtained, a clearance test with B_{12} was performed in three of them. In only one were both determinations abnormal, in two patients the clearance was prolonged, but the B_{12} concentration was normal.

Only one acute myelogenous leukemia (AML) patient demonstrated both a prolonged B_{12} clearance and elevated B_{12} concentration.

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Table I

No.	Patient	Diagnosis	Total B_{12} level (μμg./ml.) (Normal = 350–600 μμg./ml.)	B_{12} clearance
		Myelogenous leukemia		
1	M. J.	CML	1005	—
2	D. L.	CML	2280	—
3	D. S.	CML	4000	—
4	S. M.	CML	6420	—
5	K. B.	CML	324	—
6	A. S.	CML	3000	Prolonged
7	W. B.	CML	480	Prolonged
8	K. H.	CML	134	Prolonged
9	R. S.	AML	—	Prolonged
10	G. G.	AML	1135	Prolonged
11	D. B.	AML	—	Prolonged
12	A. P.	AML	320	—
13	M. W.	AML	630	—
14	R. J.	AML	280	—
		Other leukemias		
15	M. K.	ALL	—	Normal
16	M. H.	ALL	—	Normal
17	L. L.	ALL	—	Normal
18	L. R.	ALL	125	Normal
19	J. L. N.	ALL	—	Normal
20	M. L.	ALL	—	Normal
21	M. M.	Lymphosarcoma with leukemic transf.	240	Normal
22	C. P.	Erythroleukemia	600	Normal
23	S. B.	Myelomonocytic leukemia	950	Normal
24	M. W.	Leukemic reticuloendotheliosis	—	Normal
		Other diagnoses		
25	L. J.	Chediak-Higashi with lymphosarcoma	—	Normal
26	K. B.	Post-rubella syndrome	250	Normal
27	E. B.	Wiskott-Aldrich syndrome	755	Normal
28	M. B.	Hemolytic anemia	>2000	Normal
29	W. V.	Neuroblastoma	—	Normal
30	J. L.	Teratocarcinoma	—	Normal
31	W. H.	Regional enteritis	—	Normal
32	B. S.	Pancreatic insufficiency	—	Normal
33	W. N.	Agranulocytosis, fever, hepatomegaly	—	Normal
34	T. B.	Febrile disease	—	Normal
35	S. K.	Bronchiectasis and hepatosplenomegaly	—	Normal
36	T. S.	Spinocerebellar atrophy	—	Normal
37	D. O.	Progressive neurologic deterioration	—	Normal
38	D. G.	Extrahepatic biliary atresia with cirrhosis	—	Normal
39	K. B.	Normal	259	Normal

The results of other tests (i.e., Auer rods, Sudan Black B, and leukocyte alkaline phosphatase) are shown in Table II. Auer rods were found in the 6 patients with AML and in none of the patients with CML. Sudan Black B granules were positive in 5 of the patients with AML. In the two patients with AML tested, only one had a decrease of the leukocyte alkaline phosphatase concentration,[10] in the other patient it was increased.[12] In the 5 children with CML who were tested, only two (2 and 6) showed a decrease. In the remaining three it was normal.

DISCUSSION

Leukemia must be considered in the differential diagnosis of any child presenting with anemia, fever, and hepatosplenomegaly.

Table II

No.	Patient	Age	Diagnosis	Sudan Black B	Alkaline phosphatase*	Auer rods	Diagnosis made by
1	M. J.	1 yr.	CML	--†	—	0	Autopsy, 9 mo. after onset
2	D. L.	1 yr.	CML	+	55	0	Marrow 9 mo. after onset and autopsy 1 yr. after onset
3	D. S.	4 yr.	CML	+	137	0	Marrow 11 mo. after onset of disease
4	S. M.	2 yr.	CML	--	--	0	Autopsy 3 mo. after onset
5	K. B.	1 yr.	CML	[illegible]	—	0	Autopsy
6	A. S.	3 yr.	CML	--	3	0	Initial marrow
7	W. B.	8 mo.	CML	—	158	0	Autopsy 3 mo. after onset
8	K. H.	6 mo.	CML	+	65	0	Initial marrow
9	R. S.	10 yr.	AML	+	—	+	Initial marrow
10	G. G.	10 yr.	AML	+	0	+	Initial marrow
11	D. B.	8 yr.	AML	+	--	+	Initial marrow
12	A. P.	6 mo.	AML	+	211	+	Initial marrow
13	M. W.	7 yr.	AML	--	--	+	Initial marrow
14	R. J.	10 yr.	AML	+	—	+	Initial marrow

*Normal range for leukocyte alkaline phosphatase, 64–176.

†+, Study was positive; 0, study was negative; —, study was not performed.

If, in the process of eliminating other possibilities, this disease cannot be deleted, special laboratory procedures are required. Extensive studies of morphology of peripheral blood and marrow may confirm the diagnosis of myelogenous leukemia. Yet, some of the characteristic hallmarks of myelogenous leukemia such as Auer rods, low leukocyte alkaline phosphatase concentration, positive Sudan Black, and Philadelphia chromosomes may not be present.[9] As an additional diagnostic device, the vitamin B_{12} aberration reported in adult myelogenous leukemia seemed to be worthy of testing in children.

Of our 14 patients with myelogenous leukemia, 10 had either an elevated vitamin B_{12} concentration, or prolonged vitamin B_{12} clearance, or both. The remaining four had normal B_{12} levels but did not have a B_{12} clearance performed. In the six patients on whom B_{12} clearances were performed, all demonstrated prolonged clearances. Conversely, all of the 6 patients with acute lymphatic leukemia and those with the "other" types of leukemia had normal clearances. The remaining 15 patients with diverse diseases had normal clearances. One patient with a hemolytic anemia and liver necrosis had a level of serum B_{12} greater than 2000 μμg. per milliliter, but a normal clearance. A patient with biliary cirrhosis also had a normal clearance.

The pathophysiology of B_{12} metabolism in this type of leukemic process has been studied by several investigators. The B_{12} content of leukocytes from the adult chronic myelogenous leukemia patient is not elevated to the same extent as the serum B_{12} concentrations.[10] Mollin and Ross[8] have demonstrated a leukocyte B_{12} binding material which may be released into the serum and thus account for the slow clearance of this vitamin from the serum.

Normally, B_{12} migrates bound to the α_1- and α_2-globulins. Recently, Hall and Kinkler[5] have accumulated data that indicate a transcobalamine I in the serum of patients with CML; this may bind vitamin B_{12} in competition with the normal transcobalamine II. Estren and associates[4] have shown that plasma from CML labelled with radioactive B_{12} when injected into a normal recipient produces the prolonged clearance typical of the donor.

Estren and his group[4] hypothesized that a substance which they called "B_{12} transferase" aids the transfer of B_{12} from the serum B_{12} binding α-globulins to the hepatic beta-1 globulin. This would be required for the hepatic uptake which normally accounts for the majority of the B_{12} cleared from the plasma. If this hypothesis is correct, patients with myelogenous leukemia may have a deficiency of "B_{12} transferase." Certainly, fur-

ther studies will be required to define the biochemical aberration.

The survival time of adult chronic myelogenous leukemia patients is usually measured in years, where is that of children continues to be measured in months. This difference in natural history of the disease is somewhat perplexing in view of the similarity of the vitamin B_{12} abnormality in the two age groups. Future efforts to define the metabolic aberrations of this interesting compound (B_{12}) in leukemia will perhaps help identify the difference or similarities of this disease in the various age groups. It would appear from the above data that the B_{12} clearance is particularly helpful in identifying in children the rare type of childhood leukemia which is morphologically classed as myelogenous.

SUMMARY

1. The clearance of radioactive B_{12} from the plasma is reported in 31 patients. Abnormally prolonged clearances were observed in the six patients with myelogenous leukemia in whom they were measured.

2. Serum B_{12} concentrations are reported in 12 patients with myelogenous leukemia. These were elevated in 5 of 8 patients with chronic myelogenous leukemia and 1 of 4 patients with acute myelogenous leukemia.

3. The diagnostic significance of the B_{12} clearance test in myelogenous leukemia is indicated. Theories as to the basis for this abnormal B_{12} metabolism are discussed.

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VITAMIN B_{12}. Part 1*

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Historical

The problems of the anti-pernicious-anaemia factor of liver, both its chemical nature and its mode of action, have baffled biochemists and haematologists for the last quarter of a century, for it was in 1926 that Minot and Murphy, inspired by Whipple's work with dogs, found that so-called "Addisonian" or pernicious anaemia responded dramatically to oral treatment with large amounts of liver.

Space does not permit a detailed review of the early work on the fractionation of liver extracts. Indeed there is not much to add to the very complete review by SubbaRow *et al.* (1945). Many of the early suggestions about the chemical nature of the anti-pernicious-anaemia factor are now seen to have been incorrect because they were based on analyses of very impure concentrates. It is perhaps worth picking out the investigations that led to significant advances in the fractionation of liver extracts. Cohn *et al.* (1928) showed that the active principle could be extracted from liver with water, preferably at faintly acid *p*H, and that purification from proteins and other substances could be effected by adding ethanol to a concentration of about 70 per cent. Although, as we now know, vitamin B_{12} is soluble in dry ethanol, it is precipitated together with other substances from crude extracts by adding ethanol to about 95 per cent. concentration. This procedure yielded "Cohn's fraction G", which was satisfactory for parenteral administration and formed the basis for most subsequent attempts to fractionate. The same workers showed also that the active principle could be extracted from aqueous solutions with butanol, and that it was precipitated by phosphotungstic acid but not by lead acetate. All three of these steps were found useful by the present reviewer in the isolation of vitamin B_{12} from liver, and their value was earlier confirmed by other workers (West and Nichols, 1928). Dakin and his colleagues were responsible for a number of new fractionation procedures, including the precipitation of impurities with alcoholic calcium acetate and precipitation of the active principle with Reinecke acid or uranium acetate (Dakin and West, 1935; Dakin *et al.*, 1936). Of greater value were the observations that the active principle

* Part 2, dealing with vitamin B_{12} in its clinical aspects, by Dr. C. C. Ungley, will appear in the next number of *Nutrition Abstracts and Reviews*, Vol. 21, No. 1.

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could be salted out by ammonium or magnesium sulphate from neutral solutions or by sodium chloride from acid solutions. Important contributions were made by Scandinavian workers. They found that the active principle could be extracted with liquefied phenol from aqueous solutions, and that it could be adsorbed on to relatively small proportions of activated charcoal and eluted again with liquefied phenol (Laland and Klem, 1936). They found also that the active principle could be extracted from adequately purified dry concentrates by ethanol or methanol, and that it could be precipitated from such solutions with ether. There is little doubt that these Norwegian workers were the first to prepare concentrates that would give a "classical" clinical response in doses of only a few milligrams. There is no published evidence to indicate that anything more potent was obtained during the next ten years. SubbaRow, Jacobson and Prochownick (1936) showed that the active principle could be eluted from a charcoal adsorbate with hot 65 per cent. ethanol (see also SubbaRow, Jacobson and Fiske, 1935; 1936; SubbaRow and Jacobson, 1936; Jacobson and SubbaRow, 1937; 1941; SubbaRow *et al.*, 1938). The work is difficult to interpret because of claims that in addition to a "primary" factor simultaneous administration of L-tyrosine and other unidentified substances was needed to secure complete remission in pernicious anaemia. By use of a combination of published procedures for purification, a concentrate was obtained fully active in a single dose of 5·5 mg. (Emery and Hurran, 1945), and it was later claimed that a concentrate active in a single dose of 1 mg. had been prepared (Emery and Parker, 1946). It seemed at the time that so active a preparation must be nearly pure, but we now appreciate that in fact it contained only about 1 per cent. of vitamin B_{12}. No further publication of significance occurred until the announcement by Rickes *et al.* (1948*a*) of the isolation of the active principle of liver in the form of a red crystalline substance, for which they suggested the name vitamin B_{12}. A few weeks later its independent isolation in Britain was reported (Lester Smith and Parker, 1948). With one exception the whole of this work on the isolation of vitamin B_{12} was controlled by clinical tests; the exception is the work of Rickes and his colleagues, which was controlled in part by microbiological tests. This work, and the very rapid developments that have followed, have been reviewed by Lester Smith *et al.* (1950), Folkers (1950), Cuthbertson (1949*b*), Ralt (1950), Lester Smith (1949*b*; 1951), Zucker and Zucker (1950), and in an anonymous report (1950). It is evidence of the increasing interest in the field that nearly half the references cited in the present article are dated 1950 and 1951.

Isolation of Vitamin B_{12}

Since the publication of the pioneer work, the isolation of vitamin B_{12} has been reported from a number of laboratories in Great Britain, America, Holland and Switzerland. They are nearly all industrial laboratories, so it is understandable that publication of the methods of isolation has been delayed. Indeed, the only reasonably complete published account of a method of isolation from liver is that given by Fantes *et al.* (1949). The usual source material today is, however, not liver but a fermentation product of *Streptomyces griseus* (Rickes *et al.*, 1948*c*). Very little information has been released on the preparation or processing of this material, although something can be gleaned from early Merck patents.

Since the source material, whether liver or fermentation liquor, contains only about one part per million of vitamin B_{12} factors, purification involves a long sequence of steps, including at least one chromatographic separation. The account that follows is a compilation from procedures that have been found useful by one or other group of investigators.

If liver is used as source, it must first be minced and extracted with hot water or aqueous alcohol. The crude liver extract, or alternatively the fermentation liquor, may be submitted to some preliminary clarification process, such as boiling, to coagulate proteins and to release any vitamin B_{12} factors present in bound forms. It has been usual next to adsorb the active principle on activated charcoal or fuller's earth. The activity can be eluted only with some difficulty from these adsorbates; among the agents used have been liquefied phenol, hot or cold aqueous alcohols, and mixtures containing pyridine or its homologues. Recovery from the phenolic eluate can be effected by dilution with several volumes of ether and extraction with successive small portions of water; the other solvents can be removed by distillation under reduced pressure. Adsorption on a smaller amount of charcoal has sometimes been used, at the next stage or later, for further purification. Dark-coloured impurities can be precipitated with neutral or basic lead acetate, although not without some risk of loss of activity. Fractional salting out with ammonium sulphate has been employed also; some impurities may be removed with ammonium sulphate up to about one-third saturation, and the active principle is thrown down at about two-thirds saturation. A combined process of salting out and solvent extraction has been worked out independently by two groups. Thus impurities, but little of the activity, can be removed by extraction of aqueous solutions with *n*-butanol, but the active principle is extracted in the presence of rather high concentra-

tions of ammonium sulphate. The vitamin B_{12} factors are very readily extracted from aqueous solution with phenol or cresol, but these agents are unselective; better purification is effected by extraction with phenols mixed with other solvents such as butanol or toluene. Another advantageous use of solvents is for the extraction of a dry concentrate with methanol or ethanol. Further purification is conveniently effected by adding ether, which precipitates the active material from such an extract.

A number of chromatographic procedures have been applied to material partially purified by some of the methods described above. The vitamin may be adsorbed on charcoal in chromatographic fashion by running an aqueous solution of the concentrate through a bed of active charcoal, usually mixed with kieselguhr, which need be no more than one or two cm. thick. Some inactive constituents can be eluted from the adsorbate by means of 6 per cent. aqueous phenol or 10 per cent. aqueous propanol with little loss of active principle. Chromatographic columns of alumina can be used in two ways. Aqueous concentrates passing through such a column leave dark-coloured impurities behind, while the active principle passes through. If, on the other hand, a solution in about 75 per cent. methanol or ethanol is used, the vitamin B_{12} factors are adsorbed and become visible as a red band if the starting material was sufficiently pure. Silica adsorbs the vitamin B_{12} factors lightly from aqueous solutions and can be used for chromatographic purification.

Partition chromatography also has proved valuable, with moist silica, starch or kieselguhr in the columns, and *n*-butanol or solvent mixtures containing phenols for development. Bentonite too has been used for chromatographic purification.

In the British work, in which liver was the starting material, enzymic proteolysis has proved to be an essential step for breaking down persistent peptide impurities and so permitting their removal. The methods of purification employed have been almost exclusively physical in nature, because vitamin B_{12} appeared to have few chemical properties that could be exploited. The only chemical step that has proved useful, though not essential, is precipitation with Reinecke acid or with phosphotungstic acid.

When purification has proceeded to a sufficient degree, vitamin B_{12} crystallises readily from aqueous acetone in deep red needles (Lester Smith, 1948*a*; Fantes *et al.*, 1949; Ellis *et al.*, 1949*a*; Lester Smith, 1949*a*; Merck and Co., Inc., 1949).

Chemistry of Vitamin B_{12}

The constitution of vitamin B_{12} is as yet by no means fully elucidated, but already several unexpected features have come to light. The first surprise was that the molecule contains one atom each of cobalt and phosphorus (Lester Smith, 1948*b*; Rickes *et al.*, 1948*b*). Even before pure vitamin B_{12} had been isolated it was known that mild acid hydrolysis released a red acidic fragment of the molecule, which was readily extracted by butanol (Lester Smith, 1948*a*). The acid hydrolysis of vitamin B_{12} has since been studied in some detail. The first fragment to be fully characterised was 5:6-dimethylbenziminazole (Brink and Folkers, 1949; 1950; Holiday and Petrow, 1949; Beaven *et al.*, 1949). Somewhat less drastic treatment yielded 1-α-D-ribofuranosido-5:6-dimethylbenziminazole (Brink, Holly *et al.*, 1950). It is interesting to note that a very similar molecular structure occurs in riboflavin, except that the sugar chain is linked as a ribityl derivative instead of as a ribose glucoside. Still milder conditions of hydrolysis yielded a phosphorylated 1-α-D-ribofuranosido-5:6-dimethylbenziminazole (Buchanan *et al.*, 1950*a*, *b*). These three stages of degradation can be identified with γ-, β- and α-components described by Ellis *et al.* (1949*a*), Beaven *et al.* (1949), and Cooley, Ellis, Mamalis *et al.* (1950). The rate at which inorganic phosphorus is released had been studied earlier (Ellis *et al.*, 1949*c*).

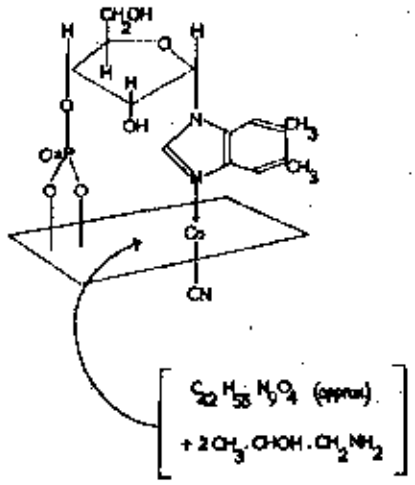
Other hydrolytic fragments were ammonia and a colourless substance with no selective ultraviolet absorption, but giving a purple colour with ninhydrin. Behaviour on paper chromatograms indicated that this substance might be 2-amino-1-propanol (Ellis *et al.*, 1949*b*, *d*), but the suggestion was later withdrawn (Cooley, Ellis and Petrow, 1950*a*, *b*; Buchanan *et al.* 1950*a*). Brink, Wolf *et al.* (1949) at first failed to detect this ninhydrin-reacting substance, but later identified it as n_g-1-amino-2-propanol (Wolf *et al.*, 1950). Chargaff *et al.* (1950) showed that two molecules of aminopropanol are present in the vitamin B_{12} molecule. They were unable to detect purines, pyrimidines or amino-acids after acid hydrolysis.

Very little has been published about the chemistry of the red fragment liberated on acid hydrolysis. It is acidic and can be readily esterified (Ellis *et al.*, 1949*a*). There is a little evidence that this part of the molecule may have a porphyrin structure: thus, on fusion with sodium hydroxide, the distillate gave pyrrole reactions (Brink, Wolf *et al.*, 1949; see also Veer *et al.*, 1950).

It has recently been disclosed in nearly simultaneous publications from Holland and the United States that vitamin B_{12} contains a cyan group attached to the cobalt atom (Veer *et al.*, 1950; Brink, Kuehl and Folkers, 1950; Kaczka *et al.*, 1950). From spectroscopic evidence it is suggested that the benziminazole N^3 also is co-ordinately linked to the cobalt atom (Beaven *et*

25 61

al., 1950). It is now possible to write the following partial formula for vitamin B_{12}:



Identification of a Group of Factors with Vitamin B_{12} Activity

Several of the vitamins, vitamin B_6 and vitamin E, for example, comprise groups of compounds closely related chemically and having similar physiological activities. The anti-pernicious-anaemia factor also is in this category. The first indication of its multiple nature was the appearance of a second red zone, containing clinically active material, on partition chromatograms of purified liver extract (Lester Smith, 1948*a*). The second red substance was separated in crystalline form and named vitamin B_{12b} by Pierce *et al.* (1949). Later work proved it to be identical with vitamin B_{12a} prepared by treatment of vitamin B_{12} with hydrogen and a platinum oxide catalyst (Kaczka *et al.*, 1949; Kaczka, Denkewalter *et al.*, 1951). It is now known that vitamin B_{12b} is identical with vitamin B_{12} except for replacement of the cyan group by hydroxyl (Brink, Kuehl *et al.*, 1950; Kaczka *et al.*, 1950). It can be obtained also by treating vitamin B_{12} with bisulphite (Fricke *et al.*, 1950), or by exposure to light (Brockman, Pierce *et al.*, 1950; Veer *et al.*, 1950). Vitamin B_{12a} or vitamin B_{12b} can readily be converted into vitamin B_{12} by treatment with cyanide. Kaczka and his colleagues (1950) have suggested the term "cobalamin" for the vitamin B_{12} molecule without the cyan group; vitamin B_{12} then becomes cyanocobalamin and vitamin B_{12a} hydroxocobalamin.

In addition to vitamin B_{12} itself and vitamin B_{12b}, two other factors, vitamins B_{12c} and B_{12d}, have been obtained from the fermentation liquors of *Streptomyces griseus* (Anslow *et al.*, 1950). Vitamin B_{12c} arises also by treatment of vitamin B_{12b} with nitrous acid, and appears to differ from vitamin B_{12} only in having a nitrite in place of the cyan group (Lester Smith *et al.*, 1951). Vitamin B_{12d} arises on removal of the nitrite group from vitamin B_{12c} as, for instance, by treatment with sulphamic acid or on distilling an acidified solution under reduced pressure. It behaves similarly to vitamin B_{12b}, and has the same absorption spectrum, but the two can be separated by partition chromatography. Vitamins B_{12b} and B_{12d}, and in some instances vitamin B_{12} itself, form loose adducts with a number of reagents, including sulphite, cyanide, thioglycollate and iodine. Some of the adducts have characteristic absorption spectra and differ from the parent factors in their partition behaviour. The cyanide adduct, which is purple in colour, presumably contains two cyan groups (Kaczka *et al.*, 1950; Lester Smith *et al.*, 1951).

Physical Constants of B_{12} Vitamins

The molecular weight of anhydrous vitamin B_{12} is about 1300. It has been determined from the percentage of cobalt present, which was found to be 4·5 (Fantes *et al.*, 1949), by X-ray crystallography (Hodgkin *et al.*, 1949), and by measurement of the elevation of the boiling-point (Brink, Wolf *et al.*, 1949). The crystals from aqueous acetone or water usually contain about 17 per cent. of water of crystallisation which can be removed completely only by heating *in vacuo*; the anhydrous material is hygroscopic. Vitamin B_{12} crystallises in needles or prisms of the orthorhombic system. Single crystal and powder X-ray photographs have been recorded (Hodgkin *et al.*, 1949). The refractive indices of air-dried crystals are α 1·616; β 1·652; γ 1·664 (Rickes *et al.*, 1948*a*).

The complex infrared absorption spectrum has been recorded by Barer *et al.* (1949; see also Brink, Kuehl *et al.*, 1950). That of vitamin B_{12b} has been determined by Pierce *et al.* (1950). The ultraviolet and visible absorption spectra of the vitamin B_{12} factors are characteristic. There is general agreement between different publications on the positions of the maxima, but the figures quoted for the intensities of absorption are variable, presumably because some of the specimens used were anhydrous and others contained moisture or were otherwise impure. Available data are given in Table 1.

The optical rotation of vitamin B_{12} can be determined only with difficulty because of the intense colour of the solutions. For vitamin B_{12} itself the following values have been recorded:

$$[\alpha]^{20°}_{4358} = -110°$$
$$[\alpha]^{22°}_{5461} = -59°$$

For the other B_{12} vitamins the following values of $[\alpha]^{23°}_{4358}$ have been recorded:

2250

#2561

Vitamin B_{12c}	+ 50°
Vitamin B_{12a}	− 20°
Vitamin B_{12d}	− 15°

Electrometric titration in aqueous solution reveals no acidic or basic group, but titration in glacial acetic acid reveals a number of weakly basic groups. Measurements of electrical conductivity and mobility, and of polarographic behaviour, have been recorded also (Fantes *et al.*, 1949; Brink, Wolf *et al.*, 1949). Vitamins B_{12} and B_{12c} are diamagnetic, which indicates that the cobalt is in the trivalent state (Diehl *et al.*, 1950; Tew, 1950, unpublished work).

TABLE I

PRINCIPAL SPECTRAL ABSORPTION MAXIMA IN ULTRA-VIOLET AND VISIBLE LIGHT OF THE B_{12} VITAMINS.

Vitamin B_{12}		Vitamin B_{12a}	Vitamin B_{12c}	Vitamin B_{12d}	Vitamin B_{12a} SO_2 adduct	Vitamin B_{12a} CN adduct
λ Max. mμ.	$E^{1\,\text{per cent.}}_{1\,\text{cm.}}$	λ Max. mμ.	λ Max. mμ.	λ Max. mμ.	λ Max. mμ.	λ Max. mμ.
278	115	274	270 (inflection)	274	275	
361	204	351	359	351	312 364	367
527 518 to 550	57 63	500 525	412 530	500 525	415 515 540	541 580

STABILITY OF B_{12} VITAMINS

Crystalline vitamin B_{12} can be heated at 100° C. for long periods without decomposition. Aqueous solutions at *pH* 4 to 7 can be autoclaved without losing more than a small part of their activity. Autoclaving or standing for some days at room temperature at *pH* 2 causes considerable, but not complete, loss of activity; destruction is more rapid at *pH* 9. Concentrates and the other vitamin B_{12} factors tend to be less stable than vitamin B_{12} itself. Heavy metals and strong oxidising and reducing agents are destructive; phenols may be safely used to preserve solutions (see Fantes *et al.*, 1949; Rickes *et al*, 1948*b*; Tromer *et al.*, 1950; Hartley *et al.*, 1950; Leng and Chow, 1950).

PREPARATION OF RADIO-ACTIVE VITAMIN B_{12}

Study of the absorption and metabolism of vitamin B_{12} would be made easier if it could be "tagged" with radio-active isotopes. The presence of cobalt and phosphorus in the molecule makes this idea attractive, but early attempts to prepare tagged vitamin B_{12} were not successful. As had been expected, no significant chemical exchange occurred with radio-active cobalt salts or phosphate (Fantes *et al.*, 1949), nor could useful amounts of the labelled vitamin be extracted from the livers of pigs or rats given radio-active cobalt (Braude *et al.*, 1949; Cuthbertson *et al.*, 1950). Chaiet *et al.* (1950) succeeded in making vitamin B_{12} containing Co^{60} by fermentation. The specific activity quoted of 0·25 μC. per mg. would, however, be inconveniently low in view of the high potency of vitamin B_{12}. The reviewer has recently succeeded in preparing vitamin B_{12} containing Co^{60} with about seventy times this specific activity.*

BOUND FORMS OF VITAMIN B_{12}

The anti-pernicious-anaemia factor appears to be present in liver as a protein complex (Cohn, Surgenor, Greene, Porsche and Lesh, 1949; Cohn, Surgenor, Greene, Hunter and Kahut, 1949). The link with protein is readily ruptured by the methods ordinarily used to prepare liver extracts, but there is evidence that the substance may still exist in a bound form in purified, unautolysed liver extracts (Wijmenga *et al.*, 1950). *Streptomyces griseus* fermentation liquors sometimes contain all or part of their vitamin B_{12} content in a bound form which is inactive microbiologically until it is split by boiling or proteolysis. Similar bound forms are present in some foods and in faecal extracts (Pennington, 1951; see also Hausmann, 1949).

Ternberg and Eakin (1949) showed that gastric juice combines with vitamin B_{12} to form a non-dialysable, microbiologically inactive complex, which can be split by heat. It is possible that this "vitamin B_{12}-combining factor" is identical with Castle's intrinsic factor which promotes the absorption of vitamin B_{12} given orally to patients with pernicious anaemia.

NON-BIOLOGICAL METHODS OF ESTIMATION

Moderately pure concentrates can be assayed colorimetrically or spectrophotometrically. A method can be based on colorimetric estimation of cobalt with one of the nitrosonaphthols, after wet ashing, either of the whole sample, or better, of a butanol extract after acid hydrolysis, to release the red cobalt-containing acidic fragment of the molecule. A colorimetric method has been devised which depends on the ready esterification of this red acid when shaken with octyl alcohol, and on the removal of coloured impurities by washing its solution in light petroleum with aqueous methanol (Fantes and Ireland, 1950). Boxer and Rickards (1950) split off the benzimidazole fragment with

* Rosenblum and Woodbury (1951) have since reported the preparation of radio-active vitamin B_{12} with a specific activity of 67 μC. per mg.

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acid, benzoylate, then hydrolyse again, and couple the resulting dimethylphenylenediamine with acetyl acetone to form a purple compound or with alloxan to form an intensely fluorescent alloxazine. The same authors are developing another method based on photolytic release of HCN from the vitamin B_{12} molecule, aspiration into alkali, and colorimetric estimation with pyrazolone reagents.

Microbiological Assay

The possibility of assaying vitamin B_{12} microbiologically was first mentioned by Shorb (1948), who used *Lactobacillus lactis* Dorner ATCC 8000. Various media and a number of different strains of *Lactobacilli* have since been recommended. Thus Caswell *et al.* (1949) used *L. lactis* ATCC 1097, v. Koeher and Schindler (1949) *L. lactis* ATCC 1175; Hoffmann *et al.* (1948) recommended *L. leichmannii* 313 ATCC 7830, and Skeggs *et al.* (1948) favoured *L. leichmannii* ATCC 4797. These organisms are all used in methods in which the amount of growth in a series of tubes containing a range of dilutions is measured, usually turbidimetrically, after overnight incubation. As an alternative, acid production can be measured after about three days' incubation.

Great difficulties have been due in part to the need of the organisms for certain unidentified substances, usually supplied in the form of tomato juice. It has recently been shown that the substances contributed by tomato juice are pyridoxamine phosphate and pyridoxal phosphate (Hendlin, Caswell *et al.*, 1950). Another difficulty has been the sensitivity of the organisms to the oxygen content of the medium; anaerobically they will grow without vitamin B_{12} (Lees and Tootill, 1951). In addition, there is a tendency for part of the vitamin B_{12} in the sample to be destroyed if it is autoclaved with the medium. It is claimed that both difficulties can be overcome by including traces of reducing agents in the medium, such as thioglycollic acid (see Shive *et al.*, 1948; Greene *et al.*, 1949; Koditschek *et al.*, 1949; Wright *et al.*, 1948; Shaw, 1949*a*, *b*, *c*; Shorb and Briggs, 1948; Lees and Emery, 1949). Techniques devised since all these points were appreciated give satisfactory results (Peeler *et al.*, 1949; Skeggs *et al.*, 1950).

For routine use the methods with a series of tubes have been largely displaced by the less sensitive but more convenient and reliable cup-plate method (Cuthbertson, 1949*a*; Foster *et al.*, 1949). Interference can be caused in all these microbiological methods by the presence of thymidine and other purine and pyrimidine deoxyribosides (Snell *et al.*, 1948; v. Koeher, 1949; Lester Smith and Cuthbertson, 1949). The cup-plate methods are more sensitive than the tube methods to interference by deoxyribosides but, since these cause the zones on the agar plates to be more diffuse, the interference is easily detected. It has been claimed that the deoxyriboside zones on agar can be eliminated by adding salt to the medium (Foster *et al.*, 1949). Alternatively, the deoxyribosides can be separated from the vitamin B_{12} factors by paper chromatography. With the tube method, the only way to assess interference is to destroy the vitamin B_{12} in the sample by heating with alkali and to repeat the test, any remaining response being attributed to deoxyribosides (Hoffmann *et al.*, 1949).

By far the most sensitive microbiological method for vitamin B_{12} is with *Euglena gracilis* var. *bacillaris* (Hutner, Provasoli, Stokstad *et al.*, 1949; Hutner, Provasoli, Schatz *et al.*, 1950). The organism can be grown on a very simple medium, but unfortunately it requires continuous illumination and an assay with it takes from four to seven days. It provides the only method sensitive enough to estimate vitamin B_{12} in body fluids (Ross, 1950).

Another organism strongly recommended recently for vitamin B_{12} assays is a mutant of *Bact. coli* (Davis, 1950; Davis and Mingiolo, 1950). It can be grown in a very simple medium, and is not sensitive to the presence of deoxyribosides, though rather high levels of methionine interfere with the test. It can be adapted to the cup-plate or the tube technique (Bessell *et al.*, 1950).

The various B_{12} vitamins give similar responses in all the microbiological assay methods. There is some indication that vitamins B_{12c} and B_{12d} do not give sharply edged zones in some cup-plate methods (Anslow *et al.*, 1950).

Vitamins B_{12}, B_{12b} and B_{12d} and the various deoxyribosides, can all be separated by paper chromatography. Their presence in preparations such as liver extracts can be demonstrated, and an approximately quantitative estimation can be effected by laying the developed papergram on a sheet of nutrient agar seeded with *Lactobacillus lactis* or *Lactobacillus leichmannii*; on overnight incubation each factor gives rise to a circular or elliptical zone of growth (Lester Smith and Cuthbertson, 1949; Cuthbertson and Lester Smith, 1949; Winsten and Eigen, 1949*a*, *b*; Woodruff and Foster, 1950; v. Koeher, 1949; v. Koeher *et al.*, 1950).

The Place of Vitamin B_{12} in Nutrition

For many years fruitless attempts were made to induce deficiency of the anti-pernicious-anaemia factor in experimental animals. It now appears that the investigations failed partly because vitamin B_{12} is rather more widely distributed in

foods than was formerly appreciated, but also because attention was focused on the haemopoietic rather than on the growth-promoting action of the vitamin. Thus attempts were made to induce a condition akin to pernicious anaemia by bleeding, by the use of substances such as phenylhydrazine or lead compounds which destroy red cells, or by an operation designed to produce a stagnant intestinal loop (Watson *et al.*, 1948).

In the end, but too late to be of any help with the isolation of vitamin B_{12}, the right technique was discovered almost accidentally. Indeed, Combs *et al.* (1950) came near to independent isolation of vitamin B_{12} by using chick growth as an assay method. Attempts in the United States to eliminate scarce and expensive animal proteins from the diets of farm animals led to the appearance of signs of deficiency. The growth rates of pigs and chicks were adversely affected, and laying hens produced eggs of low hatching capacity. Raising the level of certain sources of vegetable protein, such as soya bean, made matters worse. The signs of deficiency were prevented or cured by the addition of fishmeal or cow manure to the diet of the chicks (Hammond and Titus, 1944; Bird, 1946). Cow manure and the factor associated with animal protein and called the "animal-protein factor" were fractionated (Rubin *et al.*, 1947; Bird *et al.*, 1948), and it was shown that the factors in cow manure concentrate and fish solubles had very similar properties (Robblee *et al.*, 1948). Cary *et al.* (1946) demonstrated the presence of a rat growth factor in liver extracts effective in pernicious anaemia. Zucker and Zucker (1948*a*, *b*) coined the term "zoopherin" for a rat growth factor present in animal proteins.

By this time it was becoming probable that the different factors were identical, and the activity of cow manure suggested that they might be synthesised by micro-organisms. This idea was strengthened by the observation that the factor was apparently produced in the fermentation of food materials by organisms present in chick faeces, since chicks could manage without animal protein in their food during the warm weather, which would promote rapid fermentation of their droppings. The matter was clinched when it was shown that an organism isolated from hen faeces could synthesise a factor effective in promoting chick growth and in treating human cases of pernicious anaemia (Stokstad *et al.*, 1948). Then it was found that crystalline vitamin B_{12}, whether given orally or by injection, had the same effect for chicks as the animal-protein factor (Ott *et al.*, 1948; Lillie *et al.*, 1948).

Subsequently, however, as will be mentioned later, doubts have arisen whether vitamin B_{12} can supplement vegetable protein so that it completely replaces animal protein from natural sources, although it is undoubtedly a principal component of the animal-protein factor.

Requirements of Vitamin B_{12}

Chickens

There is ample evidence that chicks raised on vegetable protein diets, especially when the protein level is higher than normal, require vitamin B_{12}. In some laboratories, however, it has been possible to demonstrate a significant requirement of vitamin B_{12} only by using chicks hatched from the eggs of hens deprived of vitamin B_{12}. Perhaps the most dramatic evidence was that which showed that if eggs laid by deficient hens were injected with 0·5 to 1·25 μg. vitamin B_{12}, the hatching rate was significantly increased over that of untreated eggs; in addition the chicks from the treated eggs grew faster, had a lower mortality rate, and showed better feathering than those from untreated ones (Lillie *et al.*, 1949). Deficiency of vitamin B_{12} often leads to chick embryos being deformed (Olcese, Couch, Quisenberry and Pearson, 1950), and the vitamin has been found to control gizzard erosion of chicks (Mushett and Ott, 1949). On the other hand, there is a good deal of evidence suggesting that vitamin B_{12} is not the only factor that will improve these deficient diets for chicks. Dietrich *et al.* (1949) showed that vitamin C and folic acid were necessary also; a combination of vitamins C and B_{12} appeared to stimulate folic acid synthesis in the chick and, conversely, folic acid appeared to stimulate vitamin B_{12} synthesis. A number of workers have given evidence of the need for unidentified factors (Olcese, Couch and Lyman, 1950; Combs *et al.*, 1950; see also Singsen and Matterson, 1950; Carlson *et al.*, 1949).

Richardson *et al.* (1949) showed that the growth of chicks on a cottonseed or soya bean meal diet containing 40 μg. vitamin B_{12} per kg. could be improved by the addition of 0·5 or 0·8 per cent. of D-lysine. Machlin *et al.* (1950) similarly showed that 0·8 per cent. of DL-lysine and 0·1 per cent. of DL-methionine were useful supplements in addition to vitamin B_{12}. Gillis and Norris (1949), and Schaefer *et al.* (1949*a*, *b*) showed that vitamin B_{12} has a sparing action on choline or methionine and, conversely, Briggs *et al.* (1950) showed that DL-methionine (0·3 per cent.) and, to a less extent, choline have a sparing effect on vitamin B_{12}. Stokstad *et al.* (1949) showed that the vitamin B_{12} requirement of chicks on a high soya bean meal diet was satisfied by 0·3 μg. injected weekly or 1·5 μg. per kg. diet. Thus utilisation was from 50 to 75 per cent. as effective by the oral as by the parenteral route, in marked contrast with the situation in pernicious anaemia. An animal-protein factor concentrate from *Streptomyces*

aureofaciens was more effective than pure vitamin B_{12}. Since the concentrate contained a small amount of aureomycin, this antibiotic was tested (Whitehill *et al.*, 1950). The addition of 25 mg. of it per kg. diet supplemented the effect of vitamin B_{12} but, surprisingly, the antibiotic alone was more effective than vitamin B_{12} alone. It also increased the growth rate of chicks on normal diets. It was ineffective when injected, so the effect must have been on the intestinal flora. Penicillin at a level of 100 mg. per kg. diet was equally effective, though other antibiotics tested were not (see also Stokstad and Jukes, 1950). This work has recently been confirmed in Britain (Coates *et al.*, 1951).

Chicks can be used for the estimation of vitamin B_{12} in foods and other materials used as sources of the vitamin. The results must be accepted with caution, however, because they are liable to be too high if the material contains unidentified factors required by the chick, and particularly if it contains antibiotics (Stokstad *et al.*, 1949).

Pigs

Wintrobe and his colleagues have induced deficiencies in pigs by using purified diets in which folic acid antagonists or sulpha drugs were included (Cartwright *et al.*, 1948; Cartwright and Wintrobe, 1949*a*, *b*). The anaemia and other symptoms responded to folic acid and folic acid conjugates, but only partially to liver extracts or vitamin B_{12}. Similar results were obtained by Heinle *et al.* (1948; 1949). Liver extract or vitamin B_{12} gave a very poor response but, after folic acid had produced its maximum response, liver or vitamin B_{12} caused further improvement. Connor Johnson and his associates showed that liver extract or vitamin B_{12} was required by baby pigs on a synthetic milk diet containing soya bean protein (Neumann *et al.*, 1948; Connor Johnson and Neumann, 1949; Neumann *et al.*, 1950; Nesheim and Connor Johnson, 1950). During the first eight weeks they found it sufficient to inject 0·6 μg. vitamin B_{12} per kg. bodyweight daily, or to add 20 μg. vitamin B_{12} per kg. dry feed, which represents about twice the parenteral requirement. On a diet containing folic acid antagonists or sulpha drugs baby pigs required folic acid as well as vitamin B_{12} for optimum growth and haemopoiesis (Connor Johnson *et al.*, 1950). Hogan and his colleague found that baby pigs required 0·26 μg. vitamin B_{12} daily per kg. bodyweight given orally, or not more than 15 μg. per kg. feed (Anderson and Hogan, 1950; Hogan, 1950). On a deficient diet sows could not rear their young unless given vitamin B_{12} injections. The value of commercial animal-protein factor concentrates was tested for pig rearing, and it was found also that these concentrates lessened the protein requirement of the pigs (Cunha, Burnside *et al.*, 1949; Burnside *et al.*, 1949; Cunha, Hopper *et al.*, 1950). Catron *et al.*, 1950; see also Burnside *et al.*, 1950). Catron *et al.* (1949) have suggested that 10 to 20 μg. vitamin B_{12} per kg. of total ration is a suitable amount for pigs at the growing and fattening stage. In Britain, the value of liver extract for piglets has been confirmed (Brando, 1949).

It has recently been found that certain antibiotics supplement the growth-promoting effect of vitamin B_{12} for pigs in the same way as they do for chickens. Thus, inclusion of from 50 to 200 mg. aureomycin per kg. feed increased the growth rate over that obtained with an adequate amount of a vitamin B_{12} concentrate or of the crystalline vitamin (Jukes, Stokstad, Taylor *et al.*, 1950) and, although 12·5 μg. vitamin B_{12} per lb. feed caused only 10 per cent. greater gain in weight than in untreated animals, the further addition of 10 per cent. of streptomycin raised the increase in gain to 40 per cent. The same gain was produced by a Lederle animal-protein factor concentrate presumably containing aureomycin (Luecke *et al.*, 1950; see also Carpenter, 1950).

Rats

Young rats on a suitable deficient diet require vitamin B_{12} (Emerson, Wurtz and Zanetti, 1949). On account of storage of vitamin B_{12} in the liver and probably elsewhere, it has usually been found necessary to rear rats for tests from deprived mothers. A more reliable assay method has been based on the observation of Ershoff (1947) that administration of thyroxine increases vitamin requirements, although Ershoff himself has denied that the antithyrotoxic factor was vitamin B_{12} (Ershoff, 1948; 1949). On a high-protein diet containing a sulpha drug and iodinated protein rats readily became deficient and then responded to a daily dose of from 0·025 to 0·1 μg. vitamin B_{12} (Frost *et al.*, 1949). The vitamin supplement was equally effective whether given orally or by injection in single or divided doses. The results of tests made on liver extracts by this method agreed well with those of microbiological assays. Meites and Newland (1950) confirmed the finding that vitamin B_{12} counteracted the growth-inhibiting action of thyroprotein, although it had only a slight effect on growth inhibition due to diethylstilboestrol, and Sure and Easterling (1950) showed that as little as 0·3 μg. vitamin B_{12} would protect a rat against an otherwise fatal dose of 200 μg. thyroxine. Emerson, Kummer and Zanetti (1949) suggest that liver may contain another substance besides vitamin B_{12} that stimulates post-lactation growth of rats. Cuthbertson (1949*b*) found that

the deficiency induced by a vegetable diet containing L-thyroxine was not completely relieved by vitamin B_{12}, but was prevented by giving with it vitamin B_{12}, prepared by the method of Novak and Hange (1948).

Mice

The mouse has been used for vitamin B_{12} assays. Two methods are described; in one, only a deficient diet is used; in the other a thyroid preparation is included to exaggerate vitamin B_{12} requirement (Bosshardt *et al.*, 1949). It is claimed that fat has a sparing action on the vitamin B_{12} requirement of mice (Bosshardt *et al.*, 1950).

Dogs

Need for vitamin B_{12} appears to have been demonstrated in dogs. On a diet low in protein and nicotinamide and containing sulphasuxidine, they developed blacktongue and a macrocytic anaemia. After treatment with nicotinamide and folic acid the anaemia persisted; subsequently liver extract restored the blood picture to normal (Huegamer *et al.*, 1948).

Other Mammals

The possibility that rabbits may need vitamin B_{12} is suggested by the work of Thacker and Ellis (1948).

A factor either identical with vitamin B_{12} or similar in its properties is required by the fox (Schaefer *et al.*, 1948) and the mink (Tove *et al.*, 1950*a*, *b*; Tove *et al.*, 1949). No requirement for vitamin B_{12} could be demonstrated for the Syrian hamster (Scheid *et al.*, 1950).

Origin and Distribution in Nature of Vitamin B_{12}

It seems probable that the only primary source of vitamin B_{12} in nature is the metabolic activity of micro-organisms; there is no convincing evidence for its elaboration in tissues of higher plants or animals. It is synthesised by a wide range of bacteria and actinomycetes, though apparently not to any extent by yeasts or fungi (Stokstad *et al.*, 1948; Rickes *et al.*, 1948*c*; Ansbacher *et al.*, 1949; Halbrook *et al.*, 1950). Not surprisingly, the addition of about one part per million of cobalt to the culture medium increases the yield of vitamin B_{12} on fermentation with a number of micro-organisms (Hendlin and Ruger, 1950). Commercial vitamin B_{12} is now usually produced by fermentation with *Streptomyces griseus* or some other organism such as *Bacillus megatherium* (Garibaldi *et al.*, 1950).

The traces of vitamin B_{12} present in soils (Cunha, Burnside *et al.*, 1949) and some pond waters (Robbins *et al.*, 1950) doubtless arise from microbial synthesis. Such lower animals as worms and snails may well derive their vitamin B_{12} from the soil (Zucker and Zucker, 1948*c*). In the roots of higher plants traces have been detected (Robbins *et al.*, 1950), but they again are probably derived from the soil. There have been conflicting reports about the presence in grasses and other plants of material having animal-protein factor activity, or responding positively in microbiological tests for vitamin B_{12} (Hartman *et al.*, 1949*a*; Zucker and Zucker, 1948*a*, *b*; Lewis *et al.*, 1949). The microbiological activity is undoubtedly due largely, if not entirely, to the presence of deoxyribosides (Bickoff *et al.*, 1950; Peeler *et al.*, 1950), and on the balance of evidence it can be said that there remains little if any proof of the existence of significant amounts of vitamin B_{12} in vegetable foods.

There is no difficulty in accounting for the presence of vitamin B_{12} in the liver and tissue of ruminants on the basis of microbial synthesis, for it has been shown that some rumen bacteria can synthesise vitamin B_{12}, and rumen contents are known to be fairly rich in the vitamin (Zucker and Zucker, 1948*c*; Abelson and Darby, 1949). It is noteworthy that animal livers rich enough in vitamin B_{12} to be used for the manufacture of liver extracts are mostly those from ruminants. Pig livers, for example, are an uncertain source (Dedichen, 1949; Braude *et al.*, 1949; see also Mondolfo, 1949), and rat livers have been found to be low in the vitamin (Cuthbertson *et al.*, 1950). In discussing the part played by the micro-organisms in the rumen, it should be mentioned that the requirement of ruminants for cobalt appears to be partly an indirect one; that is, the cobalt is required by the rumen bacteria, which in its presence can perform the function of assisting the animal to digest its food (Gall *et al.*, 1949). Although in the presence of adequate cobalt the rumen organisms produce more vitamin B_{12}, it is doubtful whether this is the only function of cobalt; certainly vitamin B_{12}, whether given by injection or by mouth, is of no use to the animals instead of cobalt (Becker *et al.*, 1949; Marston and Lee, 1949).

It is not clear how purely herbivorous animals other than ruminants get their vitamin B_{12}, but it must presumably be through the symbiotic activity of intestinal organisms. It is known that pernicious anaemia patients may excrete large amounts of vitamin B_{12} in their faeces; they are evidently unable to absorb adequate amounts but, since pernicious anaemia appears to be due primarily to a defect in the ability to absorb vitamin B_{12}, the observation does not prove

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that normal human beings and animals cannot absorb vitamin B_{12} synthesised in the intestine (Dyke *et al.*, 1950). Hartman *et al.* (1949*c*) reported that of rats on a deficient diet, some throve, apparently because they were able to synthesise an "X factor" (probably vitamin B_{12}), while others in the same group did not. Small doses of faeces from the rats that grew induced growth in the others. All the animals failed to grow when the diet included sulphaguanidine. The experiments would appear to indicate that vitamin B_{12} can be both synthesised and absorbed when the intestinal flora is suitable.

The α : 6-dimethylbenziminazole component of the vitamin B_{12} molecule has been shown to have vitamin B_{12}-like activity for rats (Emerson *et al.*, 1950). Its action may be as a precursor of the vitamin, promoting synthesis by the intestinal organisms.

It is too early to give a detailed picture of the distribution of vitamin B_{12} in foods. It appears to be widely distributed in animal products and to be almost, if not entirely, absent from the products of the higher plants. Register *et al.* (1949) estimated vitamin B_{12} in pig and beef muscle by the growth of rats, and found about 2 and 1 μg., respectively, per 100 g., although some samples were abnormal in containing only a trace. Later, by giving thyroid to shorten the depletion period, they extended the use of their rat method to a wider range of foods (Lewis *et al.*, 1949). They found the following minimum values:

	μg. per 100 g.
Various fresh meats	1 to 3
Beef kidney and liver	15 to 20
Egg yolk	1·4
Cheese	1·4
Dried casein	1
Dried rumen contents	50
Milk	Traces

For cow's milk 2 to 3 μg. per litre is quoted, but very much less for goat's milk (Collins *et al.*, 1950).

Microbiological estimations of vitamin B_{12} in food are apt to be unreliable because of the interfering effects of deoxyribosides, and because the vitamin is often present in a bound form which is microbiologically inactive. The bound form could be released from animal tissues by enzyme digestion or heating (Scheid and Schweigert, 1950). Thompson *et al.* (1950) found tryptic digestion preferable to autolysis or digestion by pancreatin. They quoted the following values:

	μg. per 100 g.
Cooked beef	3 to 8
Fresh beef kidney and liver	42 to 47
Veal and lamb	2 to 8
Cooked pork	0·1 to 5

Peeler *et al.* (1950) recommend pancreatin digestion for the release of vitamin B_{12} from animal products. They claim good agreement between the results of microbiological and of chick assays.

The microbiological methods have been applied more extensively to the evaluation of liver extracts (Cuthbertson, Lloyd *et al.*, 1949; Girdwood and Carmichael, 1950).

Pharmacology and Mode of Action

The toxicity of vitamin B_{12} is very low despite its high potency. Winter and Mushett (1950) were unable to produce toxic effects with crystalline vitamin B_{12} in amounts of up to 100 mg. per kg. bodyweight in rats and guinea-pigs, and of up to 1600 mg. per kg. bodyweight in mice. The toxic effects reported by Traina (1950*a*) were presumably due to other substances in the concentrate he employed.

It is now clear that both vitamin B_{12} and folic acid play some part in the synthesis and metabolism of labile methyl groups and possibly of other one-carbon fragments. In conjunction with vitamin B_{12}, the methionine requirement of chicks could be met with homocystine (Jukes, Stokstad and Broquist, 1950). Similarly, rats thirty days old, but not younger, could use diets free from labile methyl compounds if they were supplemented with vitamin B_{12} as well as homocystine (Stekol and Weiss, 1950; see also Stekol *et al.*, 1950). It is concluded that vitamin B_{12} may play a decisive role in the synthesis and utilisation of labile methyl compounds. In rats and chicks vitamin B_{12} and folic acid were found to be concerned in transmethylation reactions (Salmon, 1950; Schaefer *et al.*, 1950). Methionine could be synthesised from homocystine, together with choline or betaine, by liver slices from rats given vitamin B_{12}, but liver slices from rats deficient in vitamin B_{12} were far less effective (Oginsky, 1950). Intestinal micro-organisms could play no essential part, since labile methyl groups were synthesised by the bacteriologically sterile rat, reared aseptically (Du Vigneaud *et al.*, 1950; see also Arnstein and Neuberger, 1951). For leucocyte production in the rat, a diet low in methionine and betaine did not supply sufficient labile methyl groups, but methionine alone, or betaine in conjunction with vitamin B_{12}, permitted adequate leucocyte production (Dimming *et al.*, 1950).

Possibly linked with the effect of vitamin B_{12} on methionine synthesis is the observation that some vitamin B_{12} concentrates increased the reduction of —S—S— compounds by liver slices or homogenates (Dubnoff, 1950*a*, *b*). Pure vitamin B_{12} and vitamin B_{12a} did not show the effect, which might be due to some conjugate, or otherwise modified form, of vitamin B_{12}.

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Vitamin B_{12} appears to have a lipotropic effect in rats (Drill and McCormick, 1949; McCormick and Drill, 1950; György and Rose, 1950; Schäfer *et al.*, 1950), and to have some protective effect against liver injury caused by carbon tetrachloride (Popper *et al.*, 1949; see also Mushett, 1950). Trains (1950*b*, *c*) claimed that vitamin B_{12} has an antihistamine and anti-anaphylactic effect, but the former has not been confirmed (Sharpe *et al.*, 1950).

Vitamin B_{12} appears also to have some function in connection with protein metabolism. It is suggested that vitamin B_{12} helps to metabolise protein when protein is present in the diet in very large amounts (Hartmann *et al.*, 1949*b*). It increased the biological value of casein (Henry and Kon, 1951) and seemed to enhance the utilisation of amino-acids circulating in the blood, since the blood levels were lower in chicks receiving vitamin B_{12} than in deficient chicks (Charkey *et al.*, 1950). On the other hand, vitamin B_{12} did not improve the nitrogen retention of rats (Chow and Barrows, 1950), so that when it enhances growth, the improvement is presumably related to the metabolism of carbohydrate or fat.

Finally, it is clear that vitamin B_{12} must have some function in connection with the synthesis of nucleic acids by bacteria and probably by higher animals also. Jukes, Broquist and Stokstad (1950) suggest that vitamin B_{12}, which is synthesised by many bacteria, controls the synthesis of the deoxyribosides of guanine, hypoxanthine, adenine and cytosine. The interconversion of these deoxyribosides and thymidine appears to be controlled, however, by the citrovorum factor or folinic acid (Bardos *et al.*, 1949; Brockman, Roth *et al.*, 1950), which in turn can be synthesised from folic acid by *L. leichmannii* and probably by other organisms. Presence of folic acid and vitamin B_{12} enhanced the synthesis of deoxyribosides and nucleic acid by *L. casei* (Rege and Sreenivasan, 1950; see also Roberts *et al.*, 1949), and administration of vitamin B_{12} increased liver basophilia (which implies greater formation of nucleic acid) in rats on a diet of vegetable protein (Stern *et al.*, 1949).

It is clear that many important advances have already been made in elucidating the mode of action of vitamin B_{12}. But, since pure vitamin B_{12} has been generally available for only about two years, it is hardly surprising that its haemopoietic and nutritional functions are not yet fully understood.

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Purification of Anti-pernicious Anaemia Factor. By E. Lester Smith and L. F. J. Parker. *(Research Division, Glaxo Laboratories Ltd., Greenford, Middlesex)*

Ox liver has been concentrated by the methods described by Emery & Parker (1946). Alternatively, proteolyzed liver extract made on a production scale has been readsorbed chromatographically on to charcoal and eluted with hot 65% ethanol. Material from either source has been further purified by partition chromatography on damp silica, the solvent being *n*-butanol two thirds saturated with water either alone or mixed with phenol. Alternatively *n*- or *iso*-propanol containing 10–20% of water gave excellent separations despite the complete miscibility of these solvents with water. The columns usually revealed fast-running yellow pigments, and brown pigments held fast near the top, with a pink zone between. Numerous clinical trials showed that this contained substantially all the activity. The red material could be further fractionated from coloured and colourless impurities by repetition of partition chromatography and by fractional precipitation with ammonium sulphate (between one third and two thirds saturation). Some batches of silica adsorb the pigment slightly, permitting additional purification by adsorption chromatography from aqueous solution, or better from one tenth to one half saturated ammonium sulphate, which greatly sharpened the band.

Adsorption chromatography on alumina from aqueous solution (Boots Pure Drug Co., Short & King, 1947) was useful at an early stage to remove dark pigments. For the partition chromatography, starch could be used instead of silica to avoid the complicating effect of adsorption. On starch columns, red pigments from both proteolyzed and non-proteolyzed liver separated into two pink bands with *R* values averaging 0·5 and 1·2 respectively. Non-proteolyzed liver yielded mainly the slow-moving pigment; the fast-moving one, which was also clinically active, is thought to arise from slight autolysis of the liver, because direct papain treatment of the liver yielded mainly this pigment. Repeated chromatography effected little or no further concentration of the colour beyond the stage where an optimal clinical response (Emery & Hurran, 1945) is given by about 0·3 mg. Some chemical and physical properties of such material have recently been described (Lester Smith, 1948). Following treatment with trypsin, however, chromatography on silica or charcoal gave further dramatic concentration. The substance finally crystallized from aqueous acetone as red needles. The crystalline product had 37 times the colour intensity of a batch active at 0·3 mg., giving a calculated dose of 8 μg.

It appears probable that our substance is the same as that recently described by Rickes, Brink, Koniuszy, Wood & Folkers (1948) with the suggestion that it be given the name vitamin B_{12}.

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Production, absorption, distribution and excretion of vitamin B_{12} in sheep

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1. The efficiency of production and utilization of vitamin B_{12} was studied with sheep given a cobalt-deficient diet with and without supplementary Co (1 mg/d). Vitamin B_{12} to lignin ratios in rumen contents were used to estimate minimum rates of production and these were related to faecal and urinary excretion. Tissue distribution and excretion of vitamin B_{12} were studied with [^{60}Co]cyanocobalamin and 5′-deoxyadenosyl[^{60}Co]cobalamin.

2. Labelled Co was rapidly sequestered by particulate material in the rumen and was largely excreted in the faeces. Most of the vitamin B_{12} in whole rumen contents was contained in micro-organisms, but was released on incubation at pH 2. Added cyanocobalamin was partly degraded in the rumen.

3. The vitamin B_{12} to lignin ratio in rumen contents began to decline 1–3 d after cessation of a daily Co drench. Estimated ruminal production of vitamin B_{12} on full feed was not less than 400–700 μg/d with supplementary Co and 50–110 μg/d from the Co (0·01–0·05 μg/g dry weight) in the basal diet. Production of vitamin B_{12} appeared to be limited by food intake with or without additional Co.

4. At full feed the efficiency of production of vitamin B_{12} from Co in the basal diet was about 13% while that from added Co was about 3%. Part of the vitamin B_{12} produced in the rumen was degraded before reaching the faeces and about 5% was absorbed. The minimum total requirements of sheep for vitamin B_{12} are assessed at about 11 μg/d.

5. Injected 5′-deoxyadenosylcobalamin was better retained than injected cyanocobalamin. Faecal excretion exceeded urinary excretion with both. Labelled cobalamin was selectively retained by liver (particularly by the mitochondria), kidneys, and the walls of parts of the alimentary tract. Vitamin B_{12} was secreted into the duodenum and reabsorbed in the ileum, but little secretion occurred above the duodenum and little absorption below the small intestine.

Production of vitamin B_{12} in rumen contents of sheep depends on the presence of cobalt (Hale, Pope, Phillips & Bohstedt, 1950; Hoekstra, Pope & Phillips, 1952; Hine & Dawbarn, 1954; Kercher & Smith, 1956) and this is reflected in faecal excretion (Hale *et al.* 1950; Dawbarn, Hine & Hughes, 1952; Dawbarn & Hine, 1955). Little is known, however, either of the quantities produced or of the efficiency of absorption.

Kercher & Smith (1955) estimated that retention of oral cyanocobalamin in sheep was about 3% that of injected cyanocobalamin, but large doses of injected cyanocobalamin may not be well retained. Thus, with sheep on a Co-deficient diet, 1 mg Co/d orally led to greater liver storage of vitamin B_{12} than did 25 μg cyanocobalamin/d by injection (Marston, 1970); it led to little urinary excretion but, on the other hand, 30–36% of injected cyanocobalamin (50 μg/d) was excreted in the urine (Dawbarn & Hine, 1955).

Smith & Loosli (1957) estimated that sheep given a Co-deficient diet required an additional 10 μg injected cyanocobalamin/d, but the amount provided by the diet was unknown. Marston (1970) in long-term experiments has estimated the minimum prophylactic dose of injected cyanocobalamin to be about 6 μg/d for sheep on a diet

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containing 0·03 μg Co/g (dry). The absolute requirements of sheep for vitamin B_{12} may be estimated from this dose if both the efficiency of production from the Co in the diet and the efficiency of absorption are known.

The quantities of vitamin B_{12} in rumen contents of sheep given Co-deficient diets are not negligible, and it is not necessarily true that the efficiency of absorption is the same as that when additional Co is supplied or the same as that when crystalline cyanocobalamin is given orally. The work now described was aimed at estimating these rates and efficiencies and in addition at determining the tissue distribution and excretory pattern of injected cobalamins. The total requirements of sheep for vitamin B_{12} are assessed at about 11 μg/d and, although the arguments leading to this estimate are not entirely rigorous, the results permit a reasonable description of the production, absorption and excretion of vitamin B_{12} in the sheep.

EXPERIMENTAL

Animals and diet. Australian Merino ewes, 2–6 years of age, were fed on a Co-deficient diet in cages permitting collection of excreta (Smith & Marston, 1970). A daily ration was given of 1000 g wheaten hay-chaff (0·01–0·05 μg Co/d (dry)), 50 g washed gluten (< 0·02 μg Co/g (dry)) and 10 g sodium chloride; daily food intakes were recorded and body-weights were measured each week. The ration contained 85–90% dry matter but unless otherwise stated food intakes are quoted on a moist-weight basis.

All animals received weekly drenches supplying 70 mg copper (as sulphate) and 25 ml cod-liver oil. Animals treated with Co were drenched daily at the time of feeding with 1 mg Co (as chloride) in 20 ml water. Animals given vitamin B_{12} orally received a daily drench of 500 μg cyanocobalamin in 20 ml water. Animals treated with vitamin B_{12} parenterally were injected daily with 50 μg cyanocobalamin in 0·5 ml water into the gluteus maximus muscle. Injections of [^{60}Co]cyanocobalamin (subcutaneously on the shoulder) or 5′-deoxyadenosyl[^{58}Co]cobalamin (into the jugular vein) were given at a rate equivalent to 25 μg cyanocobalamin/d in 2 ml isotonic saline.

Samples of rumen contents. These were obtained via fistulas (Jarrett, 1948) with a stainless-steel sampler mounted on a flexible handle. The hollow cylindrical sampler (10 cm × 2·2 cm diameter) contained a gate (7·5 cm × 1·8 cm) closed by a second hollow cylinder closely fitting inside the first and containing a similar gate. A lever on the handle permitted rotation of the inner cylinder through 180° to open or close the gate. The closed sampler was inserted into the rumen, the gate was opened for 1 or 2 s, then closed and the sample (15–22 g) recovered. All samples analysed comprised composites of ten such withdrawals taken from random points in the rumen. Dry-matter measurements of replicate single withdrawals from individual sheep showed the coefficient of variation of such composite samples to be about 3%. Except where otherwise stated, samples of rumen contents were taken 4 h after feeding.

Chemical estimations. Co in fodder and rumen contents was measured in samples of 15–25 g (dry) by the method of Marston & Dewey (1940). Lignin was measured by the method of Norman & Jenkins (1934*a*, *b*). The dried and ground samples of fodder or

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of 50% (v/v) ethanol-insoluble dry matter from rumen contents were refluxed in 5% (w/v) H_2SO_4 for 1 h to remove protein and hydrolysable polysaccharides, then again dried before leaving for 16 h at 18° in 72% (w/w) H_2SO_4. The results were corrected for ash content. Nitrogen was estimated by the Kjeldahl procedure of McKenzie & Wallace (1954). Dry matter in tissues was determined after heating 0·3–0·5 g samples in a drying oven for 18 h at 105°.

Assay and nomenclature of vitamin B_{12}. Vitamin B_{12} activity was determined microbiologically by four procedures (*Ochromonas malhamensis*, *Lactobacillus leichmanii*, *Escherichia coli* (tube assay) and *E. coli* (plate assay)) as described by Hine & Dawbarn (1954). All methods were standardized with cyanocobalamin. Results are expressed as vitamin B_{12} activity for the particular organism except that vitamin B_{12} activity for *Ochromonas* in any material or for *L. leichmanii* in liver (Hine & Dawbarn, 1954) are assumed to represent only cobalamins and are referred to as vitamin B_{12}.

Measurement of radioactivity. Radioactivity due to ^{58}Co or ^{60}Co was determined with an Ekco scintillation counter type N550 with an Ekco scaler type N530C (Ekco Electronics Ltd, Southend-on-Sea, Essex). Samples of faeces, alimentary tract contents and those tissues difficult to homogenize were made soluble by wet digestion in boiling nitric acid, sulphuric acid and perchloric acid, made to volume with water and the samples were counted. Soft tissues were homogenized in water for 3 min in a Servall Omnimixer (Ivan Sorvall Inc., Norwalk, Conn., USA) and samples of the homogenates counted. Urine, whole blood and bile were counted direct. Samples containing ^{58}Co were counted with 15% efficiency using a probe-type crystal for annular counting. Samples (other than fluids) of 10–20 g were made to 50 ml, and 10 ml counted. Samples containing ^{60}Co were counted with 62% efficiency using a well-type crystal. Samples (other than fluids) of 2·4 g were made to 20 ml, and 5 ml counted. All samples were counted for 10^3 s. Measured activity of ^{58}Co was corrected for radioactive decay during the experiment, but with ^{60}Co the correction was negligible and was omitted.

Materials. Crystalline cyanocobalamin was obtained from Glaxo–Allenburys (Aust.) Pty Ltd, Melbourne, and was standardized spectrophotometrically at 361 nm (Barker, Smyth, Weissbach, Munch-Petersen, Toohey, Ladd, Volcani & Wilson, 1960). ^{60}Co as cobaltous chloride and [^{58}Co]cyanocobalamin were obtained from the Radiochemical Centre, Amersham. The latter was diluted with unlabelled cyanocobalamin to a specific activity of 26 nCi/μg. 5′-Deoxyadenosyl[^{60}Co]cobalamin and also the unlabelled compound were obtained by the courtesy of Dr D. Perlman (E. R. Squibb & Sons, New Brunswick, NJ, USA). The radioactive coenzyme was chromatographed on Dowex 50 as described by Barker, Smyth, Weissbach, Toohey, Ladd & Volcani (1960). The final aqueous solution, purged of phenol, was standardized spectrophotometrically at 375 nm (Barker, Smyth, Weissbach, Toohey *et al.* 1960) and diluted with the unlabelled compound, similarly standardized to a specific activity of 72 nCi/μg. All samples of the coenzyme were carefully protected from light.

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RESULTS

Faecal and urinary excretion of ^{60}Co. A 6-year-old ewe (no. 110) was fed on the Co-deficient diet supplemented with Co for 7 months, after which Co treatment was discontinued. The final supplement of 1 mg Co contained 0·2 mCi ^{60}Co and was introduced direct into the rumen through a fistula. Faecal and urinary excretion of ^{60}Co for the following 14 d are shown in Fig. 1. Food intake over this period averaged 1016 g/d.

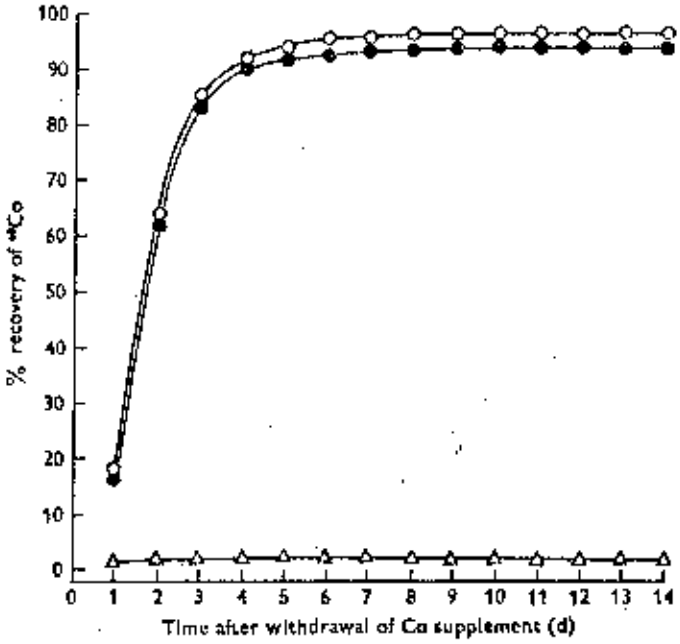

Fig. 1. Excretion of ^{60}Co by sheep after administration of 0·2 mCi ^{60}Co in 1 mg cobalt. ○, total; ●, in faeces; △, in urine.

Most (93 %) of the administered ^{60}Co was excreted in the faeces within 5 d. In the 14 d period 95·1 % appeared in the faeces and only 2·5 % in the urine. The results are in general agreement with those of Comar (1948) for cattle, and of Monroe, Sauberlich, Comar & Hood (1952) for wether lambs.

Distribution of vitamin B_{12} activity in rumen contents. Table 1 shows the results of two experiments in which vitamin B_{12} distribution was measured. In the first experiment (sample A) an animal on dry summer pasture containing adequate Co (0·15 μg Co/g (dry)) was killed and a portion of whole rumen contents strained under gravity through cheese-cloth. A little over 40 % of the weight of the sample passed freely through, and this and the residual wet fibrous matter were assayed with *L. leichmanii* for vitamin B_{12} activity, as was the initial sample. The fluid fraction contained only 4 % of the vitamin B_{12} activity, and, even on the assumption that all the residual water in the wet residue contained vitamin B_{12} activity at the same level, less than 10 %

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Original sample	Treatment	Description of separated fractions	Distribution of vitamin B_{12} activity: *Ochromonas* — Whole rumen contents (μg/100 g)	*Ochromonas* — In separated fractions (%)	*Ochromonas* — Total recovery in all fractions (%)	*L. leichmanii* — Whole rumen contents (μg/100 g)	*L. leichmanii* — In separated fractions (%)	*L. leichmanii* — Total recovery in all fractions (%)
Whole rumen contents (387 g, 15·5% dry matter) (sample A)	Strained through two thicknesses of cheesecloth under gravity	Wet vegetable matter with associated organisms (223 g, 25·4% DM)	--	--	-	6·1	96	95
		Fluid with suspended organisms (164 g, 2·1%, DM)	—	--		0·23	4	
Whole rumen contents (sample B)	Strained through stainless-steel gauze and fluid centrifuged 30 min at 21 000 g and 0°	Original whole rumen contents	3·07	100		5·47	100	
		Centrifugal supernatant fluid	-			0·21	4	
Whole rumen contents (sample B)	Strained through stainless-steel gauze, then through muslin. Retained solids repeatedly shaken in artificial saliva (pH 7, containing KCN) and strained to recover organisms. Fluid and washes combined	Washed fibrous matter	0·29	11		0·45	11	
		Combined strained fluid fractions	2·37	89	87	3·67	89	75
Suspended micro-organisms in rumen fluid diluted with artificial saliva (pH 7, containing KCN) (combined strained fluid fractions of sample B)	Centrifuged 30 min at 21 000 g and 0°.	Supernatant fluid	0·11	5	92	0·34	9·1	98
		Sediment	2·07	95		3·26	91·5	
	Saturated with toluene, incubated 90 min at 39° and pH 6·6, then chilled and centrifuged 30 min at 21 000 g and 0°.	Supernatant fluid	0·53	24	92	1·61	43	102
		Sediment	1·64	76		2·14	57	
	Saturated with toluene, adjusted to pH 2 with hydrochloric acid, incubated 90 min at 39°, then chilled and centrifuged 30 min at 21 000 g and 0°	Supernatant fluid	2·34	94	105*	3·45	93	101
		Sediment	0·15	6		0·26	7	

DM, dry matter. * Recovery of added cyanocobalamin 101 %.

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of the activity in whole rumen contents could be regarded as freely detached from fibrous solids.

Distribution of vitamin B_{12} activity between micro-organisms and fluid in rumen contents (obtained through a fistula) of a sheep eating 1050 g/d of the Co-deficient diet supplemented with 1 mg Co/d is shown by the results of the analysis of sample B. The sample was taken 4 h after feeding and drenching with Co. With the *L. leichmanii* assay only 4% of the vitamin B_{12} activity remained in the supernatant fraction after straining and high speed centrifugation. From the *Ochromonas* assay almost 90% of the

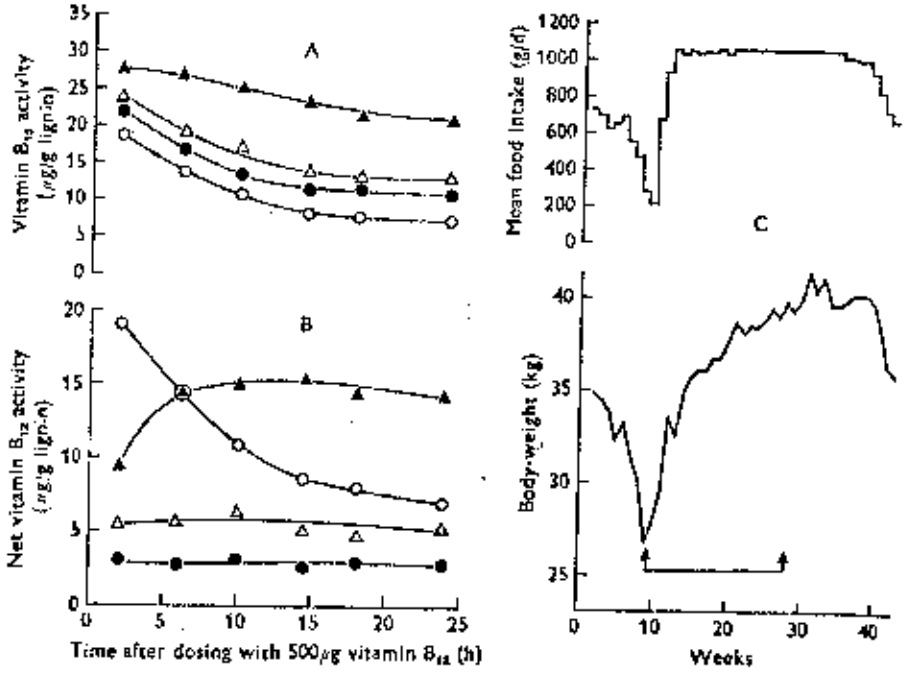

Fig. 2. Degradation of cyanocobalamin introduced into the rumen of sheep. C shows the response in food intake and body-weight of a severely vitamin B_{12}-deficient animal to a daily drench of 500 μg cyanocobalamin started at the first arrow and discontinued at the second. A and B show the results of microbiological estimations of vitamin B_{12} activity in rumen contents after the first dose. Activity was estimated by *Ochromonas malhamensis* (○), *Lactobacillus leichmanii* (●), *Escherichia coli* (tube method) (△), and *E. coli* (plate method) (▲). The total activities are shown in A, and in B the total activity for *Ochromonas*, together with the net activities for the other organisms after subtracting the *Ochromonas* activity.

vitamin B_{12} could be released from fibrous solids by repeated mechanical shaking (four times each for 4 min) in McDougall's (1948) artificial saliva (containing 0·005% KCN and adjusted to pH 7 with H_3PO_4). Most (95%) of this activity was sedimented on centrifuging and was presumably associated with bacteria. The washed fibrous solids were seen by microscopic examination to contain some residual adherent bacteria.

Incubation for 90 min at 39° (pH 6·6 in the presence of toluene) led to release of

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but one-quarter of the vitamin B_{12} from the bacteria in the fluid phase, but when incubated at pH 2 (HCl) almost all (94 %) of the vitamin B_{12} was released.

Degradation of cyanocobalamin introduced into the rumen. An animal (no. 9047) was fed on the Co-deficient diet until it was severely deficient of vitamin B_{12} and was then treated orally with 500 μg cyanocobalamin/d. The response is shown in Fig. 2C. Treatment was stopped after 4 months, and 7 weeks later the vitamin B_{12} reserves created by this treatment had been depleted to the point where appetite again failed. (Recovery was immediate on subsequent treatment with 50 μg cyanocobalamin/d by injection.)

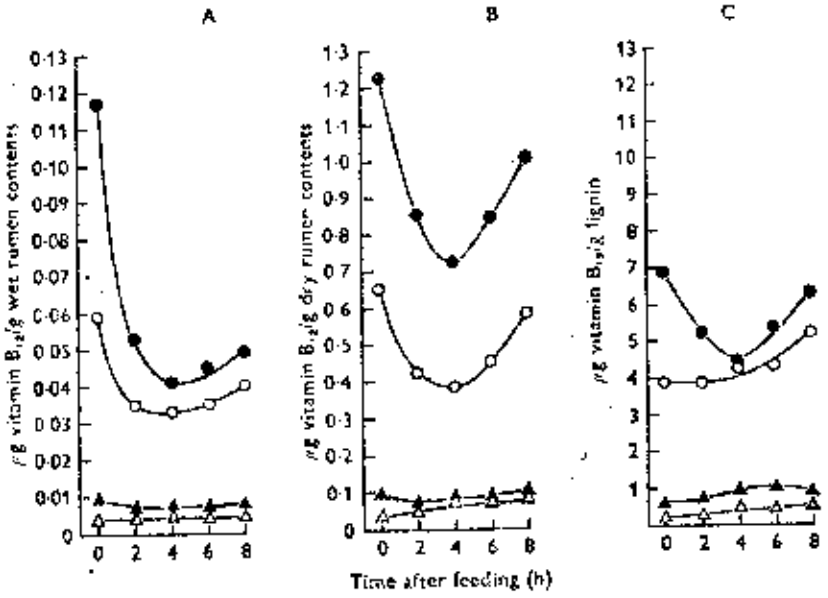

Fig. 3. *Ochromonas* vitamin B_{12} activity in whole rumen contents of sheep after feeding, expressed as μg cyanocobalamin/g wet matter (A), dry matter (B) or lignin (C), both with and without supplementary cobalt. ○, animal no. 110, +Co; ●, no. 193, +Co; △, no. 110, −Co; ▲, no. 193, −Co.

Although the daily drench of 500 μg cyanocobalamin permitted absorption of sufficient vitamin B_{12} for the animal's needs, little remained for storage. Using the values of Marston (1970) for prophylactic doses of injected cyanocobalamin on this diet, one may calculate that absorption probably lay between 6 μg and 12 μg/d, or between 1 % and 3 % of the dose.

Degradation of cyanocobalamin in the rumen was detected in samples of rumen contents taken through a fistula at intervals for 24 h after the first dose. This dose was introduced directly into the rumen and the animal was starved for 24 h. The ratios of vitamin B_{12} activity to lignin for the four assay procedures are shown in Fig. 2A, and in Fig. 2B the same results for vitamin B_{12} measured with *Ochromonas* are compared with the activity of factors other than cobalamins active for the other organisms. These were calculated by subtracting the *Ochromonas* vitamin B_{12} to lignin ratios from the corresponding ratios obtained by the other methods.

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The rapid decline in the ratio of *Ochromonas* vitamin B_{12} to lignin over the first 10 h was accompanied by an increase in net *E. coli* (plate) vitamin B_{12} to lignin ratio, but there was little change in either net *E. coli* (tube) or net *L. leichmanii* vitamin B_{12} to lignin ratio.

Production of vitamin B_{12} in the rumen. No completely satisfactory method has been devised for measuring the rate of passage of an unabsorbed substance produced in the rumen of a fed animal. In the present work approximate rates of production of vitamin B_{12} have been calculated from *Ochromonas* vitamin B_{12} to lignin ratios in the rumen 4 h after feeding, by multiplying by the daily intake of lignin. This method is subject to criticism, and the results, particularly in animals receiving Co, must be regarded as minimum values.

Fig. 3 and Table 2 show the vitamin B_{12} activity in rumen contents at different times during the first 8 h after feeding, both with and without supplementary Co. Both animals were stabilized for at least 14 d with or without Co before the measurements were made. Use of the lignin ratio reduced but did not eliminate the effects of rate of consumption of food, and in animals receiving Co the vitamin B_{12} to lignin ratio tended to reach a minimum value 4–6 h after feeding.

In the experiment shown, the hay-chaff contained 86% dry matter and 0·03 μg Co/g (dry) and lignin was 11·1% of the dry matter. From the lignin intake and the vitamin B_{12} to lignin ratio 4 h after feeding, the minimum rates of production of vitamin B_{12} for animals 110 and 193 respectively were 406 and 423 μg/d when Co was given and 46 and 92 μg/d when Co was withheld. The results for the Co-depleted rumen are also regarded as minimum values because part of the vitamin B_{12} in rumen contents is not associated with lignin-bearing solids (Table 1) and Hogan & Weston (1967) and Weston & Hogan (1967) have shown that the rate of turn-over of water through the rumen is faster than that of fibrous solids. The latter error applies to all measurements reported but is probably small. Table 1 shows that vitamin B_{12} is fairly closely associated with lignin-bearing solids in the rumen and suggests that lignin is a reasonably appropriate marker in terms of distribution.

Fig. 4 shows the effects of withdrawal of Co on vitamin B_{12} production in the rumen of two sheep (no. 9029 and no. 9047) previously stabilized for 16 weeks and 2 weeks respectively on 1 mg Co/d. Periodic samples were taken for 112 d, during which time the mean food intake of no. 9029 was 920 g/d and of no. 9047 was 800 g/d. Calculated from lignin intakes, the minimum rate of vitamin B_{12} production fell from 600–750 μg/d to about 50 μg/d within 5 d of withdrawal of Co. The latter value was maintained for the remainder of the experiment and reflects synthesis from the Co in the fodder (0·01 μg Co/g (dry)). In animal 9047 the vitamin B_{12} to lignin ratio did not fall until the 2nd day after the final drench.

In a further experiment with no. 9029, Co was withdrawn after daily drenching for 4 months. The full ration of 1050 g/d was consumed and vitamin B_{12} production fell from a minimum of 480 μg/d to 50 μg/d at 6 d and 65 μg/d at 14 d after withdrawal of Co. The diet contained 0·04 μg Co/g (dry).

Production of other factors with vitamin B_{12} activity in rumen contents. The increases in *Ochromonas* vitamin B_{12} to lignin ratios in the rumens of both Co-deficient and

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Table 2. *Vitamin B_{12} activities in rumen contents of sheep during 8 h after feeding*

Animal no.	Treatment	Time after feeding (h)	Food intake over preceding 2 h (g)	Lignin content of dry matter in rumen (%)	Vitamin B_{12} activity: *Ochromonas* (μg/g) (dry)	*E. coli* (plate) (μg/g) (dry)	*E. coli* (tube) (μg/g) (dry)	*L. leichmanii* (μg/g) (dry)
193	1 mg cobalt/d	0	0	17·9	1·23	13·9	2·5	—
		2	686	16·3	0·86	9·8	1·8	—
		4	364	16·6	0·73	9·0	1·6	—
		6	0	15·8	0·85	10·0	1·9	
		8	0	16·0	1·01	12·6	2·1	—
193	Rumen depleted of Co	0	0	19·7	0·102	0·61	0·33	0·27
		2	756	17·3	0·078	0·79	0·30	0·23
		4	294	16·9	0·097	1·03	0·37	0·31
		6	0	15·6	0·090	1·16	0·41	0·34
		8	0	15·8	0·108	1·25	0·45	0·35
110	1 mg Co/d	0	0	19·5	0·66	13·4	2·9	2·1
		2	548	16·4	0·43	7·5	1·9	1·4
		4	349	15·8	0·39	6·8	1·6	1·2
		6	133	16·0	0·46	7·6	1·8	1·5
		8	16	17·5	0·59	9·6	2·3	1·9
110	Rumen depleted of Co	0	0	19·0	0·039	0·30	0·12	—
		2	499	17·2	0·051	0·51	0·20	—
		4	315	15·6	0·074	0·96	0·37	—
		6	169	15·9	0·072	0·89	0·33	—
		8	13	16·2	0·082	1·22	0·43	—

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Co-supplemented sheep, shown in Fig. 3, clearly demonstrate synthesis of vitamin B_{12}. In both instances simultaneous synthesis of other cobamides may be inferred from Table 2 where the ratios to *Ochromonas* vitamin B_{12} of the activity for the other test organisms may be calculated and shown to be maintained. In Table 3 are summarized the results of all measurements made of vitamin B_{12} activity in rumen contents in the present series of studies. In addition to the summarized results of Table 2, the values in

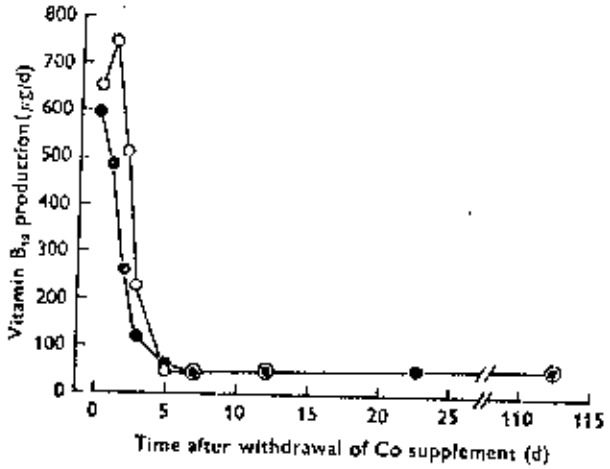

Fig. 4. Minimum rates of production of vitamin B_{12} in the rumen of sheep after withdrawal of a cobalt supplement, estimated from *Ochromonas* vitamin B_{12} to lignin ratios in rumen contents 4 h after feeding. Both animals received 1 mg Co/d by mouth before withdrawal on day 0. ●, animal 9029; ○, animal 9047.

Table 3 include the published results of Hine & Dawbarn (1954) and Dawbarn, Hine & Smith (1957). As in Fig. 2, the results for *E. coli* (plate), *E. coli* (tube) and *L. leichmanii* have been expressed as net activities by subtracting the *Ochromonas* values in order to express the activities of factors other than the cobalamins. In Table 3 the Co-treated animals had received 1 mg Co/d for at least 14 d and the Co-depleted animals had been deprived of supplementary Co for at least 7 d. Both groups subsisted on the Co-deficient diet (0·01–0·04 μg Co/g (dry)). Pasture-fed animals consumed a mixed grass-legume diet containing about 0·15 μg Co/g (dry).

Characteristic differences occurred not only between animals with and without Co (1 mg/d) on the Co-deficient diet, but also between Co-treated animals and grazing animals on sound pastures. Some of these features were noted by Hine & Dawbarn (1954).

Rumen contents of Co-deficient animals were characterized by low activities of vitamin B_{12} measured by all methods. The Co-treated animals showed a high net *E. coli* (plate) activity, and although this was not significantly higher than that in grazing animals, the ratio net *E. coli* (plate) to *Ochromonas* was significantly higher than that in either Co-deficient or pasture-fed animals. The high activity for *Ochromonas* in rumen contents of pasture-fed animals, although not significantly greater than that in Co-treated animals, contributed to the significantly lower ratios net *E. coli* (plate) to

Table 3. *Vitamin B_{12} and vitamin B_{12}-like substances in the rumen contents of sheep*

(Values from Table 2 and from the published results of Hine & Dawbarn (1954) and Dawbarn *et al.* (1957); mean values with their standard errors for the number of observations shown in parentheses)

Vitamin B_{12} activity	Cobalt-treated (Co+) (μg/g) dry	Cobalt-depleted (Co−) (μg/g) dry	Pasture-fed (P) (μg/g) dry	Significance of differences between Co+ and Co−	P and Co−	Co+ and P
Net *E. coli** (plate)	11·06 ± 1·53 (8)	0·577 ± 0·077 (6)	7·28 ± 1·28 (5)	$P < 0·01$	$P < 0·01$	NS
Net *E. coli** (tube)	1·62 ± 0·22 (8)	0·20 ± 0·032 (6)	1·04 ± 0·15 (5)	$P < 0·01$	$P < 0·01$	NS
Net *L. leishmanii**	0·890 ± 0·123 (7)	0·117 ± 0·032 (5)	0·78 ± 0·18 (5)	$P < 0·01$	$P < 0·05$	NS
Ochromonas	0·814 ± 0·088 (8)	0·075 ± 0·005 (6)	2·32 ± 0·67 (5)	$P < 0·01$	$P < 0·05$	NS

	Ratios of activities† Co+	Co−	P	Significance of differences Co+ and Co−	P and Co−	Co+ and P
Net *E. coli* (plate) / *Ochromonas*	13·84 ± 1·33 (8)	7·65 ± 0·86 (6)	3·96 ± 0·75 (5)	$P < 0·01$	$P < 0·05$	$P < 0·01$
Net *E. coli* (tube) / *Ochromonas*	2·19 ± 0·45 (8)	2·65 ± 0·44 (6)	0·60 ± 0·13 (5)	NS	$P < 0·01$	$P < 0·05$
Net *L. leishmanii* / *Ochromonas*	1·27 ± 0·27 (7)	1·52 ± 0·39 (5)	0·36 ± 0·09 (5)	NS	$P < 0·05$	$P < 0·05$

* Obtained by subtracting *Ochromonas* activity from total activity.
† Means of individual ratios.

Ochromonas, net *E. coli* (tube) to *Ochromonas* and net *L. leichmanii* to *Ochromonas* in the former, and this feature was characteristic of the grazing animals.

Ruminal production and faecal and urinary excretion of vitamin B_{12} by pair-fed Co-deficient and Co-treated ewes at low food intake. Two fistulated ewes (no. 9151 and no. 9029) were fed on the Co-deficient diet until food intake fell, when no. 9029 was treated with Co and pair-fed with no. 9151 (Smith & Marston, 1970). After 8 weeks, when food intake was 330 g/d, samples of rumen contents were taken and faeces and urine collected daily from the following day for 14 d. Further samples of rumen contents were taken on the 15th day.

Table 4. *Estimated minimum rates of ruminal production of vitamin B_{12} in pair-fed vitamin B_{12}-deficient and cobalt-treated ewes*

Animal no.	Treatment	Food intake (g/d) (dry)	Lignin intake (g/d)	Vitamin B_{12} in rumen contents (μg/g lignin)	Estimated minimum rate of production of vitamin B_{12} in rumen (ng/d)
9151*	Standard Co-deficient diet	220	23·1	0·37	9
9029	1 mg Co/d and pair-fed with 9151	216	22·6	5·6	127

* Severely vitamin B_{12}-deficient.

The mean value of the vitamin B_{12} to lignin ratio in the rumen contents was used to estimate ruminal vitamin B_{12} production over the 14 d collection period, and faecal and urinary excretion of vitamin B_{12} was determined from pooled samples of the excreta (Dawbarn & Hine, 1955). The wheaten hay-chaff used contained 11·0% lignin and 0·04 μg Co/g, both on a dry-weight basis.

Results are shown in Tables 4 and 5. In the deficient animal, daily faecal excretion of vitamin B_{12} was greater than estimated ruminal production, but both quantities were small. In the Co-treated animal, faecal excretion of vitamin B_{12} was somewhat less than the minimum estimated ruminal production. In both instances urinary excretion of vitamin B_{12} was small.

Production of vitamin B_{12} in relation to soluble Co concentration in the rumen. A 5-year-old ewe (no. 110) was stabilized for several months at full food intake with supplementary Co before Co was withdrawn. For several days before the final Co supplement, samples of rumen contents were taken 4 h after feeding for estimation of Co, lignin and vitamin B_{12}. The final supplement (1 mg Co) contained 1·0 mCi ^{60}Co and was introduced direct into the rumen at the time of feeding. Samples of rumen contents were taken 4 h later and then daily for 8 d 4 h after feeding. Immediately after taking each of these samples a sample of rumen fluid was withdrawn with a syringe and centrifuged for 30 min at 0° and 13000 g. The supernatant fluid was essentially free from micro-organisms and was used to estimate ^{60}Co and dry matter.

The samples of whole rumen contents were analysed for total Co, ^{60}Co, dry matter, lignin and *Ochromonas* vitamin B_{12}.

The measured water content of the two kinds of sample together with the lignin concentrations in whole rumen contents allowed the ^{60}Co values to be expressed as ^{60}Co to lignin ratios for each. On this basis the rate of decline of ^{60}Co with time was

Table 5. *Vitamin B_{12} excretion in relation to food intake of pair-fed vitamin B_{12}-deficient and cobalt- or vitamin B_{12}-treated ewes*

Expt no.	Animal no.	Treatment	Period of pair-feeding or recovery (weeks)	Food intake* (g (dry)/d)	Excretion of vitamin B_{12}† Faeces (μg/d)	Urine (μg/d)
1	9134	Vitamin B_{12}-deficient	—	180	34	—
	7049	1 mg Co/d and pair-fed with 9134	27	168	86	—
2	9151	Vitamin B_{12}-deficient	—	220	18	0·07
	9029	1 mg Co/d and pair-fed with 9151	8	216	92	0·76
3	5009	Vitamin B_{12}-deficient	—	285	21	0·09
	7019	50 μg cyanocobalamin/d by injection and pair-fed with 5009	15	275	57	15·2
4	9090	Vitamin B_{12}-deficient	—	365	44	0·11
	7009	50 μg cyanocobalamin/d by injection and pair-fed with 9090	9	355	66	17·7
5	0077	Co-depleted in rumen‡	—	727	127	0·26
	0113	1 mg Co/d and pair-fed with 0077	4	727	260	3·6
6	0113	Co-depleted in rumen‡	—	723	120	0·35
	0077	1 mg Co/day and pair-fed with 0113	2	723	300	2·7
7	5009	Vitamin B_{12}-deficient	—	196	5	0·17
	5009	500 μg cyanocobalamin/d by mouth	6–32 d	509	120	0·24

* From the values of Smith & Marston (1970).
† From the values of Dawbarn & Hine (1955).
‡ Not deficient of vitamin B_{12} in their tissues.

found to be accurately exponential in both (except for the first supernatant sample taken 4 h after ^{60}Co was given). The half-time for decline of ^{60}Co in the supernatant fraction (20·9 h) was somewhat longer than that in whole rumen contents (17·5 h) and in consequence, although only 1·2% of the ^{60}Co was present in the supernatant fraction 28 h after the dose, 8 d later the amount had risen to 3%. This may have been due to a greater proportion of the supernatant ^{60}Co being present as cobamides as the Co concentration fell. These may not have been so tightly bound by the micro-

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organisms as was ionic Co. In the first sample (4 h), 3% of the ^{60}Co in whole rumen contents was in the supernatant fraction. Under somewhat similar conditions Tosic & Mitchell (1948) found about 20% of administered Co in the supernatant fraction 10 h after feeding.

The concentration of Co in the supernatant fraction was calculated from the ^{60}Co content by assuming the specific activity (μCi ^{60}Co/μg Co) to be the same as that measured in corresponding samples of whole rumen contents. These values, expressed as μg Co/g water, are shown in Fig. 5, together with the values for vitamin B_{12} and total Co in whole rumen contents expressed as ratios to lignin.

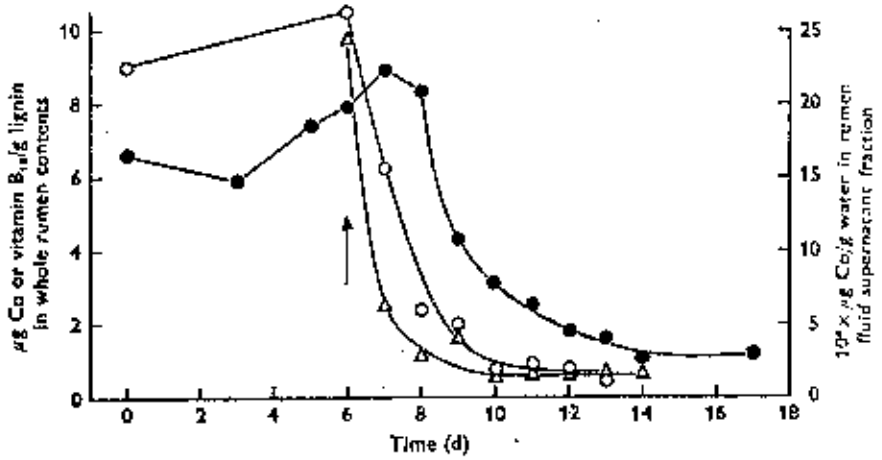

Fig. 5. Concentrations of cobalt and vitamin B_{12} in rumen contents of sheep before and after withdrawal of a Co drench (1 mg/d) on day 6. ○, total Co; △, Co in solution; ●, *Ochromonas* vitamin B_{12} expressed as cyanocobalamin.

Although both total Co and supernatant Co fell rapidly after the final dose, vitamin B_{12} concentrations did not fall until 2 d later when supernatant Co was less than 0·5 ng/ml and total Co less than 20 ng/g of wet rumen contents. The final concentration of Co in the depleted rumen at 8 d was steady at less than 0·2 ng/ml in the supernatant fraction and about 5 ng/g wet rumen contents (the value for the supernatant fraction was previously reported incorrectly as 20 pg/ml; Marston, Allen & Smith, 1961). The Co content of the chaff used was 0·53 μg Co/g lignin and this value was approached in the depleted rumen.

Calculated from daily lignin intakes, the estimated minimum rate of production of vitamin B_{12} in this experiment fell from 730 μg/d when Co was given to 110 μg/d 8 d after it was withdrawn.

Efficiency of conversion of Co into vitamin B_{12} in the rumen. From the Co to lignin and vitamin B_{12} to lignin ratios in whole rumen contents (Fig. 5) the fraction of the total Co present as vitamin B_{12} (cobalamins) may be calculated. These values are shown in Fig. 6 as a function of the Co to lignin ratio. Although the errors in this estimation became considerable at low Co concentrations, the efficiency of conversion of Co into vitamin B_{12} was obviously higher when Co was depleted.

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It was clear from early work (Hale *et al.* 1950; Hine & Dawbarn, 1954) that ruminal synthesis of vitamin B_{12} was more efficient from small quantities of dietary Co than from supplementary Co, and the present results suggest an approximately linear decrease in efficiency from about 15% in the rumen of animals depleted to about 3% when 1 mg Co/d was given.

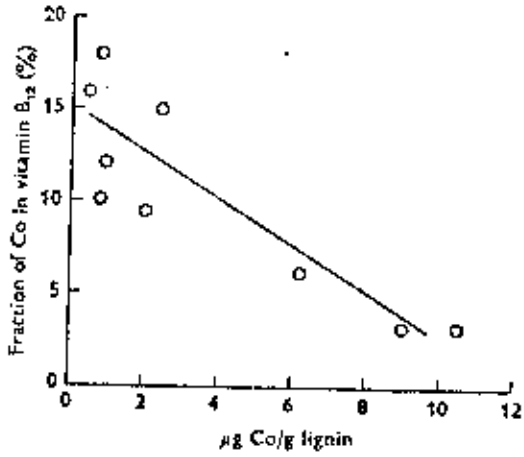

Fig. 6. Relationship between the cobalt concentration in rumen contents of sheep and the percentage present as cobalamins, derived from the values shown in Fig. 5.

Excretion of vitamin B_{12} in faeces and urine. Dawbarn & Hine (1955) have reported values for mean daily faecal and urinary excretion of vitamin B_{12} by pair-fed Co-deficient and Co-treated or vitamin B_{12}-treated ewes over 14 d periods. Nitrogen and combustible energy retention for the same periods were reported (except for the fistulated animals 9151 and 9029) by Smith & Marston (1970). The combined results for food intake and vitamin B_{12} excretion are summarized in Table 5. In the vitamin B_{12}-deficient animals the severity of the deficiency is indicated by food intake which had fallen from about 940 g (dry)/d. In Expts 5 and 6, both animals were restricted to the food intakes shown, and in periods without Co these animals were not seriously depleted of vitamin B_{12} in the tissues (Smith & Marston, 1970).

Also reported by Dawbarn & Hine (1955) and shown in Table 5 are faecal and urinary excretion of vitamin B_{12} by a severely deficient animal (no. 5009) before and after treatment with 500 μg cyanocobalamin/d by mouth. The animal responded to this treatment with increased food intake and body-weight gains, but when the treatment was withdrawn after 9 months it immediately relapsed and had evidently retained barely sufficient vitamin B_{12} for its daily needs. Steady levels of excretion were attained by the 5th day, and the means of the values 6, 13 and 32 d after treatment was begun are given in Table 5. If the requirement of injected cyanocobalamin is about 6 μg/d (Marston, 1970), only about 1% of the dose can have been absorbed even though a little more than 20% appeared in the faeces.

As noted by Dawbarn & Hine (1955), urinary excretion in animals fed on this diet

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with or without supplementary Co was seldom more than 1% of faecal excretion, but when cyanocobalamin was injected (50 μg/d) 30–36% was excreted in the urine and most of the remainder in the faeces.

Table 6. *Distribution of ^{58}Co in tissues and alimentary tract contents after subcutaneous injection of 500 μg [^{58}Co]cyanocobalamin (13 μCi) over 20 d into a vitamin B_{12}-deficient sheep*

Organ, tissue or section of alimentary tract	Tissues: Wet weight (g)	Tissues: Specific activity (nCi/g) (wet)	Tissues: Percentage of total administered dose	Contents of alimentary tract: Wet weight (g)	Contents of alimentary tract: Dry matter (%)	Contents of alimentary tract: Specific activity (nCi/g) (dry)	Contents of alimentary tract: Percentage of total administered dose
Liver	401	5·35	16·5	—	—	—	—
Kidneys	81	8·42	5·3	—	—	—	—
Spleen	55	1·45	0·6	—	—	—	—
Lungs	385	1·03	3·1	—	—	—	—
Brain	95	0·40	0·3	—	—	—	—
Heart	158	0·35	0·4	—	—	—	—
Skeletal muscle	—	0·13	—	—	—	—	—
Bone marrow	—	< 0·03	—	—	—	—	—
Blood	—	0·08	—	—	—	—	—
Gall bladder	2	0·72	—	—	—	—	—
Gall bladder contents	2·4	0·59	—	—	—	—	—
Oesophagus	37	0·27	< 0·1	—	—	—	—
Rumen	472	2·90	10·5	3150	16·4	0·12	0·5
Reticulum	119	2·05	1·9	330	12·6	< 0·05	< 0·1
Omasum	133	3·06	3·2	161	17·2	0·33	< 0·1
Abomasum	178	0·81	1·2	286	10·4	0·54	0·1
Duodenum	9	1·18	< 0·1	12	6·2	3·06	< 0·1
Ileum 1	40	2·24	3·8	67	10·5	1·81	0·8
2	43	1·21		98	10·3	2·19	
3	20	1·42		73	10·9	1·47	
4	25	1·49		75	7·6	2·19	
5	75	1·71		140	8·0	2·24	
6	33	1·19		93	7·5	1·42	
7	41	1·53		109	6·6	0·44	
8	44	1·51		92	7·0	0·41	
Caecum	144	0·82	0·9	580	11·4	0·64	0·4
Colon 1	58	0·71	1·3	75	14·5	0·55	0·3
2	53	0·63		58	18·2	0·68	
3	58	0·86		50	23·5	0·86	
4	80	0·58		44	21·7	0·68	
Rectum	89	0·40	0·3	34	27·4	0·51	< 0·1

Excretion and tissue distribution of [^{58}Co]cyanocobalamin injected into a vitamin B_{12}-deficient sheep. A vitamin B_{12}-deficient ewe (no. 830, body-weight 24 kg, food intake 440 g/d) was injected subcutaneously with 25 μg (0·65 μCi) [^{58}Co]cyanocobalamin/d for 20 d and was killed on the 21st day. Food intake increased to 880 g/d and body-weight to 27 kg during treatment.

Daily measurements of faecal and urinary ^{58}Co showed that during the 21 d 19·9% of the labelled vitamin B_{12} was excreted in the faeces and 13·3% in the urine. In a previous similar experiment lasting for 7 d (animal no. 377), 7·2% of the injected

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label appeared in the faeces and 8·4% in the urine. Net retention of injected vitamin B_{12} by the tissues of deficient animals was therefore high, but the distribution of excreted vitamin B_{12} between faeces and urine was similar to that of repleted animals (Table 5).

Table 7. *Distribution of ^{60}Co in tissues and alimentary tract contents after intravenous injection of 304 μg 5′deoxyadenosyl[^{60}Co]cobalamin (21·9 μCi) over 10 d into a vitamin B_{12}-deficient sheep*

Organ, tissue or section of alimentary tract	Tissues: Specific activity (nCi/g) (wet)	Tissues: Dry matter (%)	Contents of alimentary tract: Dry matter (%)	Contents of alimentary tract: Specific activity (nCi/g) (dry)
Liver	19·6	24·0	—	—
Kidneys	17·0	17·6	—	—
Spleen	5·0	23·6	—	—
Pancreas	3·6	21·0	—	—
Submaxillary gland	3·9	21·0	—	—
Adrenals	3·4	19·1	—	—
Ovary	4·2	16·0	—	—
Thyroid	5·2	21·8	—	—
Lung	2·0	13·9	—	—
Heart	1·0	11·6	—	—
Skeletal muscle	0·2	19·0	—	—
Bile	0·03	—	—	—
Rumen	4·3	17·5	12·6	0·3
Reticulum	4·1	14·9	—	—
Abomasum	2·2	21·7	9·9	1·2
Duodenum	3·7	18·1	6·5	9·3
Ileum 1	3·3	14·9	8·8	15·7
2	3·5	16·6	11·6	12·6
3	3·8	16·5	6·1	10·4
4	2·9	13·1	8·1	5·1
5	0·8	8·8	8·6	3·3
6	0·3	9·0	8·7	3·5
7	3·5	15·3	8·4	2·5
8	2·8	13·0	8·5	1·3
Caecum	2·3	18·9	14·1	1·7
Colon 1	3·0	15·2	17·7	1·0
2	2·7	21·0	22·2	1·0
3	2·1	20·3	22·6	0·8
Rectum	1·8	32·2	23·8	1·1

The distribution of labelled vitamin B_{12} in tissues and alimentary tract contents of animal 830 was determined at slaughter. Immediately after death the small and large intestines were ligated in sections and the contents of each section gently extruded and collected. The contents of the remainder of the alimentary tract were separated and collected without ligation. After extrusion of contents, the sections of gut were rinsed out with isotonic saline and retained for analysis. Table 6 shows the results for alimentary tract contents on a dry-weight basis and for tissues on a wet-weight basis.

Excretion and tissue distribution of 5′-deoxyadenosyl[^{60}Co]cobalamin in a vitamin B_{12}-deficient sheep. A vitamin B_{12}-deficient ewe (no. 519, body-weight 27 kg, food intake 260 g/d) was injected intravenously with 30·4 μg (2·19 μCi) 5′-deoxyadenosyl[^{60}Co]cobalamin/d for 10 d and killed on the 11th day. Food intake increased to 640 g on the

10th day of treatment and body-weight to 30 kg. Samples of tissues and alimentary tract contents were collected at slaughter as before and in addition a sample of liver was chilled and homogenized in 0·25 M-sucrose for centrifugal separation of intracellular components (Smith, Osborne-White & Russell, 1965).

Table 8. *Subcellular distribution of ^{60}Co in liver after intravenous injection of 5′-deoxyadenosyl[^{60}Co]cobalamin injected into a sheep*

(A sample of the liver referred to in Table 7)

Starting material	Derived fraction	^{60}Co recovered from 14·7 g liver (nCi)	Nitrogen recovered from 14·7 g liver (mg)	Activity (nCi/mg N)
Whole liver	Total	270	393	0·69
Homogenate in 0·25 M-sucrose	Nuclear-free homogenate (600 g supernatant)	171	233	0·73
	Nuclear fraction (600 g sediment)	95·2	176	0·54
Nuclear-free homogenate	Washed mitochondria	89·2	58·4	1·53
	Mitochondrial wash fluid	2·4	19·7	0·12
	Microsomes	4·4	40·9	0·11
	Soluble fraction	68·5	125·8	0·55
Washed mitochondria	Centrifugal supernatant from mechanically disrupted mitochondria*	59·3	21·9	2·71

* Mitochondria disrupted at top speed in a Servall Omnimixer for 5 min before centrifuging for 1 h at 54000 g.

Table 7 shows the distribution of labelled cobalamins in tissues and tract contents, and Table 8 shows the distribution in the liver homogenate. At the end of the 10 d period 5·7% of the injected labelled material had been excreted in the faeces and 2·5% in the urine. The liver retained 25·6% of the injected coenzyme, and a large proportion of this (52% of the activity of the nuclear-free homogenate) was firmly retained within the mitochondria. There was no evidence of accumulation by microsomes. The nuclear fraction had a lower activity relative to nitrogen than did the whole homogenate and although this fraction was heavily contaminated with unbroken cells and cell debris, it is concluded that ^{60}Co did not accumulate in the nuclei.

DISCUSSION

With animals receiving 1 mg Co/d on full or near-full feed, the six estimates of minimum production rates of vitamin B_{12} ranged from 410 to 730 μg/d, the lower values corresponding in general to high rates of feeding during the 4 h before sampling. These values represent a range of minimum efficiencies of conversion of Co into vitamin B_{12} from 1·7% to 3·1% based on total daily Co intakes. From Fig. 6 the fraction of ruminal Co present as vitamin B_{12} under these conditions was about 3%. The agreement between this value and the highest of the estimated minimum efficiencies provides the basis for an assessment of ruminal vitamin B_{12} production at about

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200 μg/d. In the single ewe studied at low food intake (Table 4), the same Co supplement produced a similar vitamin B_{12} to lignin ratio in rumen contents, suggesting that vitamin B_{12} production was limited by food intake rather than by Co at this level. The rough correlation between food intake and daily faecal excretion of vitamin B_{12} in animals receiving Co (Table 5) supports this contention.

In the Co-depleted rumen the minimum estimates of vitamin B_{12} production ranged from 46 to 110 μg/d on full feed, the variation depending in part on the Co content of the diet. Based on dietary Co intakes, the corresponding efficiencies ranged from 5% to 18% (mean 12%), and from Fig. 6 the fraction of Co present as vitamin B_{12} in rumen contents was about 15%. From these values the efficiency of conversion of dietary Co into vitamin B_{12} with the feed used is assessed at 13 ± 5%, and this did not appear to be altered at low food intake.

The efficiency of production of vitamin B_{12} from Co was undoubtedly affected by the nature of the diet. Measured by microbiological assay, rumen contents from pasture-fed animals contained a significantly greater proportion of their total vitamin B_{12} activity as cobalamins than did animals on the experimental diet either with or without Co. This is consistent with the electrophoretic results of Dawbarn *et al.* (1957), but the high *E. coli* (plate) activity in animals on the experimental diet supplemented with Co is unexplained. Of the cobamides and cobinamides known to be present in rumen contents (Porter, 1953, 1957) only Factor B (cobinamide) leads to this kind of response (Smith, 1965); Dawbarn *et al.* (1957) found little Factor B in rumen contents of Co-treated animals.

The estimated efficiency of absorption of cyanocobalamin given orally (1–3%) is consistent with previous estimates (Kercher & Smith, 1955; Marston, 1970) but cyanocobalamin in the rumen was fairly rapidly destroyed and little more than 20% of the daily dose appeared in the faeces. From the *E. coli* (plate) response, Factor B appeared to be a major product of degradation of cyanocobalamin in the rumen, and the virtual absence of Factor B from rumen contents of pasture-fed or Co-treated animals (Dawbarn *et al.* 1957) suggests that bacterially bound vitamin B_{12} in the rumen may not be so readily degraded. This is particularly apparent in pasture-fed animals where high *Ochromonas* vitamin B_{12} levels were accompanied by relatively low net activities for the other test organisms.

Destruction of vitamin B_{12} produced from Co in the rumen cannot be reliably assessed from faecal excretion because of possible synthesis in the lower gut (Kercher & Smith, 1956), but daily faecal vitamin B_{12} in Co-treated animals was about half the estimated ruminal production. In the absence of supplementary Co, faecal excretion of vitamin B_{12} may have been affected in addition by a net loss of vitamin B_{12} from the tissues. In severely deficient animals at low food intake, mean daily faecal vitamin B_{12} represented 12% of dietary Co but in animals not depleted of tissue vitamin B_{12} this fraction was 21% and probably exceeded ruminal production. Destruction of ruminal vitamin B_{12} undoubtedly occurred during passage through the alimentary tract, but it may not have been as extensive as that of cyanocobalamin given by drench.

Absorptive efficiencies of vitamin B_{12} may be calculated for both Co-treated and Co-deficient animals. By extrapolation from liver storage of vitamin B_{12} in sheep on

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graded doses of injected cyanocobalamin, Marston (1970) estimated that about 34 μg vitamin B_{12}/d were absorbed in animals receiving 1 mg (or 10 mg) Co/d. (This is a maximum value that neglects excess urinary excretion of injected cyanocobalamin.) From the present estimate of ruminal production (700 μg/d), this represents an absorptive efficiency of about 5%. For animals on a diet containing 0·03 μg Co/g (dry), the minimum prophylactic dose of injected cyanocobalamin was about 6 μg/d, and of oral Co was about 40 μg/d. On the assumption that 13% of dietary Co was converted into vitamin B_{12}, the efficiency of absorption was again about 5%.

Using these estimates, an assessment may now be made of the total requirements of sheep for vitamin B_{12} either from the minimum requirement for Co (70 μg/d, Marston 1970) or from the minimum requirement for injected cyanocobalamin on a diet containing 0·03 μg Co/g (dry). The total requirement is assessed at about 11 μg/d.

The site of absorption of vitamin B_{12} appears to be the small intestine, as in other animals. Substantial amounts of labelled vitamin B_{12} were secreted into the duodenum and then reabsorbed throughout the length of the ileum. Transitorily high levels of vitamin B_{12} activity for *Ochromonas* or *L. leishmanii* have been reported in the small intestine of sheep (Hine & Dawbarn, 1954; Kercher & Smith, 1956).

After injection of labelled cobalamins the specific activities (μCi/g (dry)) of tract contents immediately above and below the small intestine were about the same, indicating that most of the vitamin B_{12} secreted into the duodenum was reabsorbed. This is in marked contrast to the poor absorption of vitamin B_{12} coming from the rumen and presumably released from bacteria in the acid conditions of the abomasum (Table 1). The poor absorption of vitamin B_{12} coming from the rumen is unexplained but, in view of the high efficiency of reabsorption, it would not appear to be due to competition for intrinsic factor by other cobamides.

The distribution of labelled cobalamins in alimentary tract contents provides no evidence for absorption of vitamin B_{12} below the small intestine and shows that little injected vitamin B_{12} entered the rumen. The activities in bile in the two experiments were widely divergent and with injected 5′-deoxyadenosyl[^{60}Co]cobalamin the activity was much lower than that in the duodenum and upper ileum. No conclusions can be reached as to the means of secretion of vitamin B_{12} into the duodenum.

The distribution in tissues of the two labelled cobalamins was similar and in general resembled that in other animals, with liver and kidneys the chief sites of retention (Reizenstein, 1959; Gräsbeck, Ignatius, Järnefelt, Lindén, Mali & Nyberg, 1961; Rosenblum, Reizenstein, Cronkite & Meriwether, 1963). The relatively high amounts of labelled cyanocobalamin in kidneys may reflect the higher urinary excretion of this substance. Injected labelled 5′-deoxyadenosylcobalamin was strongly retained by the liver, and the distribution in liver cells was similar to that in rat liver (Newman, O'Brien, Spray & Witts, 1962). A feature of interest is the relatively high activity found in the walls of the rumen. The epithelium of this tissue, like liver mitochondria, actively metabolizes propionate (Pennington & Sutherland, 1956; Smith *et al.* 1965), a reaction sequence that involves 5′-deoxyadenosylcobalamin (Marston & Smith, 1961).

It is probable that faeces, rather than urine, is normally the major vehicle of excretion of vitamin B_{12} released from the tissues (Dawbarn & Hine, 1955), the balance between

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absorption and excretion being established in the small intestine. Urinary excretion of injected labelled 5′-deoxyadenosylcobalamin was substantially less than that of cyanocobalamin and liver retention was higher. It is possible that the relatively high urinary excretion of cyanocobalamin reflects a slower uptake by tissues of this form of vitamin B_{12}.

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Methylmalonic Acid and Coenzyme A Concentrations in the Livers of Pair-Fed Vitamin B_{12}-Deficient and Vitamin B_{12}-Treated Sheep

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The concentrations of CoA in the livers of severely vitamin B_{12}-deficient ewes were about 2-6 times those in pair-fed animals treated with vitamin B_{12}. When the feeding rates of the pair-fed animals were closely similar, the concentrations of methylmalonic acid in deficient livers were about twice those in vitamin B_{12}-sufficient livers. The molar concentrations of CoA present were more than three times those of methylmalonic acid in both deficient and treated animals, and it is concluded that the elevated concentrations of CoA in the deficient livers were not primarily due to accumulation of methylmalonyl-CoA.

Increased concentrations of pantothenic acid or CoA have been reported in the livers of vitamin B_{12}-deficient chicks (Evans, Groschke & Butts, 1951), rats (Boxer, Shonk, Gilfillan, Emerson & Oginsky, 1955) and sheep (Dawbarn, Forsyth & Kilpatrick, 1968), but the cause of the increase is unknown. Accumulation of methylmalonyl-CoA occurs during propionate metabolism in liver homogenates of vitamin B_{12}-deficient rats (Smith & Monty, 1959) and sheep (Marston & Smith, 1961), and has been attributed to depletion in the liver of 5′-deoxyadenosyl-cobalamin, the specific coenzyme for methylmalonyl-CoA mutase (EC 5.4.99.2).

The unusual stability of the thiol ester bond of methylmalonyl-CoA (Stadtman, Overath, Eggerer & Lynen, 1960) could permit its accumulation in the liver, and the present work was undertaken to discover whether the elevated concentrations of CoA in the livers of vitamin B_{12}-deficient sheep could be attributed to this cause.

MATERIALS AND METHODS

Animals. Six Australian Merino ewes, aged 17 months (mean body wt. 32kg.), were fed on a cobalt-deficient diet in open-air pens. The daily ration consisted of 1kg. of wheaten-hay chaff (cobalt content 0·03 μg./g. dry wt.), 50g. of washed commercial gluten (cobalt content <0·02 μg./g. dry wt.) and 10g. of NaCl, supplemented weekly with 70mg. of copper (as $CuSO_4$ in water) and 25ml. of cod liver oil. After 14–16 weeks of this treatment, the mean body weight was 23kg. and the appetite of the sheep began to fail, and from that time food intake and body weight progressively declined. From 23 weeks, when food intakes were 400–600g./day and mean body weight was 25kg., two of the animals (nos. 385 and 318) were treated with vitamin B_{12} (250μg./week) injected intramuscularly in water, and pair-fed with animals nos. 305 and 348 respectively (Expts. 2 and 3 in Table 1). From 32 weeks, when food intake of the deficient animals was 300–400g./day and mean body weight was 29kg., animal no. 392 was treated similarly with vitamin B_{12} and pair-fed with animal no. 357 (Expt. 1). Five weeks later, the latter pair of animals was killed, and after a further 5 weeks the former two pairs were killed.

Animals were pair-fed on a daily basis. As a routine food was given *ad libitum* to deficient animals at 10.00 hours and removed and weighed on the following morning at 09.00 hours, the treated animal being scheduled 1 day later than its deficient counterpart. This procedure was followed in Expt. 1, the deficient animal (no. 357) being killed at 14.00 hours after starvation for 5hr. and the treated animal at the same time on the following day. The treated animal, however, consumed all its ration within 4hr. on the day before being killed and so was without food for 24hr. In Expts. 2 and 3 the feeding procedure was varied to ensure comparable food intakes with time of the members of each pair. In these experiments the animals were fed for only 8hr. on the days on which they were killed and on the 2 days immediately beforehand. Food intakes of the deficient animals were measured hourly and the appropriate quantities were given hourly to the respective treated animals 23hr. later. On the day of slaughter food was first given to deficient animals at 06.00 hours and intakes were checked hourly until they were killed at 14.00 hours. A similar schedule was followed on the next day for the respective treated animals.

Determination of CoA. CoA in liver was determined in duplicate at each of three dilutions by the method of Kaplan & Lipmann (1948) as modified by Novelli (1955). A sample of CoA (70–75% pure) was obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.) and found to contain 340 Lipmann units (Kaplan & Lipmann, 1948)/mg. Based on the equivalence of 1 Lipmann unit at 0·6μg. of pantothenic acid (Novelli, 1955) the sample was about 71% pure, and CoA in liver was determined by comparison with this standard. The values obtained for CoA in livers of vitamin B_{12}-sufficient sheep (Table 2) were similar to those for total

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CoA measured by Hawkins *et al.* (1963), obtained by *Lactobacillus plantarum* assay of bound pantothenic acid [12·1 ± 4 (S.E.M.) μg. of CoA/g. liver].

Determination of methylmalonic acid. Methylmalonic acid in liver was determined by isotope dilution of a small amount of methyl[1-^{14}C]malonic acid of high specific radioactivity added to a homogenate in 0·7N-NaOH (which would hydrolyse methylmalonyl-CoA); methyl[^{14}C]malonic acid was then recovered and purified and its specific radioactivity determined.

After the animal had been killed by cutting its throat, the liver was rapidly removed and samples were taken for determination of CoA and vitamin B_{12}. The remainder (170–432 g.) was weighed, quickly cut into pieces with scissors and homogenized in a Waring Blendor for 3 min. at top speed in 750 ml. of 0·7N-NaOH. The homogenate was washed into a 2l. flask and 5·6 μmoles (8·3 μc) of methyl-[1-^{14}C]malonic acid were added. The suspension was heated to 80° and mechanically stirred for 30 min. at this temperature, the pH being maintained above 12. The flask contents were then adjusted to about pH 4 with 5M-H_2SO_4 and the flask was heated in a boiling-water bath for 5 min. after reaching 90°. After being chilled, the suspension was centrifuged, the sediment was resuspended in water and again centrifuged, and the supernatants were combined and filtered. The filtrate (about 1l.) was adjusted to pH 9 with 5N-NaOH and extracted with three 250 ml. portions of butan-1-ol to remove unsaponified lipid. The solution was adjusted to pH 2 with 5M-H_2SO_4 and extracted with five 250 ml. portions of butan-1-ol. About 90% of the ^{14}C was recovered in the butan-1-ol, the remainder being discarded. The methyl[1-^{14}C]malonic acid was extracted from the butan-1-ol into an excess of aqueous NaOH with a mechanical emulsifier (Vibro-Mischer; A.-G. für Chemie-Apparatebau, Zurich, Switzerland) and the butan-1-ol was washed similarly with two 5 ml. portions of water. The aqueous phases (recovered by centrifuging) were combined (25–30 ml.) and adjusted to pH 10 with 0·25M-H_2SO_4 before being evaporated to dryness *in vacuo* (complete removal of butan-1-ol was essential before column chromatography). The dried salts were dissolved in 1 ml. of 1·5M-H_2SO_4 and chromatographed on a column (40 cm. × 1·2 cm. diam.) of Celite with chloroform and chloroform–butan-1-ol mixtures. Fractions (10 ml.) were collected and ^{14}C was measured in 0·1 ml. samples. Chromatographic and counting procedures were as described by Smith, Osborne-White & Russell (1967). A single radioactive peak was eluted in butan-1-ol–chloroform (1:9, v/v) corresponding to the elution position of methylmalonic acid and lactic acid. The tubes containing most of the ^{14}C were combined, the solvent was evaporated and methylmalonic acid and lactic acid were separated by paper chromatography. The free acids were applied as a streak in 1 ml. of water to Whatman 3MM paper and chromatographed (descending) for 2 hr. with ethanol–aq. NH_3 (sp.gr. 0·88)–water (8:1:1, by vol.) (Jones, Dowling & Skrabe, 1953). After being dried in air, the paper was cut into longitudinal strips, the single radioactive peak in each strip was located with a scanning device and the appropriate areas were cut out and eluted with water. After the combined eluates had been evaporated to dryness, the residue was dissolved in 1 ml. of 1·5M-H_2SO_4 and chromatographed on Celite as described above. The eluate was collected in fractions (10 ml.), ^{14}C determined in 0·1 ml. samples and the remainder titrated under N_2 with 0·1M-NaOH (low carbonate content) by using a micro-burette. The purity of the recovered labelled material was indicated by the constancy of the specific radioactivity in successive fractions. The original methyl[1-^{14}C]malonic acid was standardized in the same way, the dilution due to unlabelled methylmalonic acid in the liver being calculated from the fall in specific radioactivity.

Methylmalonic acid in blood (700 ml.) was determined similarly, except that the initial alkaline hydrolysis was omitted and half the above quantity of labelled methylmalonic acid was used.

Preparation of methyl[1-^{14}C]malonic acid. This was prepared from diethyl[1-^{14}C]malonate and unlabelled methyl iodide by malonic ester synthesis in the presence of stoicheiometric quantities of sodium ethoxide (Weiner, 1949). Reagents were added to the reaction tube in an atmosphere of dry N_2, with dessicated equipment at 0°. To a pear-drawn-out Pyrex test tube (11 mm. internal diam.) was added 10·7 mg. (67 μmoles) of diethyl[1-^{14}C]malonate, which was washed in by 215 μl. of anhydrous ethanol. This was followed by about 71 μmoles of sodium ethoxide (3 ml. of a solution of 4·0 g. of metallic sodium in 100 ml. of anhydrous ethanol), and 5 μl. (about 80 μmoles) of methyl iodide (freshly distilled from silver wool). The tube was stoppered, rapidly sealed in a flame and immersed in a water bath at 80° for 2 hr. After being cooled, the tube was opened and the esters were saponified with 5 ml. of 0·1N-NaOH at 100° for 30 min. The solution was washed through a column of Dowex 50 (H^+ form) to remove Na^+, and evaporated to dryness. The residue was taken up in 1 ml. of 1·5M-H_2SO_4 and chromatographed on Celite as described above. A radioactive peak was obtained in butan-1-ol–chloroform (1:9, v/v) corresponding to the elution position of methylmalonic acid and well separated from that of malonic acid. Recovery of methyl[1-^{14}C]malonic acid was 68%, based on consumption of [1-^{14}C]malonic ester, and the specific radioactivity (1·6 μc/μmole) was identical with that of the [1-^{14}C]malonic ester.

Measurement of ^{14}C. Scintillation counting of ^{14}C in effluent fractions from Celite columns was as described by Smith *et al.* (1967). Samples (0·1 ml.) of fractions (10 ml.) were counted in 5 ml. of Bray's (1960) liquid-scintillator solution. Counting efficiency with effluent containing butan-1-ol–chloroform (1:9, v/v) was 40·2%.

Assay of vitamin B_{12}. Vitamin B_{12} activity in liver and blood serum was determined by microbiological assay with *Lactobacillus leichmanii* (Dawbarn & Rine, 1954; Dawbarn, Rine & Smith, 1957).

Materials. Diethyl[1-^{14}C]malonate was obtained from The Radiochemical Centre, Amersham, Bucks. CoA and acetone-dried powder of pigeon liver were obtained from Sigma Chemical Co. Other materials were as described by Smith *et al.* (1967) or were reagent-grade chemicals.

RESULTS

The vitamin B_{12} status of the animals at the time of slaughter is shown in Table 1. Deficiency symptoms appear in sheep fed on this diet when the liver vitamin B_{12} concentration has declined to less than 0·15 μg./g. (Marston, 1961). The three deficient animals used in the present experiments show

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Table 1. *Liver and serum vitamin B_{12} concentrations in pair-fed vitamin B_{12}-deficient and vitamin B_{12}-treated cows*

In each case the treated animal was fed on the cobalt-deficient diet for the same period as the corresponding deficient animal, and was treated with vitamin B_{12} and pair-fed after both animals had become deficient. Food intakes of all animals began to fall after 14–16 weeks on the experimental diet, from an initial value of 1050 g./day. Initial body weights were about 32 kg.

Expt. no.	Animal no.	Treatment	Age when killed (months)	Food intake for week before slaughter (g./day)	Body wt. (kg.)	Liver wt. (g. fresh wt.)	Vitamin B_{12} Content: Liver (μg./g. fresh wt.)	Vitamin B_{12} Content: Serum (ng./ml.)
1	357	Cobalt-deficient diet for 37 weeks	26	250	21·3	256	0·035	—
	392	Cobalt-deficient diet for 37 weeks; treated with 250 μg. of vitamin B_{12}/week and pair-fed with animal no. 357 for final 5 weeks	26	250	20·3	208	0·65	—
2	305	Cobalt-deficient diet for 42 weeks	27	215	16·9	269	0·027	0·35
	385	Cobalt-deficient diet for 42 weeks, treated with 250 μg. of vitamin B_{12}/week and pair-fed with animal no. 305 for final 19 weeks	27	216	17·8	199	0·83	4·0
3	348	Cobalt-deficient diet for 42 weeks	27	339	19·4	468	0·015	0·25
	318	Cobalt-deficient diet for 42 weeks, treated with 250 μg. of vitamin B_{12}/week and pair-fed with animal no. 348 for final 19 weeks	27	339	24·0	259	0·78	7·2

low vitamin B_{12} concentrations well below this value, and marked loss of appetite and body weight occurred. Vitamin B_{12} concentrations in serum also reflected the severe depletion that had occurred. The pair-fed animals treated with vitamin B_{12} showed liver vitamin B_{12} concentrations well above 0·15 μg./g., but losses in body weight, due to the reduced food intakes, were similar.

Table 2 shows the concentrations of methylmalonic acid and CoA found in the livers. Methylmalonic acid concentrations were elevated in the livers of vitamin B_{12}-deficient animals in comparison with those of pair-fed controls, and in the single case studied a similar difference was found in the concentrations in whole blood (Expt. 1). The large difference in concentrations of methylmalonic acid in the liver of these two animals was probably due in part to the longer period of starvation of the vitamin B_{12}-treated animal, brought about by the much more rapid consumption of its ration. When food intake was adjusted on an hourly basis on the day of slaughter and for 2 days beforehand, the difference between deficient and treated animals was smaller (Expts. 2 and 3). In the latter experiments methylmalonic acid concentrations in livers of deficient animals were about twice those in pair-fed animals treated with vitamin B_{12}.

It is not known to what extent the various acyl-CoA compounds in the livers were hydrolysed to free CoASH during preparation of the samples for CoASH determination by the method of Kaplan & Lipmann (1948). If it is assumed that hydrolysis was complete and that all the methylmalonate found was present in the liver as methylmalonyl-CoA, then in Expts. 2 and 3 the latter substance contained no more than 20% of the total CoA in either deficient or treated animals. In Expt. 1, where the concentration of methylmalonic acid in the liver of the deficient animal was higher, the corresponding maximum concentration of methylmalonyl-CoA was 31% of the CoASH found. It is probable, however, that hydrolysis of acyl-CoA compounds was not complete, and from the appearance of the free acid in blood it is also probable that part of the methylmalonic acid found in the liver was present in the free state. In either case the fraction of total CoA that could be accounted for as methylmalonyl-CoA would be less.

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Table 2. *Methylmalonic acid and CoA in the livers of pair-fed vitamin B_{12}-deficient and vitamin B_{12}-treated sheep*

In Expts. 2 and 3 the intakes of the pair-fed animals were adjusted on an hourly basis on the day of slaughter and for 2 days beforehand. The vitamin B_{12} status of the animals is shown in Table 1.

Expt. no.	Animal no.	Treatment	Food intake immediately before slaughter	Methylmalonic acid in whole blood (nmoles/ml.)	Methylmalonic acid in liver (nmoles/g. fresh wt.)	CoA concentration found (nmoles/g. fresh wt.)
1	357	Vitamin B_{12} deficient	Consumed 278 g. over 23 hr., then starved 5 hr. before slaughter	118	126	415
	392	Vitamin B_{12}-treated and pair-fed with animal no. 357	Consumed 278 g. over 4 hr., then starved 24 hr. before slaughter	5	13	160
2	305	Vitamin B_{12}-deficient	Consumed 253 g. over 8 hr. immediately before slaughter; intakes checked hourly	—	73	420
	385	Vitamin B_{12}-treated and pair-fed with animal no. 305	Consumed 283 g. over 8 hr. immediately before slaughter; intakes equivalent to animal 305 on hourly basis	—	34	166
3	348	Vitamin B_{12}-deficient	Consumed 266 g. over 8 hr. immediately before slaughter; intakes checked hourly	—	64	442
	318	Vitamin B_{12}-treated and pair-fed with animal no. 348	Consumed 266 g. over 8 hr. immediately before slaughter; intake equivalent to animal 348 on an hourly basis	—	26	157

DISCUSSION

The concentrations of methylmalonic acid in the livers at slaughter were presumably affected by the quantities of food consumed over the few hours immediately preceding death. Ruminal production of short-chain volatile fatty acids, including propionic acid, rapidly follows the consumption of food (Gray, Walker, Pilgrim & Jones, 1957) and is followed by their absorption and metabolism. However, apart from the low concentration of methylmalonic acid in the liver of the vitamin B_{12}-treated animal that was starved (no. 392), there was no clear indication of this in the results. Thus the highest concentration was found in the deficient animal no. 357 after 5 hr. starvation, and from body weight and liver vitamin B_{12} concentration this was the least severely affected of the deficient animals. When food intake was adjusted on an hourly basis and animals were fed for only 8 hr. each day, this was accompanied by a decreased daily food intake by one deficient animal (Expt. 3) and an increased daily food intake by the other (Expt. 2). The concentrations of methylmalonic acid found, however, were similar in the livers of both these animals after 8 hr. of feeding and both values were lower than that of animal no. 357.

On the other hand the concentrations of CoA found were very similar in livers of all three deficient animals, and in no case did the maximum possible concentration of methylmalonyl-CoA exceed one-third of the total CoA. It is concluded that the extra CoA in the livers of vitamin B_{12}-deficient sheep was not in the form of methylmalonyl-CoA.

We record our thanks to the late Dr H. R. Marston for suggesting this work and for providing the pair-fed animals. Thanks are due to Dr M. C. Dawburn, formerly of this Division, for the vitamin B_{12} analyses.

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Serum Levels of Vitamin B_{12} Following Intramuscular Injection of a Depot Preparation

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IN AN earlier study[1] we presented clinical evidence showing that cobalamin administered intramuscularly in oil with aluminum monostearate was excreted into the urine over a prolonged period. This was in contrast with cobalamin administered in an aqueous medium. In the latter instance essentially all of the administered vitamin appeared to be recovered in the urine within twenty-four hours.

In view of the interesting effect of the oil-aluminum monostearate combination in prolonging the availability of the vitamin to the organism following injection, it appeared worthwhile to determine the extent to which the blood level of the vitamin might reflect the influence of the depot vehicle. Data on this point from studies with dogs are presented here.

METHODS

Control blood samples were taken from two groups of four dogs each apportioned as equally as possible on a weight basis. Following this, the vitamin in sterile water (Ducobee® "1000," 1,000 μg. vitamin B_{12} per ml.) or in sesame oil with 2 per cent aluminum monostearate (Ducobee Depot, 1,000 μg. vitamin B_{12} per ml.) was administered to each of the dogs intramuscularly (rectus femoris) in a dose of 15 μg. per kg. On a weight basis this amount approximated that given in the earlier clinical trial. Blood samples were then taken at one, two, three, four, seven, nine, eleven and fifteen days.

Blood samples, 8 to 10 ml. from the jugular vein, were taken from the dogs the morning before feeding so that they represent fasting samples. They were chilled for one hour to promote shrinking of the formed clot. They were then centrifuged for a half hour and the clear serum was taken for assay.

For estimation of the cobalamin content of serum, 1 to 2 ml. aliquots were diluted 1:5 with pH 4.6 acetate buffer following the procedure of Gaffney et al.,[2] steamed for a half hour, cooled to room temperature and centrifuged. An aliquot was diluted 1:2 with 0.33M Na_2HPO_4 to bring it to neutrality and then further diluted as needed for microbiologic assay by the U.S.P. procedure using L. leichmannii as the test organism. The extent of growth of the organism was based upon titration with 0.05N alkali of the lactic acid produced over a seventy-two-hour incubation period at 37°C. A comparison of the lactic acid production in the tubes containing the serum samples with the acid in the tubes containing the reference vitamin B_{12} permitted calculation of the vitamin B_{12} content of the serum samples.

To determine whether the depot preparation would exercise any untoward effect upon continued injection, it was administered twice weekly to two dogs (beagle) and two monkeys (rhesus) for a total of forty-six injections to each over a period of 170 days. Each of the four extremities were utilized in rotation. Hematologic studies were carried out on the animals before medication was started and at six-week intervals thereafter. At the end of the injection period, all of the animals were

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TABLE I

Serum Vitamin B_{12} Levels in Two Groups of Four Dogs Each Following the Intramuscular Administration of the Vitamin in Water or in a Depot Oil and Aluminum Monostearate Preparation

Determination	Dog No.	Weight (kg.)	Serum Vitamin B_{12} Following Its Intramuscular Injection (mμg. per ml.)								
			Initial	Day							
				1	2	3	4	7	9	11	15
Aqueous Preparation											
	1	7.8	0.33	1.94	0.735	0.72	0.62	0.50	0.41	0.53	0.475
	2	7.0	0.39	1.60	0.676	0.51	0.64	0.42	0.39	0.39	0.32
	3	13.0	0.22	1.88	0.62	0.66	0.52	0.38	0.36	0.32	0.21
	4	12.6	0.33	1.79	0.72	0.64	0.54	0.36	0.33	0.34	0.23
Average			0.30	1.82	0.76	0.63	0.58	0.43	0.37	0.40	0.31
Standard error			±0.03	±0.06	±0.17	±0.04	±0.03	±0.04	±0.02	±0.05	±0.06
vs. initial			...	...	...	...	p = <0.001	p = 0.04	p = 0.07	p = 0.11	Not significant
Depot Preparation											
	5	5.8	0.36	2.50	1.07	1.75	1.02	0.765	0.71	0.785	0.51
	6	9.8	0.30	3.60	1.57	1.47	1.32	0.86	0.795	0.775	0.40
	7	11.6	0.33	2.50	1.62	1.10	0.95	0.46	0.34	0.35	0.22
	8	13.8	0.24	3.90	2.82	2.00	1.67	1.20	0.71	0.65	0.34
Average			0.31	3.17	2.00	1.58	1.22	0.82	0.64	0.62	0.38
Standard error			±0.02	±0.35	±0.29	±0.19	±0.14	±0.15	±0.10	±0.10	±0.07
vs. initial			...	...	...	...	...	p = 0.012	p = 0.014	p = 0.02	p = 0.4
vs. aqueous			...	...	...	p = 0.0014	p = 0.0025	p = 0.04	p = 0.03	p = 0.09	Not significant

examined grossly, and one of the monkeys was sacrificed for tissue section studies. The injection sites were imbedded in paraffin, sectioned at 6 μ, and stained with hematoxylin and eosin.

RESULTS

The results of the microbiologic analyses of the serum samples for vitamin B_{12} following the intramuscular injections of each of the preparations into the dogs are summarized in Table I. The average pretest serum vitamin B_{12} levels of 0.30 to 0.31 mμg. per ml. fall into the range of those noted for human beings by Chalmeras and Shinton[5] by assay with Euglena gracilis[4] and by Thompson and Hecht[6] by the procedure of Gaffney et al.[3]

From the average response to the administration of each of the vitamin B_{12} source preparations summarized in Table I and in Figure 1, it is seen that the dogs exhibited markedly elevated serum vitamin B_{12} levels. Those given the vitamin in an aqueous menstruum clearly had elevated levels through four days at which time their serum vitamin B_{12} values averaged 0.58 mμg. per ml. Since two or three of the four dogs of the group had serum vitamin B_{12} levels below 0.40 mμg. per ml. after four days, the significance of the average being above the pretest level may be questioned ($p = 0.04$ or higher).

It is interesting that the serum vitamin B_{12} levels of the four dogs given the vitamin as a depot preparation not only exhibited elevated levels over a longer period, between eleven and fifteen days, but also that the levels at-

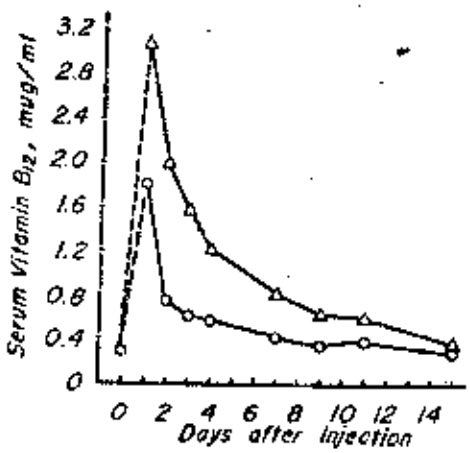

FIG. 1. Average serum vitamin B_{12} levels in dogs following the intramuscular injection of the vitamin in an aqueous (○- ○) or depot (△- △) preparation.

tained averaged two to three times those of the dogs given the vitamin in an aqueous medium. This may reflect the fact noted earlier[1] that excess vitamin is quickly excreted following its administration in water. Release of the vitamin administered in oil containing aluminum monostearate took place more slowly as shown by prolonged urinary excretion. This is in accord with the above result which demonstrates elevated serum levels for a period of time about threefold longer than that following administration of the vitamin in the usual aqueous medium.

Of interest equal, perhaps, to the relative rates of excretion following the administration of the vitamin in an aqueous or depot preparation is the correlation between the data of this study and those of Buckwalter and Dickison.[4] Studying blood levels following the administration of procaine penicillin in oil and aluminum monostearate, these investigators noted that the vehicle supported elevated blood levels of the antibiotic for about twelve days. This is in striking agreement with the data given in Table I in which it is seen that the vitamin was clearly maintained at elevated levels for more than eleven (although less than fifteen) days.

With regard to the effects of the repeated injections in dogs and monkeys we may summarize briefly by noting that there was no apparent evidence of pain, redness or swelling at any time in any of the injection sites. No significant changes were observed in the total red and white blood cell counts, the differential counts, the hematocrit or in the hemoglobin concentration of the blood of the monkeys or the dogs. Microscopically, necrosis of muscle tissue was not seen although droplets of oil were observed in the connective tissue between the muscle bundles. Thus, it is seen that no adverse effects could be discerned and the preparation was completely tolerated as evidenced both by gross and microscopic examination of the sites of injection.

In conclusion, we may say that oil and aluminum monostearate serve as an effective depot vehicle for the intramuscular administration of vitamin B_{12} just as they do for procaine penicillin.

SUMMARY

Serum levels of vitamin B_{12} were determined in two groups of four dogs each following the intramuscular administration of vitamin B_{12} in water or in a depot oil and aluminum monostearate preparation.

Confirming earlier published observations based on the urinary excretion of the vitamin following its administration in each of these vehicles, the studies with the dogs showed that the vitamin administered in a depot form maintained elevated levels for a period about three times that following the administration of the vitamin in the usual aqueous medium.

It was noted, in addition, that the serum levels which followed the administration of the vitamin in the depot preparation were two to three times those which followed the administration of the vitamin in water.

Tolerance to injection of the preparation was established. No untoward blood or tissue effects were seen in dogs or monkeys following forty-six injections of the preparation over a 170-day period.

Accordingly, it is clear that administration of vitamin B_{12} in oil with aluminum monostearate results in prolonged and elevated serum levels of vitamin B_{12}.

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