VITAMIN B_{12}

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Absorption, Distribution, and Elimination of a Long-Acting Vitamin B_{12} Preparation

By KNUD KRISTENSEN* and TAGE HANSEN

The prolonged effect of various long-acting vitamin B_{12} preparations has been examined on rabbits regarding absorption, distribution, and elimination after intramuscular injection.

RECENT investigations (13) have shown that patients with pernicious anemia have considerably greater requirements for vitamin B_{12} than have previously been assumed. More recently, there has been considerable interest in preparations that can meet the requirements more adequately and in a more satisfactory way than the aqueous solutions of vitamin B_{12} used previously. The authors have investigated the characteristics of various preparations made on the basis of various principles. A preparation containing cyanocobalamin–tannin complex suspended in aluminum monostearate oil gel[1] was studied in detail.

The clinical value of this preparation is reflected in papers by Bastrup Madsen *et al.* (1, 5), Schwartz *et al.* (19, 20), Moulopoulos (14, 15), an editorial (8), Nielsen and Vedso (16), Schrumpf (18), and Gough *et al.* (10).

EXPERIMENTAL

Materials and Methods

Preparations A–G were investigated (Table I). After intramuscular injection (i.m.) in rabbits the following were investigated: (a) liberation of vitamin B_{12} ^{60}Co from the site of injection; (b) absorption of vitamin B_{12} ^{60}Co by the liver; and (c) excretion of vitamin B_{12} ^{60}Co in urine and feces.

For preparations F and G the investigations were supplemented by radioactivity counts and microbiological determinations of vitamin B_{12} in liver, kidney, and femoral muscle about 3 months after the start of trial.

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[1] Marketed as Behrocex (Dumex Ltd., Copenhagen, Denmark).

Preparations

A number of preparations were made using vitamin B_{12} labeled with ^{60}Co. Their composition is shown in Table I. The products were prepared according to methods described in a British patent (6).

The distribution and excretion of vitamin B_{12} after parenteral administration of preparation B and of aqueous solutions of cyanocobalamin were investigated in rats and in healthy subjects by Davis *et al.* (7), Thompson and Hecht (22), Astudillo *et al.* (3), and Gross *et al.* (9), but no records have been found of investigations on patients with pernicious anemia. The authors have not investigated any preparations of vitamin B_{12} suspended in oil or vitamin B_{12} suspended in 2% monostearate oil gel; the latter was described by Arnold *et al.* (1, 2), Sobell *et al.* (21), and Heinrich and Gabbe (11). It does not appear to possess any retarded action of interest for clinical use.

Preparations F and G correspond to a marketed suspension of cyanocobalamin–tannin complex in aluminum monostearate oil gel,[1] except that labeled vitamin B_{12} (^{60}Co) was used instead of ordinary cyanocobalamin. Spectrophotometric and microbiological checks were made on the preparations. The radiochemical purity of the labeled compounds was confirmed by paper chromatography and subsequent counts and by microbiological determinations of the vitamin B_{12} activity on agar plates with *Lactobacillus leichmannii*, as described by Winsten and Eisen (23), among others.

Animal Material.—Twelve white male rabbits were used. The initial weights were from 2.7 to 3.0 Kg. The rabbits were anesthetized with sodium amobarbital (30 mg./Kg.) supplemented by ether during the radioactivity counts.

Standards and Methods of Measurement.—The radioactivities above the site of injection and above the liver were determined on a scintillation detector shielded by 5 cm. of lead with a 30 mm. round opening upon which the object to be measured was placed. Feces and organs were homogenized before

TABLE I.—DOSAGE SCHEDULE OF VARIOUS VITAMIN B_{12} PREPARATIONS

Prepn.	Form of Vitamin B_{12}	Vehicle [a]	Vitamin B_{12} Concn., mcg./ml.	B_{12}-Co Concn., gc./ml.	Quantity Injected, ml.
A	Cyanocobalamin	Aqueous 0.9% NaCl soln.	500	0.18	0.30
B[a]	B_{12}-zinc-tannate	Aqueous suspension	500	0.18	0.30
C	B_{12}-zinc tannate	Suspended in 2% aluminum monostearate oil gel	500	0.5	0.25
D[a]	B_{12}-tannate	Suspended in 2% aluminum monostearate oil gel	500	0.5	0.25
E	B_{12}-tannate	Suspended in sesame oil	500	0.5	0.25
F[b]	B_{12}-tannate	Suspended in 2% aluminum monostearate oil gel	1000	1.0	0.50
G[b]	B_{12}-tannate	Suspended in 2% aluminum monostearate oil gel	1000	1.0	0.50

[a] Trademarked preparation is Depinar. [b] Trademarked preparation is Retolvex.

counting. A scintillation detector with a well-type crystal was used for counting radioactivity in urine and liquid samples of feces and organs.

The microbiological vitamin B_{12} activity was determined by the *L. leichmannii* method (12) as modified by Noer (17).

Before and after each measurement the scintillation detector was checked against a standard and corrected accordingly.

In order to determine the absolute content of radioactive vitamin B_{12} in samples of urine, feces, and organs, a known quantity of the injection preparation in question was added after the samples had been counted. Thus, the counts of the samples and of the standards were carried out under the same conditions.

Trial Methods.—Blank values were determined on urine and feces collected for 24 hr. from the rabbits in specially constructed metabolism cages. After being anesthetized, the rabbits were strapped to the counting table with the right femoral muscle above the detector.

After the background had been counted, the preparation to be tested was injected intramuscularly into the right femoral muscle directly above the detector. The doses employed are shown in Table I. Immediately afterward, the radioactivity at the site of injection was screened at various sites around the injection site, and the counts were carried out at the site of highest activity. Immediately afterward, the radioactivity in the hepatic region was determined in the manner described above.

Counts of the activity at the site of injection and in the hepatic region were carried out at irregular intervals for 3 months, and their relative values, as percentages of the initial count, were calculated.

During the first 3–4 days after the injection, urine and feces were collected as 24-hr. samples.

In the tests made with preparations F and G, the rabbits were killed with ether after the test period of about 3 months; liver, kidney, and femoral muscle were dissected free, and their contents of radioactive material were determined. In addition to the organs mentioned above, the spleen, heart, bladder (containing urine), small intestine (duodenum, jejunum, ileum), large intestine (cecum, including vermiform appendix, colon, rectum), and contents of both large and small intestine were collected from rabbit 3. (See Table II.) Liver, kidneys, and muscle from the right femur were taken from the control rabbit, which had been kept on the same diet as the test animals for 3 months, in order to obtain a basis of comparison and a standard for the content of vitamin B_{12}. A microbiological determination of vitamin B_{12} was carried out on all homogenates of liver, kidney, and femoral muscle.

RESULTS

Measurement Above the Site of Injection.—Figure 1 shows the increase of prolonged effect obtained by combining the different principles. It will be seen that a clearly prolonged effect is only obtained by suspending a vitamin B_{12} compound in

TABLE II.—PERCENTAGE RADIOACTIVITY ABOVE SITE OF INJECTION

Rabbit	Prepn.	Std.	Days After Injection 2	3	4	7	9	10	11	15	18	21	25	28	30
1	F	100	69.8		65.7				60				47.9		
2	F	100		76.2			68.2					52.7			
3	G	100	86.5					85		81.5				72	
4	G	100		61.1		55.1			50		40.5				34.6

TABLE II.—(*Continued*)

Rabbit	Prepn.	Std.	Days After Injection 37	39	44	46	53	59	66	78	87	95
1	F	100		34			22.7			9.7		
2	F	100	38.9						10.9		7.6	
3	G	100			51.6							
4	G	100				25.5		19.4				4.1

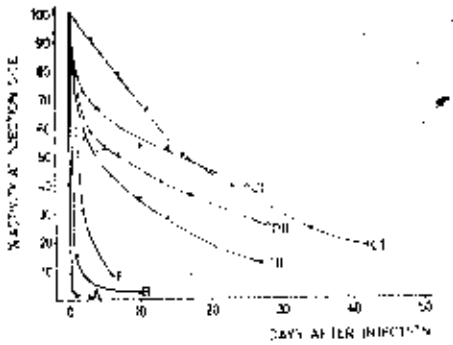

Fig. 1.—Absorption of radioactive vitamin B_{12} from the site of injection. Key: A, aqueous solution of vitamin B_{12}; B, aqueous suspension of B_{12}-zinc-tannate; C_I and C_{II}, B_{12}-zinc tannate suspended in a 2% aluminum monostearate oil gel; D_I and D_{II}, B_{12} tannate suspended in a 2% aluminum monostearate oil gel; E, B_{12}-tannate suspended in oil.

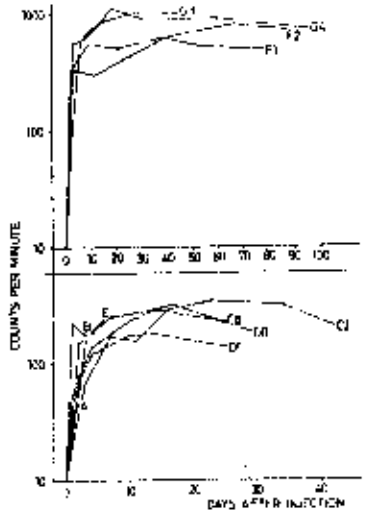

Fig. 2.—Radioactive vitamin B_{12} above the liver. Key: (bottom) A, aqueous vitamin B_{12} solution; B, aqueous suspension of B_{12}-zinc-tannate; C_I and C_{II}, B_{12}-zinc-tannate suspended in a 2% aluminum monostearate oil gel; D_I and D_{II}, B_{12}-tannate suspended in a 2% aluminum monostearate oil gel. E, B_{12}-tannate suspended in oil. Key: (top) F_I and F_{II}, B_{12}-tannate suspended in a 2% aluminum monostearate oil gel; G_I and G_{II}, B_{12}-tannate suspended in a 2% aluminum monostearate oil gel.

aluminum monostearate oil gel. Table II shows the prolonged effect of two preparations of vitamin B_{12}-tannate in aluminum monostearate oil gel (F and G). Rabbit 3, which died during narcosis about 1.5 months after the beginning of the investigation, differed from the other three. During the period of investigation the rabbit did not move about much. This could have been the reason for the small liberation of vitamin B_{12} from its intramuscular depot.

Measurement Above the Liver.—Figure 2 gives an impression of the storage of vitamin B_{12} in the liver, obtained by combining the different principles. The graphs for preparations F and G show considerably higher counts because of the larger amount of radioactivity injected (see Table I).

The counts shown in Fig. 2 were obtained by deducting the value expressing a scattered radiation from the depot of the femoral muscle from the counts corrected for background. The value of scattered radiation decreases in proportion to the activity at the site of injection; accordingly, the correction will be:

(corr. counts per minute above liver immediately after injection) × (% activity at site of injection on the day in question) divided by 100.

Because of the somewhat inaccurate method of measurement, only the shapes of the graphs, not the absolute values should be compared.

Excretion in Urine.—Table III shows the difference in excretion in urine after administering the various preparations. The differences in urinary excretion between preparation D and preparations F and G may be explained partly by the larger quantity of F and G injected, and partly by a minute amount of free vitamin B_{12} in the preparations.

Excretion in Feces.—The excretion in the feces was <1%/24 hr.

Content of Vitamin B_{12} in Liver, Kidneys, and Femoral Muscle.—At the time when the rabbits were killed the vitamin B_{12} contents of liver, kidneys, and femoral muscle were measured, both by radioactivity measurements and by microbiological determinations. Table IV shows the results. The counts immediately show that the content of vitamin B_{12} in liver and kidneys of rabbits treated with preparations F and G has increased compared with that of the control rabbit. The content of vitamin B_{12} in the right femur is due to vitamin B_{12} not yet liberated from the site of injection, and a reasonable agreement with the results obtained by radioactivity measurements above the site of injection was found. The difference between vitamin B_{12} measured microbiologically and vitamin B_{12} determined by radioactivity gives us the level of vitamin B_{12} in the rabbits before the injection and is of the same order of magnitude as those obtained microbiologically from the control rabbit.

Radioactive Vitamin B_{12} Content in Other Organs and Tissues (Rabbit 3).—*Spleen.*—No measurable vitamin $B_{12}{}^{60}Co$ (<1% of dose administered).

Heart.—No measurable vitamin $B_{12}{}^{60}Co$ (< 1% of dose administered).

Contents of Intestine.—Less than 1% of dose administered.

TABLE III.—URINARY EXCRETION OF VARIOUS VITAMIN B_{12} PREPARATIONS

Rabbit	Prepa	1st. 24 hr., %	2nd. 24 hr., %	3rd. 24 hr., %	4th. 24 hr., %
	A	60	<5	<2	...
	B	10–15	<5	<2	...
	C	<1	<1	<1	...
	D	<1	<1	<1	...
	E	19	11	2	...
1	F	7	6	<1	<1
2	F	13	1	<1	<1
3	G	2	4	<1	<1
4	G	6	1	<1	<1

TABLE IV.—VITAMIN B_{12} CONTENTS OF LIVER, KIDNEYS, AND RIGHT FEMORAL MUSCLE

Prepn.	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Rabbit	1	2	3	4	5 (control)
Days after inj.	78	87	14	95	
Liver					
Weight, Gm.	184	151	71	168	121
Vitamin B_{12}, microbiologically, total, mcg.	66.8	65.3	36.2	81.5	30.1
Total vitamin B_{12} radioactivity, mcg.	28.9	23.6	23.9	19.1	...
Kidneys					
Weight, Gm.	13.7	10.9	13.5	14.8	10.3
Vitamin B_{12}, microbiologically, total, mcg.	6.2	7.5	6.0	7.1	4.2
Total vitamin B_{12} radioactivity, mcg.	3.2	3.0	2.5	4.4	...
Right Femoral Muscle					
Weight, Gm.	101	126	98	131	112
Vitamin B_{12}, microbiologically, total, mcg.	67	26	...	30	8
Total vitamin B_{12} radioactivity, mcg.	60	23	275	24	...
	12% of dose	4.6% of dose	55% of dose	4.8% of dose	
Radioactivity above site of injection, % (see Table II)	9.7	7.6	54.6	4.1	...

Small Intestine (Jejunum, Duodenum, Ileum).—Less than 1% of dose administered.

Large Intestine (Cecum, Including Vermiform Appendix, Colon, Rectum).—Less than 1% of dose administered.

Bladder (Containing Urine).—Less than 1% of dose administered.

DISCUSSION

These investigations on rabbits clearly reveal that preparations of B_{12}-tannate or B_{12}-zinc-tannate in a 2% aluminum monostearate oil suspension release vitamin B_{12} from an intramuscular depot over a much longer period of time than does B_{12}-zinc-tannate in an aqueous suspension or B_{12}-tannate in an oil suspension or vitamin B_{12} in a water solution (Fig. 1).

Vitamin B_{12} preparations in a 2% aluminum monostearate oil suspension can release vitamin B_{12} for the body for more than 1 month compared with a few days for the other types of preparations investigated.

By this prolonged release one avoids the urinary excretion that always follows injections of vitamin B_{12} in excess of serum vitamin B_{12} binding capacities. This also means that vitamin B_{12} is available for storage in vitamin B_{12} depots over a longer period of time (Fig. 2). Due to the techniques used, only the shapes of the curves are comparable. A more detailed study of 2 different vitamin B_{12} preparations in a 2% aluminum monostearate oil suspension showed vitamin B_{12} was released from the intramuscular depots for more than 2 months, with some of the injected material still left at the injection site as vitamin B_{12} as determined by microbiological methods. Table IV shows there is good agreement between direct measurements above the site of injection and measurements of the dissected femoral muscle.

Furthermore, it is shown (Table IV) that the released vitamin B_{12} was deposited in the liver and kidneys, as could be expected, and that the liver and kidneys from rabbits injected with B_{12}-tannate in a 2% aluminum monostearate oil suspension contained a considerably greater amount of vitamin B_{12} in comparison with the control.

SUMMARY

By investigations carried out on rabbits, the properties of various vitamin B_{12} preparations with prolonged effect are demonstrated, including the liberation from intramuscular depots and the storage in the natural depots of the body. After intramuscular injection of B_{12}-tannate in aluminum monostearate oil gel the observations were: (*a*) a prolonged liberation of vitamin B_{12} from the depot injected into the muscle; (*b*) an increased vitamin B_{12} activity in liver and kidneys, taken as a sign of storage in the natural depots of the body; and (*c*) minimum excretion in urine.

REFERENCES

(1) Arnold, A., *et al.*, *Am. J. Clin. Nutr.*, **10**, 561 (1962).
(2) Arnold, A., *et al.*, *Ann. Pharmacotherapy Res. Fecal.*, **9**, 1835 (1978).
(3) Asturdillo, M., *et al.*, *Rev. Clin. Espan.*, **78**, 681 (1961).
(4) Dustrup-Madsen, P., *et al.*, *Ugeskrift Læger*, **124**, 1087 (1962).
(5) *Ibid.*, **128**, 261 (1966).
(6) Brit. pat. 980650.
(7) Davis, R. L., *et al.*, *Proc. Soc. Exptl. Biol. Med.*, **101**, 211 (1959).
(8) Editorial, *Ugeskrift Læger*, **124**, 1716 (1962).
(9) Glass, G. B. J., *et al.*, *Blood*, **18**, 511 (1961).
(10) Gough, J., *et al.*, *Lancet*, **ii**, 1311 (1961).
(11) Heinrich, H. C., and Gibbe, E. E., *Arzneimittel-Forsch.*, **11**, 1087 (1961).
(12) Hoff Jørgensen, E., "Microbiological Assay of Vitamin B_{12}," in Glick, D., ed., "Methods of Biochemical Analysis," vol. 1, Interscience Publishers, Inc., New York, N. Y., 1957, pp. 81–113.
(13) Kristensen, K., *et al.*, *Ugeskrift Læger*, **124**, 1681 (1962).
(14) Meulengracht, E., *Ibid.*, **124**, 1693 (1962).
(15) Meulengracht, E., *Lancet*, **i**, 454 (1963).
(16) Nielsen, B. G., and Vedsø, S., *Ugeskrift Læger*, **125**, 1446 (1963).
(17) Nørr, B., "Vækstfaktoren for *Lactobacillus leichmannii* 313 i Halvog Helsyntetiske Substrater," thesis in "Collected Papers from Farmacon," vol. 3, 1960, Copenhagen, Denmark, 1956–1959, 120 pp.
(18) Schaudopf, A., *Tidsskr. Norske Lægeforen.*, **83**, 1511 (1963).
(19) Schwartz, M., *et al.*, *VII Intern. Kongr. Int. Med.*, **1962**, 561.
(20) Schwartz, M., *et al.*, *Lancet*, **ii**, 1181 (1962).
(21) Sibell, S. D., *et al.*, *Am. J. Clin. Nutr.*, **8**, 214 (1960).
(22) Thompson, R. H., and Hecht, R. A., *Biol.*, **7**, 311 (1950).
(23) Winsten, W. A., and Elgen, B., *J. Biol. Chem.*, **181**, 161 (1949).

J. Amer. Chem. Soc. 77(1):252

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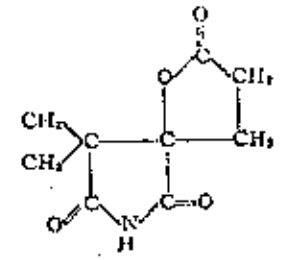
VITAMIN B_{12}. XXIV. DL-3,3-DIMETHYL-2,5-DIOXO-4-HYDROXYPYRROLIDINE-4-PROPIONIC ACID LACTONE AND DL-3,3-DIMETHYL-2,5-DIOXOPYRROLIDINE-4-PROPIONIC ACID, NEW DEGRADATION PRODUCTS

Sir:

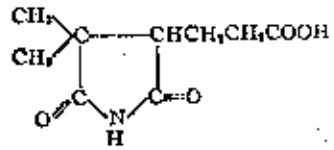
The oxidation of acid-hydrolyzed vitamin B_{12} with sodium chromate in acetic acid has given new pyrrole-like degradation products which have been identified by degradation and synthesis. These pyrrole-like products constitute the first detailed organic structural evidence concerning the heterocyclic nature of the red cobalt complex which remains after hydrolysis.

The chloroform-soluble portion of the oxidation reaction mixture was subjected to countercurrent distribution between chloroform and water. Material with a distribution coefficient near unity yielded a crystalline, weakly acidic substance (I) of m.p. 151–152°, of the composition $C_8H_{11}NO_4$. The compound showed infrared absorption bands at 3.02, 5.65, and 5.80 μ, in agreement with an imide structure containing a γ-lactone group. Hydrolysis converted the imide to a lactone dicarboxylic acid, m.p. 167–168°. The material from the chloroform–water distribution which was excessively soluble in the aqueous phase was redistributed between water and ether to yield a second imide (II), m.p. 143–144°, of the composition $C_8H_{13}NO_4$. Hydrolysis of the imide yielded a tricarboxylic acid, m.p. 152–153°.

Fusion of the imide (I) with potassium hydroxide gave succinic and isobutyric acids. When this imide was distilled with zinc dust, a basic product was obtained which gave a positive Ehrlich pyrrole test. Of two possible structures for the imide (I), DL-3,3-dimethyl-2,5-dioxo-4-hydroxypyrrolidine-4-propionic acid lactone was found to be correct by synthesis.



I

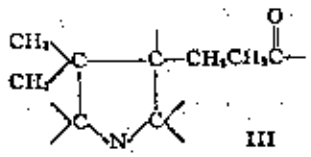


II

The sodium enolate of ethyl isobutyrate was treated with β-carbethoxypropionyl chloride. The resulting diethyl 2,2-dimethyl-3-oxo-adipate, on addition of hydrogen cyanide and hydrolysis of the cyanohydrin diethyl ester so produced, gave a compound identical with the lactone dicarboxylic acid. Pyrolysis of the ammonium salt of this synthetic lactone acid yielded the imide (I).

The composition and melting point of the tricarboxylic acid were found to correspond closely to those properties of 2-methyl-pentane-2,3,5-tricarboxylic acid.[1] A synthetic specimen of this acid was prepared[1] and found to be identical to that obtained by degradation. This synthetic acid on treatment with one mole of ammonia yielded, after pyrolysis, DL-3,3-dimethyl-2,5-dioxo-4-hydroxypyrrolidine-4-propionic acid, which was found to be identical to the imide (II).

The imide grouping of the degradation products I and II is most probably the result of the oxidation reaction. The imides are probably derived from a moiety or moieties in the vitamin B_{12} molecule having a pyrrolidine, pyrroline or pyrrolenine nucleus as represented by the partial structure III. Imides I and II are optically inactive. In view of the



III

polyamide character of vitamin B_{12},[2] and our observation that the yield of acidic oxidation products is increased by prior hydrolysis, the presence of the free carboxyl group of partial structure III

(1) Perkin and Thorpe, *J. Chem. Soc.*, **85**, 128 (1904).

(2) J. B. Armitage, J. R. Cannon, A. W. Johnson, S. F. J. Parker, B. S. Smith, W. H. Stafford and A. R. Todd, *Ibid.*, 3840 (1953); H. Diehl and J. L. Billinghoe, *Iowa State College Journal of Science*, **27**, 431 (1953).

as an amide in the parent molecule is a distinct possibility.

Other investigators[3] have reported the isolation of oxalic, succinic, methyl succinic, and dimethyl malonic acids in addition to four unknown ones by the permanganate oxidation of acid-hydrolyzed vitamin B_{12}. Dimethyl malonic and succinic acids might be derived from moieties having structure III.

These imides I and II and their bearing upon the organic structure and coördination linkages of vitamin B_{12} are being studied further.

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RECEIVED NOVEMBER 16, 1954

(3) H. Schmid, A. Ebnother and P. Karrer, *Helv. Chim. Acta*, **36**, 65 (1953).

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Nutritional vitamin B_{12} deficiency in an infant

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MEGALOBLASTIC ANEMIA secondary to dietary deficiency of vitamin B_{12} in young infants is exceedingly rare in this country. This paper describes an infant with megaloblastic anemia secondary to insufficient vitamin B_{12} in breast milk. The patient's mother was a vegetarian. This case is reported to emphasize the importance of administration of exogenous vitamin B_{12} to infants breast-fed by mothers who are vegetarians.

From the Department of Pediatrics, University of Cincinnati, The Children's Hospital, and The Children's Hospital Research Foundation.
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Dr. Lampkin is a recipient of Special Fellowship Award No. CA 42900 from the National Cancer Institute.
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CASE REPORT

The patient was a 10-month-old Caucasian boy who was admitted to The Children's Hospital because of anemia. He was born at term; the birth weight was 8 pounds and 5 ounces. From birth, the child was breast-fed without supplementation with other foods or vitamins. For approximately one month before admission the patient had had a progressive decrease in activity and an increase in irritability. The mother was a Seventh Day Adventist and for 12 years had been a vegetarian who excluded almost all animal protein from her diet. She had had 7 pregnancies during the past 12 years. Three pregnancies resulted in viable infants, and 4 ended during the first trimester. During her pregnancies she took multiple vitamins which contained 1 μg of vitamin B_{12} per capsule. The vitamin capsules were discontinued at the termination of each pregnancy.

On physical examination, the infant was pale and irritable. The results of the remainder of

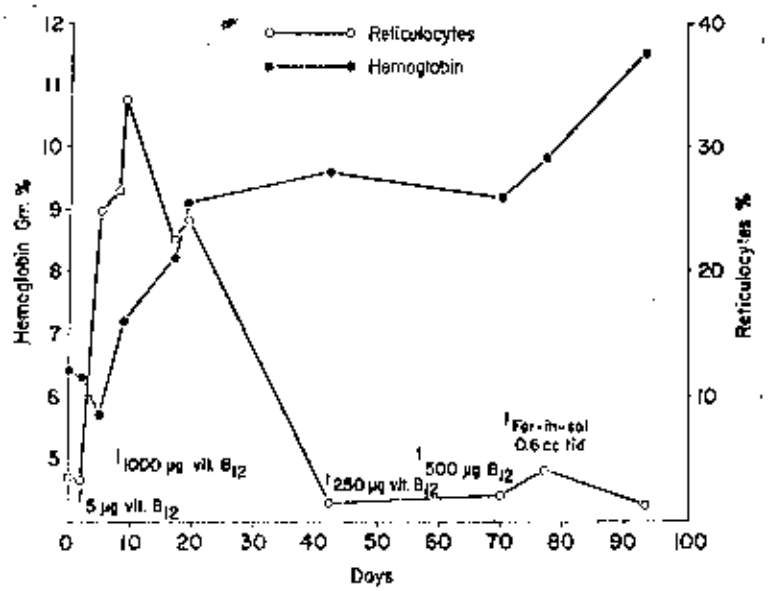

Fig. 1.

the physical examination were within normal limits. Initial laboratory data were as follows: hemoglobin, 6.4 Gm. per cent; hematocrit, 21 per cent; red blood cell count, 1.5 million per cubic millimeter. The red cell indices were calculated to be: mean corpuscular volume, 99 $c\mu$; mean corpuscular hemoglobin, 30 $\mu\mu g$; mean corpuscular hemoglobin concentration, 30 per cent. The white blood cell count was 4,700 per cubic millimeter with 13 per cent neutrophils. The platelet count was 128,000 per cubic millimeter, and the reticulocyte count was 3.1 per cent. On blood smear, there were macrocytes, microcytes, occasional nucleated red blood cells which were megaloblastic, and giant platelets. Neutrophils were not hypersegmented. The diagnosis of megaloblastic anemia was confirmed by examination of the bone marrow aspirate.

Five micrograms of vitamin B_{12} was given intramuscularly because of the probability from history that the child had a vitamin B_{12} deficiency. The infant continued to be breast-fed and no change was made in the mother's diet until response to therapy had been observed. Within 24 hours after the injection, the patient was less irritable and more active. Seventy-two hours after injection, erythroid hyperplasia with only minimal megaloblastic changes was present in a bone marrow aspirate. The hemoglobin and reticulocyte response to therapy with vitamin B_{12} are shown in Fig. 1. Reticulocytosis began on the third day after the injection of vitamin B_{12} and was maximal on the seventh day. A progressive increase in the hemoglobin concentration to 9.1 Gm. per cent was seen 17 days after starting vitamin B_{12} therapy. A Schilling test was done and was normal (Table I). As seen in Table I, prior to therapy the serum vitamin B_{12} level was low, and the serum folate level was high. Forty days after starting B_{12} therapy, the patient's hemoglobin was only 9.6 Gm. per cent. An additional 750 μg of vitamin B_{12} was given without a reticulocyte response. At this time the patient's serum iron studies were indicative of iron deficiency. The values for serum iron and per cent saturation of iron were higher than normal prior to vitamin B_{12} therapy (Table I). Following the administration of iron, the hemoglobin value increased to the normal range and has remained so for the past 2 years. The serum vitamin B_{12} and folate levels returned to normal after vitamin B_{12} therapy (Table I).

In spite of a normal bone marrow, the absolute number of neutrophils remained less than 1,000 per cubic millimeter, but more than 500 for 8 months. Coincident with a second degree burn of his left foot, the absolute neutrophil count increased above 1,500 and has been normal for the past 2 years. A white blood count

Table I. Special laboratory studies

Test	Patient: Before treatment	Patient: 68 days after treatment	Mother
Serum vitamin B_{12} (pg./ml.)	90	520	200
Serum folate (ng./ml.)	32	10.2	12.4
Serum iron (γ %)	270	65	
Total iron-binding capacity (γ %)	310	543	
% saturation	88	12	
Breast milk			0.080
Schilling test*		10.2	23.0

Serum and breast milk vitamin B_{12} determinations were done using *Lactobacillus leichmannii*.[7] (Normal range of values in serum in Dr. John Will's laboratory in Cincinnati is 150 to 500 pg. per milliliter. Reported normal range of values in breast milk is 0.10 to 1.5 μg per liter[7]). Serum folate determinations were done using *Lactobacillus casei*; normal range of values is 5 to 21 ng. per milliliter.[3]

*Per cent excretion of 0.5 μc Co^{58} labeled vitamin B_{12}. Greater than 8 per cent is normal. Test was done on patient 11 days after start of treatment.

and differential had never been obtained in the patient before his admission to The Children's Hospital.

As seen in Table I, the maternal serum B_{12} was at the lower level of the normal range, and the vitamin B_{12} concentration in the breast milk was low. The mother's serum folate level and the Schilling test (Table I) were normal.

There was no family history of anemia or neutropenia, and complete blood counts in the father, mother, and sister were not abnormal. Since the diagnosis of megaloblastic anemia in the infant, the mother has been taking 1 μg of vitamin B_{12} daily by mouth. Eight months ago, an infant boy was born. This infant has been breast-fed since birth and began to receive solid food at 2 months of age. There is no evidence of megaloblastic anemia in this infant.

DISCUSSION

The rapid clinical and hematologic responses to 5 μg of vitamin B_{12} demonstrated that the infant had a deficiency of that vitamin. Serum obtained for a vitamin B_{12} level on admission, but reported later, confirmed this deficiency. The normal result of the Schilling test excluded pernicious anemia and intestinal malabsorption of vitamin B_{12} in the infant. The low vitamin B_{12} concentration in the breast milk established that the deficiency in the infant was on a nutritional basis. Nutritional vitamin B_{12} deficiency has been reported in a breast-fed infant whose mother had pernicious anemia.[4] Pernicious anemia and intestinal malabsorption of vitamin B_{12} were excluded in the mother of our infant by the Schilling test. Therefore, the low-vitamin B_{12} level in the mother's breast milk was attributed to decreased oral intake of vitamin B_{12}.

The elevated serum folate level in the infant decreased to a normal value after the vitamin B_{12} deficiency was corrected. Such a finding is not uncommon in patients with a vitamin B_{12} deficiency.[5]

Neutropenia is frequently seen in megaloblastic anemia secondary to a vitamin B_{12} deficiency. However, persistence of neutropenia after adequate therapy is unusual, and the cause for the neutropenia in this child is unclear.

We thank Dr. John Will for assaying the sera for vitamin B_{12} and folic acid and are indebted to Shankman Laboratories, Los Angeles, California, for assaying the breast milk for vitamin B_{12}.

REFERENCES

1. U. S. Pharmacopoeia, 14th revision, Mack Publishing Company, Easton, Pa., on authority of U. S. Pharmacopoeial Convention, Washington, D. C., 3rd suppl. to U. S. P. IX, pp. 15-19, 1951; 4th suppl. to U. S. P. IX, pp. 7-9, 1954.
2. Castle, W. B., Ellenbogen, C. A., May, C. D., Welch, A. D., Zuelzer, W. W., and Butler, A. M.: Vitamin B_{12} and folic acid in infant nutrition, J. A. M. A. 146: 1028, 1951.
3. Herbert, V.: The assay and nature of folic acid activity in human serum, J. Clin. Invest. 40: 81, 1961.
4. Lampkin, B. C., Shore, N. A., and Chadwick, D.: Megaloblastic anemia of infancy secondary to maternal pernicious anemia, New England J. Med. 274: 1160, 1966.
5. Herbert, V., and Zalusky, R.: Interrelation of vitamin B_{12} and folic acid metabolism: Folic acid clearance studies, J. Clin. Invest. 41: 1263, 1962.
6. Harris, J. W.: The red cell, Cambridge, Mass., 1963, Harvard University Press, p. 150.

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RETENTION OF CRYSTALLINE VITAMIN B_{12} BY HEALTHY MALE INDIVIDUALS FOLLOWING INTRAMUSCULAR INJECTION[1]

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In the preceding paper in this series (Yamamoto, Barrows, Lang and Chow, '51) it was reported that stock colony rats which were maintained on diets providing enough vitamin B_{12} (from milk proteins, 6.5%, and whole liver, 1%) to be apparently adequate for their growth requirements could retain significant amounts of the vitamin following subcutaneous injection. It is, therefore, of interest to ascertain whether healthy human subjects with liberal intakes of diets containing vitamin B_{12} from natural sources (meat, milk) can likewise retain significant quantities following intramuscular administration. To this end, a group of healthy male subjects were given varying doses of vitamin B_{12} by injection. The urinary outputs preceding and following the injection were collected quantitatively. The amount of vitamin B_{12} retained was estimated by computing the difference between the injected dose and the determined activity in the urine samples. The results of this study are reported here.

MATERIALS AND METHODS

Vitamin B_{12}

A sterile solution of crystalline vitamin B_{12} was employed.[2] Its concentration, determined by microbiological assay shortly

[1] The authors wish to acknowledge a grant-in-aid from Sharp and Dohme, Inc., and the National Vitamin Foundation.

[2] It was kindly supplied by Merck and Company.

before this experiment, was 100 μg per milliliter. For the injections either this solution or one prepared from it by dilution with an equal volume of sterile saline solution was used.

Plan of experiment

Twenty healthy male subjects of from 14 to 45 years of age were included in this study. They were divided, at random, into 5 groups. One group, receiving no injection of vitamin B_{12}, served as controls. (Some individuals were used in this group and in one other.) The urinary excretion of vitamin B_{12} by individuals in this group provided basal excretion values. In addition, urine specimens from several individuals in the other groups were collected 24 hours prior to injection; these pre-injection samples were also used to establish basal excretion levels.

Individuals, according to the group to which they were assigned, received single intramuscular injections of vitamin B_{12} solution in amounts equivalent to 20, 30, 50, or 75 μg. These dosages were chosen on the basis of data from previous experiments (Conley, Krevans, Chow, Barrows and Lang, '51) which suggested the possible retention of approximately 20 to 40 μg of vitamin B_{12} by healthy humans. After the injection, which took place during the early part of the morning, the subjects were encouraged to increase fluid intake by drinking coffee. Urine samples were collected from each subject for 24 hours after injection, each in two fractions representing the first 8 hours and the following 16 hours. The volumes of the 8-hour samples ranged from 225 to 1,800 ml, averaging 650 ml. The volumes of the second specimens varied from 455 to 2,370 ml, with an average of 945 ml. All samples were assayed microbiologically for vitamin B_{12} activity, using the method of Skeggs, Nepple, Valentik, Huff, and Wright ('50).

RESULTS

The results of the experiment are given in table 1. It can be seen that the random distribution resulted in relatively

TABLE 1

The retention of vitamin B_{12} by healthy subjects after intramuscular injection

GROUPS	B_{12} INJECTED	NO. OF SUBJECTS	AGE		BODY WT.		MICROBIOLOGICAL ACTIVITY IN URINE[1]				MICROGRAMS B_{12} RETAINED	
			Range	Ave.	Range	Ave.	− 48 hr.	− 24 hr.	+ 8 hr.	+ 24 hr.	Found	Calculated[2]
	μg											
A	0.0	5	14–45	31	150–180	160	< 0.15	< 0.15	..	..	...	...
B	20	5	27–42	33	120–165	145	...	< 0.15 (2)[3]	1.3	< 0.15	18.7 ± 0.2	17.3
C	30	5	27–31	29	155–200	168	...	< 0.15 (1)[3]	1.7	< 0.2	28.3 ± 0.9	24.8
D	50	5	27–34	29	135–180	158	...	< 0.1 (1)[3]	9.8	< 0.2	40.2 ± 5.1	39.6
E	75	4	26–29	28	155–180	174	...	< 0.1 (2)[3]	19.6	< 0.2	55.4 ± 2.7	56.0

[1] Microbiological activity is expressed as equivalent of crystalline vitamin B_{12} in micrograms.

[2] Number in parentheses indicates the number of individuals in the group who participated in the collection of urine during the pre-injection period.

[3] Compare Chesterman, Cuthbertson and Pegler ('51).

comparable average age and body weights in the several groups. (We do not believe that in these experiments variations in age or weight were significant.) The basal excretion of the subjects in group A was consistently less than 150 mμg of vitamin B_{12} in 24 hours or, on the average, less than 50 mμg during an 8-hour period. Values of the same order of magnitude were also obtained from the few individuals whose urinary specimens were collected during the 24 hours preceding the injection. Since the total activity found during the basal period represents a negligible amount in comparison with even the smallest injected dose (20 μg) it was not considered in calculating the quantity of B_{12} retained by the body.

Paper chromatographs were run on all of the 8-hour post-injection samples. In each instance spots with virtually the same Rf value as that associated with vitamin B_{12} in the same system were observed.

Several points of interest are worth noting. In the first place, regardless of the amount of vitamin injected the microbiological activity in the urine specimens reached a maximum within 8 hours after injection. Only a negligible amount was present in the specimens collected during the following 16 hours. In the second place, subjects in group B receiving 20 μg of vitamin B_{12} intramuscularly excreted approximately 1 μg of vitamin B_{12} activity, indicating a retention of as much as 19 μg. With an injected dosage of 30 μg (group C), the microbiological activity in the urine of these subjects remained at substantially the same level; thus, the amount of vitamin B_{12} retained by this group of subjects was as much as 29 μg. With a further increase in the dosage, to 50 μg (group D) and to 75 μg (group E), the urinary excretion of microbiological activity was substantially elevated, with resultant net average retentions of 40 and 55 μg of B_{12}, respectively.

These data, therefore, demonstrate that substantial amounts of vitamin B_{12} can be retained by healthy human males following intramuscular administration to individuals whose dietary

history indicates presumably adequate oral intake of this vitamin. The amount of vitamin B_{12} retained appears to be dependent on or related to the amount injected. An empirical equation describing the relationship between administered dosages and excretion was suggested by Chesterman, Cuthbertson and Pegler ('51) as being $E = -1 - 1.2D$ %, where E = amount vitamin B_{12} excreted and D = amount injected. Within the presently reported range of administered dosages, our data are in quite good agreement with this equation, as is shown in the last column of table 1.

DISCUSSION

Berk et al. ('48) demonstrated that oral administration of vitamin B_{12} in small doses failed to bring about therapeutic response in patients with pernicious anemia, unless gastric juice from healthy individuals was also administered at the same time. If vitamin B_{12} is required by the normal human, and it is difficult to imagine otherwise, the daily requirement is probably adequately met in healthy individuals by diets which contain components, such as milk or meat products, rich in vitamin B_{12}. This conclusion is also substantiated by the small frequency with which reported clinical deficiency of this vitamin (pernicious anemia) is encountered.* In view of our previous findings that orally administered vitamin B_{12}, even in massive doses of the crystalline material or as naturally occurring complexes in meat or milk products, is poorly absorbed in normal subjects, or in normal laboratory animals (Chow, Barrows and Lang, '50; Chow, Rosenblum, Silber, Woodbury, Yamamoto and Lang, '51), it is conceivable that healthy subjects are not "saturated" with vitamin B_{12}. This hypothesis is in agreement with the findings reported in this communication which demonstrate the retention of sub-

*We recognize that the pernicious anemia syndrome can be regarded as the result of intrinsic factor insufficiency and is, perhaps, more accurately described in those terms than as vitamin B_{12} deficiency.

stantial quantities, roughly proportional to the administered parenteral dose.

Calculation of the amount retained was made by taking the difference between the administered dosage and the amount found in the urine. This implicitly assumes that administered vitamin B_{12} is not excreted in an altered form or as a split product not available to the test organisms used for microbiological assay. The reasonableness of these assumptions is supported not only by our paper chromatography studies but by the data reported elsewhere (Yamamoto, Barrows, Lang and Chow, '51; Chow, Rosenblum, Silber, Woodbury, Yamamoto and Lang, '51) on the distribution coefficient of the excreted vitamin following administration to rats of vitamin B_{12} tagged with radioactive Co^{60}. Their validity is further strengthened by the finding that certain tissues, such as the muscle, liver, kidneys, and so forth, of rats injected subcutaneously with the radioactive vitamin contain large amounts of Co^{60} which, in some cases, has been demonstrated to be explicitly associated with vitamin B_{12} (Rosenblum, Chow, Condon and Yamamoto, '52). These considerations therefore led us to the conclusion that the difference between the injected and the excreted vitamin represents the amount retained by the tissues.

Although our procedure for the measurement of retention is similar to a "saturation test" (Kline and Eheart, '44) for vitamin C, which is considered valuable as a means of determining adequacy of ascorbic acid intake, we have no data at the present time to indicate that the retention values reported here for vitamin B_{12} represent the physiological requirements of healthy humans. Nevertheless, our results demonstrate the ability of healthy males to retain large quantities of vitamin B_{12} following a single, intramuscular administration; amounts presumably many times larger than those estimated to be present in normal diets. The normal subjects used in these experiments were not saturated with vitamin B_{12} in spite of a presumably adequate intake in food.

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SUMMARY

Crystalline vitamin B_{12} was administered intramuscularly to groups of healthy male subjects at the following levels: 20, 30, 50, and 75 μg. Urinary collections from each subject were made during 24 hours after injection, each in two fractions representing the first 8 hours and the following 16 hours. The vitamin B_{12} microbiological activities in the urine specimens were measured. These data permitted calculation of the amount retained, by taking the difference between the injected and the excreted vitamin. It was found that more than 50 μg of vitamin were retained daily by these subjects, depending on the dosage administered.

LITERATURE CITED

BERK, L., W. B. CASTLE, A. D. WELCH, R. W. HEINLE, R. ANKER AND M. FESTEIN 1948 The etiologic relationship of achylia gastrica to pernicious anemia. X. Activity of vitamin B_{12} as food (extrinsic) factor. New England J. Med., *239:* 911–913.

CHESTERMAN, D. C., W. F. J. CUTHBERTSON AND H. F. PEGLER 1951 Vitamin B_{12} excretion studies. Biochem. J., *48:* 1.

CHOW, B. F., L. BARROWS AND C. A. LANG 1950 The microbiological activity of vitamin B_{12} in the urine of normal rats following the oral and subcutaneous administration of this vitamin. J. Nutrition, *42:* 405.

CHOW, B. F., C. ROSENBLUM, R. H. SIGLER, D. T. WOODBURY, R. YAMAMOTO AND C. A. LANG 1951 Oral administration of vitamin B_{12} containing cobalt[60] to rats. Proc. Soc. Exp. Biol. Med., *76:* 393.

CONLEY, C. L., J. R. KREVANS, B. F. CHOW, C. H. BARROWS AND C. A. LANG 1951 Observations on the absorption, utilization and excretion of vitamin B_{12}. J. Lab. Clin. Med., *38:* 84.

KLING, A. B., AND M. S. J. EHRART 1944 Variation in the ascorbic acid requirements for saturation of nine normal young women. J. Nutrition, *28:* 413.

ROSENBLUM, C., B. F. CHOW, G. P. CONDON AND R. YAMAMOTO 1952 To be published.

SHEGGS, H. R., H. M. NEPPLE, K. O. VALENTIK, J. W. HUFF AND L. D. WRIGHT 1950 Observations on the use of *Lactobacillus leichmanni* 4797 in the microbiological assay of vitamin B_{12}. J. Biol. Chem., *184:* 271.

YAMAMOTO, R., C. H. BARROWS, C. A. LANG AND B. F. CHOW 1951 Further studies on the absorption of vitamin B_{12} following oral and parenteral administration. J. Nutrition, *45:* 507.

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SUPPLEMENTAL FOLIC ACID THERAPY IN PERNICIOUS ANEMIA: THE EFFECT ON ERYTHROPOIESIS AND SERUM VITAMIN B_{12} CONCENTRATIONS IN SELECTED CASES

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THE concurrent use of folic acid and of vitamin B_{12} or purified liver extract has been suggested for complete maintenance therapy in certain patients with pernicious anemia on the basis that presumably minimal amounts of both vitamin B_{12} and folic acid are required for normal hematopoiesis.[1-4] It has been repeatedly demonstrated that folic acid alone not only is inadequate treatment for pernicious anemia but also may be responsible for accelerated development of neurological disturbances.[2, 5-9] In contrast, folic acid has no detectable harmful hematological or neurological effects in normal subjects,[7, 8] and when given together with parenteral vitamin B_{12} or liver extract usually produces no ill effects in patients with pernicious anemia.[8, 10] The study here reported was undertaken in order to assess the need for supplemental folic acid in the maintenance therapy of certain patients with pernicious anemia selected on the basis that they had not achieved normal blood values following prolonged therapy with amounts of vitamin B_{12} seemingly adequate for this purpose in the majority of other patients under observation.

MATERIALS AND METHODS

Thirty-five patients were studied who had been diagnosed as having pernicious anemia on the basis of achlorhydria, macrocytic anemia with abnormal bone marrow, and characteristic hematological response to purified liver extract or vitamin B_{12}. All had been maintained in remission for two years or more with monthly injections either of 15 μg of crystalline vitamin B_{12} or of purified liver extract (Liver Injection U.S.P.) containing 15 μg of vitamin B_{12}. The individuals in this group, representing about one-fifth of the cases of pernicious anemia receiving maintenance therapy in an outpatient clinic at the Boston City Hospital, were chosen because they had had hemoglobin levels of less than 12.5 Gm. per cent for one year or more. In the past, some of these patients had been given larger doses of vitamin B_{12} or of purified liver extract without noticeable benefit.

During the period of this study all the patients were given larger doses of their customary maintenance therapy. Twenty-four of the thirty-five patients selected were thereafter treated every two weeks for up to one year with intramuscular injections of 30 μg of crystalline vitamin B_{12},† and eleven were so treated with Liver Injection U.S.P.,†

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†Kindly supplied by Mr. G. R. Walden of Eli Lilly & Company.

containing, according to the manufacturer, 20 μg of vitamin B_{12}. After each patient had received the increased dosage of vitamin B_{12} as such, or as liver extract, for six months (Period 1), each was given, in addition, 5 mg. of folic acid orally daily throughout a second six-month period (Period 2).

Red cell, hemoglobin, and serum vitamin B_{12} levels were determined from samples of venous blood. Red cell counts were performed using standardized pipettes and counting two chambers filled from two separate pipettes. Hemoglobin determinations were made using a photoelectric colorimeter according to the method of Evelyn.[11] Red cell indices were calculated.[12] Serum vitamin B_{12} concentrations were estimated by a microbiologic technique using *Euglena gracilis* according to a modification[13] of the method of Ross. Strict precautionary measures were observed in order to preclude accidental contamination of samples with vitamin B_{12}. Control red blood cell levels and indices, and serum vitamin B_{12} concentrations were determined at the start of the study. Red cell levels and indices were repeated every two months throughout the study. Assays of serum vitamin B_{12} from blood samples drawn two weeks after the last previous injection of vitamin B_{12} or of liver extract were repeated at six and at twelve months. A change in hemoglobin concentration of more than 10 per cent or in serum vitamin B_{12} concentration of more than 20 per cent was considered to be individually significant. Complete data were obtained on twenty-seven patients of the original group of thirty-five.

RESULTS

Effect on Hemoglobin Levels.—The patients described varying degrees of subjective improvement after beginning to receive the increased dosage of vitamin B_{12} as such, or as liver extract. None experienced symptomatic relapse while taking the additional folic acid. One patient who had glossitis that did not improve after increased vitamin B_{12} therapy described marked regression of lingual symptoms while taking folic acid. The laboratory data obtained are summarized in Table I. Of the twenty-two patients receiving crystalline vitamin B_{12}, only five manifested a significant and sustained increase in hemoglobin six months after starting on the larger doses of vitamin B_{12}. Of the eleven patients who received purified liver extract, none showed significant hemoglobin increments. In no instance did a significant fall of hemoglobin concentration occur during Period 1. Supplemental folic acid therapy during Period 2 caused no significant change, either up or down, in the hemoglobin levels of any patient. One female patient, who proved to have an associated iron deficiency, received folic acid for four months without showing any change. However, after receiving ferrous sulfate, a significant rise in her hemoglobin level occurred.

Effect on Serum Vitamin B_{12} Levels.—The serum vitamin B_{12} levels of the patients on whom this determination was made at the outset, after six, and after twelve months are shown in Table I and Fig. 1. The initial total serum vitamin B_{12} concentration was less than 100 μμg per milliliter in twenty of the thirty-five patients. At the end of Period 1, after each patient had received 30 μg of crystalline vitamin B_{12} or 20 μg of vitamin B_{12} as Liver Injection U.S.P. every two weeks for six months, only two of thirty-one patients who were successfully followed had serum vitamin B_{12} levels of less than 100 μμg per milliliter. At the end of Period 2, after one year of increased vitamin B_{12} therapy and after six months of additional folic acid therapy, none of twenty-nine patients on whom data were obtained had a serum vitamin B_{12} level below 100 μμg per milliliter.

There is, however, no indication that these increments in serum vitamin B_{12} levels were accompanied by increases in hemoglobin or by decreases in mean corpuscular volume (Fig. 2) in the cases studied. As in patients who showed no rise in hemoglobin level, in the five patients in whom a rise occurred

TABLE I. HEMATOLOGICAL AND MICROBIOLOGIC DATA ON THIRTY-FIVE PATIENTS WITH PERNICIOUS ANEMIA WHO RECEIVED INTRAMUSCULAR VITAMIN B_{12} AND SUPPLEMENTAL ORAL FOLIC ACID THERAPY

	Initial levels		Period 1: after six months of increased parenteral vitamin B_{12} therapy		Period 2: after six months of supplemental oral folic acid therapy (5 mg. per day)	
Patient	Hemoglobin (gm./100 ml.)	Total serum vitamin B_{12} ($\mu\mu$g/ml.)	Hemoglobin (gm./100 ml.)	Total serum vitamin B_{12} ($\mu\mu$g/ml.)	Hemoglobin (gm./100 ml.)	Total serum vitamin B_{12} ($\mu\mu$g/ml.)
Vitamin B_{12} 30 μg Every Two Weeks						
1	8.6	1,050	8.8	1,150	9.3	545
2	10.4	106	11.4	305	11.6	330
4	11.0	277	11.5	772	11.8	321
5*	9.2	48	15.3	961	14.3	310
7*	11.4	107	13.1	600	13.3	210
8	11.6	381	12.5	1,300	12.9	482
9	11.5	37	12.5	348	12.9	232
10	12.1	43	13.2	168	—	—
11	11.3	60	—	—	11.1	205
12	11.8	421	12.6	377	12.8	168
13	9.6	52	9.2	143	11.3	144
17	13.0	424	11.7	393	11.8	330
18	12.4	52	12.4	221	12.2	185
19	12.4	207	13.5	900	12.8	205
21	12.7	10	13.0	311	12.6	218
23	11.4	15	11.9	412	11.3	110
24*	13.5	28	14.9	108	15.2	320
25	12.4	810	12.4	570	13.3	240
26*	12.3	44	14.4	325	14.5	253
27	11.0	770	11.2	1,210	—	—
30*	12.4	84	15.3	207	14.6	500
33	11.2	268	11.8	330	11.8	145
34	11.6	53	—	—	—	—
35	11.7	68	12.2	405	12.7	275
Liver Injection U.S.P. (of Vit. B_{12} 30 μg) Every Two Weeks						
3	11.8	151	12.8	645	12.6	175
6	11.8	60	12.2	135	12.8	202
14	12.0	186	13.1	360	13.6	235
15	12.4	19	12.8	97	12.5	120
16	11.4	142	11.8	271	12.0	214
20	12.4	98	13.1	—	12.5	208
22	12.6	31	13.5	128	14.2	131
28	12.3	575	12.4	960	13.1	745
29	12.1	46	12.6	—	—	—
31	11.7	76	12.5	191	12.5	—
32	11.0	35	11.8	78	—	—

*Considered to show a significant increase in hemoglobin level.

the serum vitamin B_{12} level returned to a normal range from an initial level of less than 100 $\mu\mu$g per milliliter. Of sixteen patients with initially low levels who were still under observation at the end of Period 1, fourteen had vitamin B_{12} levels within the normal range after six months of increased

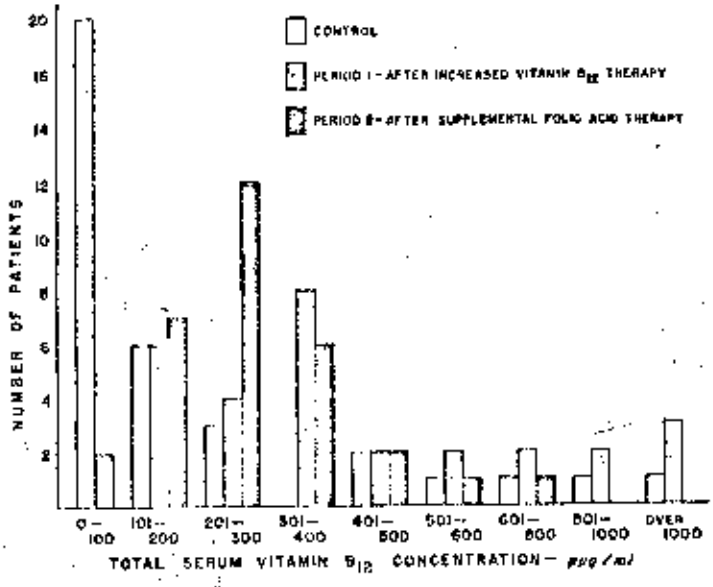

Fig. 1.—Serum vitamin B_{12} levels in relation to period of observation. Patients with pernicious anemia in relapse have serum vitamin B_{12} levels of less that 100 μμg per milliliter. Number of patients on whom estimations were obtained: Control, 35; Period 1, 31; Period 2, 29.

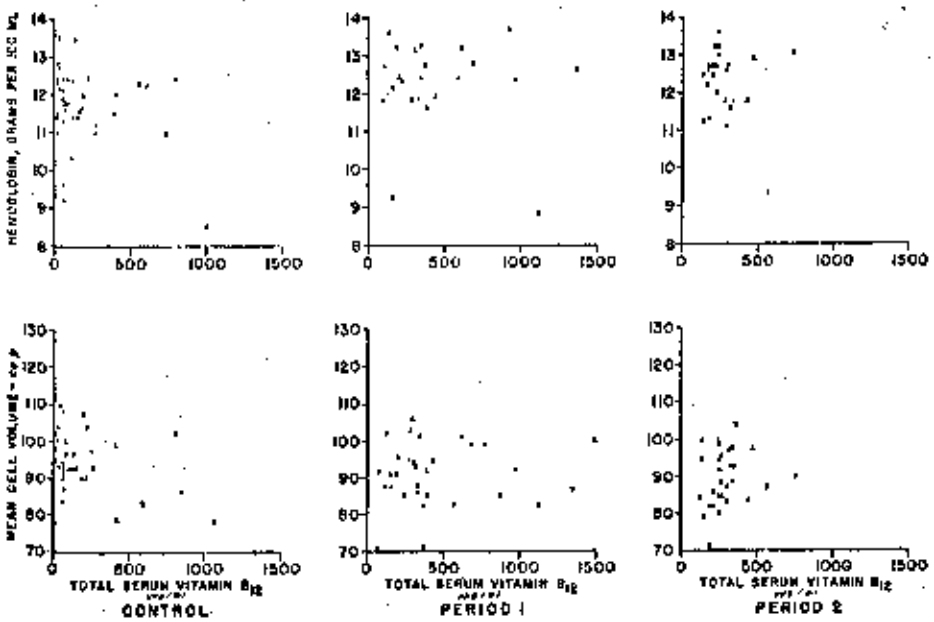

Fig. 2.—Hemoglobin level and mean cell volume in relation to serum vitamin B_{12} level.

vitamin B_{12} therapy. Concerning the remaining two patients, each of whom received a total of 40 μg of vitamin B_{12} per month as liver extract, one had a normal vitamin B_{12} level at the end of Period 2 and the other did not return to the clinic after the end of Period 1. The actual range of serum vitamin B_{12} concentrations varied greatly at the end of each period. Initial control levels ranged from 10 to 1,050 $\mu\mu g$ per milliliter with a mean of 198 $\mu\mu g$ per milliliter for the entire group. The twenty-four patients who had received crystalline

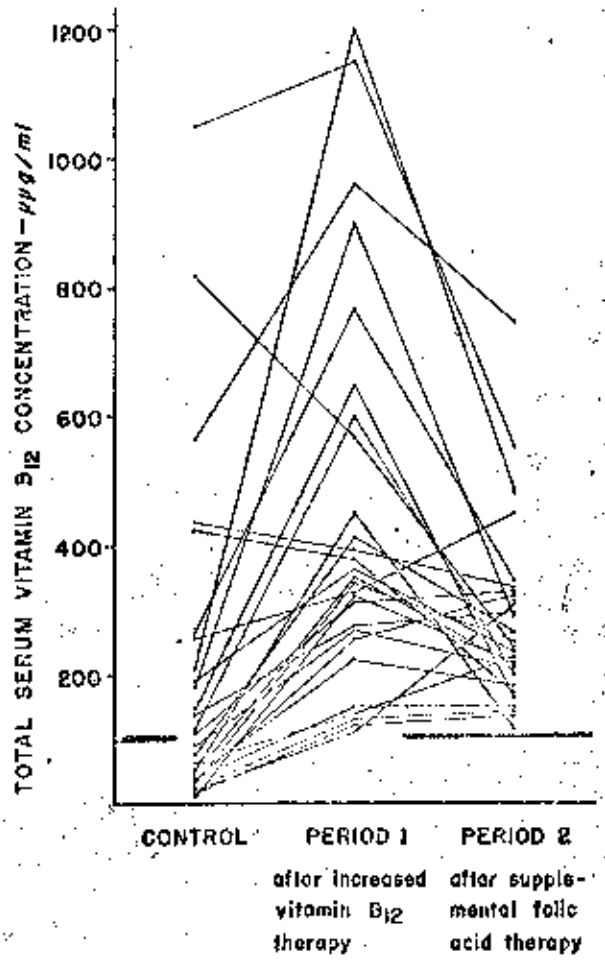

Fig. 3.—Individual serial responses of the serum vitamin B_{12} level in twenty-seven patients with pernicious anemia in relation to period of observation. Note the number of control vitamin B_{12} values below 100 $\mu\mu g$ per milliliter.

vitamin B_{12} averaged 225 $\mu\mu g$ per milliliter, and the eleven patients who, before these observations, had been receiving purified liver extract averaged 137 $\mu\mu g$. At the end of Period 1, the range for the thirty-one patients on whom data were obtained was 78 to 1,840 $\mu\mu g$ per milliliter with a mean of 455. The

subgroup who received crystalline vitamin B_{12} had an average of 527 $\mu\mu g$ per milliliter, and the patients who received purified liver extract had an average of 319. Of the thirty-one patients followed six months, twenty-six had increases in serum vitamin B_{12} concentration greater than 20 per cent and one had an increase of between 10 and 20 per cent; two showed no significant change; and two had levels between 10 and 20 per cent below their initial concentration.

Vitamin B_{12} estimations were made on twenty-nine of the original thirty-five patients at the end of Period 2, following six months of folic acid therapy given in addition to the larger vitamin B_{12} dosage. Vitamin B_{12} levels were not obtained before folic acid was started in two of these twenty-nine patients; hence in only twenty-seven patients were vitamin B_{12} levels obtained serially. The serum vitamin B_{12} concentration at the end of Period 2 was normal in the two not studied on all three occasions. The average level for the entire group at the end of Period 2 was 276 $\mu\mu g$ per milliliter, and the range was 116 to 745. For the twenty-one patients receiving crystalline vitamin B_{12} the average was 285 $\mu\mu g$ per milliliter, and for the eight remaining patients on purified liver extract, 254. At the end of Period 2 three patients showed an increase in serum vitamin B_{12} concentration greater than 20 per cent, three an increase of 10 to 20 per cent, and three no change. The most consistent change after administration of supplemental folic acid was the fall in vitamin B_{12} concentration. This occurred in eighteen of the twenty-seven patients on whom serial vitamin B_{12} levels were obtained at control times and at the end of both Periods 1 and 2. In two the vitamin B_{12} level fell 15 to 20 per cent, and in sixteen it fell more than 20 per cent. In this group, fourteen of the twenty patients receiving crystalline vitamin B_{12} and four of the seven patients treated with liver extract showed such a drop. In Fig. 3 are shown the vitamin B_{12} concentrations in twenty-seven individual patients initially and at the end of Periods 1 and 2.

DISCUSSION

The purpose of this study was to evaluate the hematopoietic effectiveness of folic acid as a supplement to vitamin B_{12} therapy in a group of patients with pernicious anemia, who were selected because they had hemoglobin levels in the low normal or mildly anemic range as compared with those of other patients on similar therapy. It was thought that a further rise in hemoglobin subsequent to the addition of folic acid to an optimal dosage schedule of vitamin B_{12} would indicate that folic acid deficiency existed. Chodos,[9] Ross,[8] and Meacham[14] have reported their experiences with vitamin B_{12} and folic acid in the therapy of pernicious anemia. Spray[15] has reported abnormal folic acid utilization in pernicious anemia in relapse. Girdwood[16] has suggested that vitamin B_{12} deficiency may lead to actual or to apparent folic acid depletion and has reported one patient who had deficiency both of folic acid and of vitamin B_{12}. Spies[8] has indicated the possible coexistence of folic acid and vitamin B_{12} deficiency, and Ungley[17] has described two such patients.

Ritz[14] has suggested that suboptimal oral doses of folic acid and vitamin B_{12} have a synergistic action in inducing a response in patients with pernicious anemia in relapse. Reisner[10] has indicated that such therapy in relapse results in enhancement of the hematopoietic effect. It has been well established that folic acid, although not complete therapy for pernicious anemia, does regularly initiate and maintain for varying periods a hematopoietic response. Thus, under various circumstances response to folic acid, although not necessarily indicating a predominant deficiency of folic acid, has been observed in pernicious anemia.

That therapy with folic acid alone can be detrimental, resulting in neurological changes with or without hematopoietic relapse, is similarly well established.[2-5] However, so long as vitamin B_{12} is given together with folic acid, no harmful effects ordinarily ensue.[6, 10] The clinical course of the patients here reported confirms this observation in that none of these patients who received folic acid and vitamin B_{12} manifested any undesirable neurological or hematological change. In fact, the glossitis of one patient improved while she was receiving supplemental folic acid although the hemoglobin level showed no change.

Effect on Hemoglobin Levels.– Prior to the present study patients attending this clinic had been receiving approximately 15 μg of vitamin B_{12} (either as the crystalline vitamin or as purified liver extract) every four weeks for periods up to six years without manifest neurological or hematological relapse. At the end of Period 1, six months after the institution of the increased doses of vitamin B_{12} (20 or 30 μg every two weeks), five of thirty-three patients showed an increase in hemoglobin concentration greater than 10 per cent. This indicates that although maintenance with injections of 15 μg of vitamin B_{12} every four weeks was adequate for normal hemoglobin synthesis in most of these patients (and presumably in the approximately 125 other patients attending this clinic), it was not so for all. It has been suggested[11] that maintenance with 30 μg of vitamin B_{12} per month is probably optimal therapy. Observations of the serum vitamin B_{12} and hemoglobin levels of these clinic patients indicate that 15 μg of vitamin B_{12} every four weeks is suboptimal.

None of the patients here reported had further increment in hemoglobin concentration at the end of Period 2, six months after the institution of supplemental folic acid therapy, thus indicating that concomitant folic acid lack was not a limiting factor in hemoglobin synthesis in these particular patients. Nor was there any decline in hemoglobin concentration to suggest that folic acid might interfere with normal hematopoiesis in pernicious anemia treated with vitamin B_{12}.

Effect on Serum Vitamin B_{12} Levels.– The microbiologic determination of serum vitamin B_{12} has proved to be a useful aid in the diagnosis of the vitamin B_{12} deficient state.[13, 20] Patients with pernicious anemia previously untreated or in relapse have serum vitamin B_{12} levels of less than 100 $\mu\mu g$ per milliliter, whereas normal subjects and patients with miscellaneous diseases have serum

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concentrations above this value. Twenty patients with pernicious anemia who had been receiving injections of 30 μg of vitamin B_{12} every four weeks were selected at random from the clinic; all but two patients at the end of Period 1 and all patients at the end of Period 2 had serum vitamin B_{12} levels greater than those of patients with untreated pernicious anemia. Serum vitamin B_{12} levels of less than 100 μμg per milliliter initially detected in twenty of the thirty-five patients so tested indicate that treatment with 15 μg of vitamin B_{12} each month is probably suboptimal maintenance therapy in the sense that it is inadequate to replenish body stores. Hence, on this basis we may conclude that the intramuscular injection of 15 μg of vitamin B_{12} every four weeks is inadequate therapy in terms of tissue saturation. Serum vitamin B_{12} levels of patients receiving injections of 30 μg of vitamin B_{12} every four weeks and of those receiving 20 or 30 μg every two weeks for eight to twelve months were above the relapse range. Meacham[14] suggested that 30 μg of vitamin B_{12} every four weeks is adequate and, possibly, optimal maintenance therapy. The serum vitamin B_{12} levels, as indicated previously, are in agreement with the suggestion that 30 μg of vitamin B_{12} monthly is an adequate dosage schedule for most patients.

Neurological relapse in exclusively folic acid-treated pernicious anemia patients occurs in approximately 50 per cent of cases within two years.[2] The rapidity with which relapse develops may correlate with the amount of folic acid given. Clinical observation of patients receiving oral liver preparations in the early days of pernicious anemia therapy suggests that the incidence of neurological relapse was not so frequent as in folic acid-treated patients. Of thirty patients in this clinic who received no antipernicious anemia therapy,[21] none developed neurological relapse in a period of two years, although about half experienced hematological relapse. This suggests that folic acid may have a neuropathic effect, either directly or by altering vitamin B_{12} utilization. The present finding that during Period 2 the daily addition of 5 mg. of folic acid orally to the maintenance therapy with intramuscular vitamin B_{12} was followed in eighteen of twenty-seven patients by a significant drop in serum vitamin B_{12} concentration offers some support to the latter concept. Similarly, studies of the acute effect of large doses of folic acid in monkeys receiving a diet deficient in vitamin B_{12} and folic acid also present suggestive, although not conclusive, evidence that folic acid may cause a fall in serum vitamin B_{12} concentration.[22] Whether this represents a general effect of vitamin imbalance, such as may occur when single members of the vitamin B complex group are administered in the presence of mixed deficiency,[23] or is a specific result of some antagonistic interaction of folic acid and vitamin B_{12},[24] cannot be stated. Nonetheless, it does suggest the theoretical possibility that over a protracted period the combined use of suboptimal amounts of vitamin B_{12} and large amounts of folic acid might result occasionally in the development of neural dysfunction. Chodos[9] described one patient with subacute combined degeneration who improved only after folic acid was omitted from combined folic acid and vitamin

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B_{12} therapy, and two patients with pernicious anemia and other organic disease who manifested progression of neurological disturbance while receiving both vitamin B_{12} and folic acid.

SUMMARY

1. Administration of 30 μg of vitamin B_{12} intramuscularly every two weeks increased the hemoglobin levels of only five of a group of twenty-two patients previously receiving 15 μg of crystalline vitamin B_{12} every four weeks. None of eleven patients who received 20 μg of vitamin B_{12} in the form of Liver Injection U.S.P. every two weeks was so benefited.

2. Supplemental folic acid did not alter hemoglobin levels; therefore folic acid deficiency as a limiting factor in hematopoiesis was shown not to exist in twenty-nine patients given adequate therapy with vitamin B_{12}.

3. The serum vitamin B_{12} level in twenty of thirty-five patients receiving 15 μg of vitamin B_{12} once every four weeks was less than 100 $\mu\mu g$ per milliliter, which is characteristic of patients with pernicious anemia in relapse. It is greater than 100 $\mu\mu g$ per milliliter in most patients receiving 30 μg of vitamin B_{12} once every four weeks. Inasmuch as the serum vitamin B_{12} concentration probably reflects tissue saturation, treatment with the larger dose of vitamin B_{12} appears to be more nearly adequate.

4. Administration of folic acid adversely affected the serum vitamin B_{12} level of eighteen of twenty-seven patients receiving 20 or 30 μg of vitamin B_{12} intramuscularly every two weeks, possibly by accelerating the utilization of vitamin B_{12}. Therefore, supplementation of inadequate intramuscular vitamin B_{12} therapy with folic acid, in addition to being unnecessary for nearly all patients, may actually be inadvisable unless larger doses of vitamin B_{12} are given.

We wish to express our appreciation to the Misses Mary Lou Hanson, Carol Sue Smith, and Eleanor M. Fleming for technical assistance, and to Mrs. Ann Marker, R.N., for her untiring efforts in managing the Out-Patient Clinic.

REFERENCES

1. Diaz Rivas, F., Morales, F. H., and Meyer, L. M.: The Oral Use of Combined Vitamin B_{12} and Folic Acid in Tropical Sprue, Ann. Int. Med. 36: 1076-1085, 1952.
2. Ross, J. F., Belding, H., and Paegel, B. L.: The Development and Progression of Subacute Combined Degeneration of the Spinal Cord in Patients With Pernicious Anemia Treated With Synthetic Pteroylglutamic (Folic) Acid, Blood 3: 68-90, 1948.
3. Spies, T. D., Garcia Lopez, G., Milanes, F., Lopez Toca, R., and Reboredo, A.: Folic Acid and Vitamin B_{12} in Anemia, Gen. Practitioner 11: 37, 1950.
4. Ungley, C. C.: Vitamin B_{12} and Other Dietary Factors in Megaloblastic Degeneration of the Cord, Proc. Roy. Soc. Med. 43: 537-541, 1950.
5. Heinle, R. W., and Welch, A. D.: Folic Acid in Pernicious Anemia, J. A. M. A. 133: 730-741, 1947.
6. Vilter, C. F., Vilter, R. W., and Spies, T. D.: The Treatment of Pernicious and Related Anemias With Synthetic Folic Acid. I. Observations on the Maintenance of a Normal Hematologic Status and on the Occurrence of Combined System Disease at the End of One Year, J. Lab. & Clin. Med. 32: 262-273, 1947.
7. Harvey, E. A., Hummel, L., and Murphy, W. P.: Absence of a Toxic Effect of Folic Acid on the Central Nervous System of Persons Without Pernicious Anemia, New England J. Med. 242: 446-447, 1950.

8. Weissberg, J., McGavack, T. N., Vogel, M., and Koningsberg, S.: The Effect of Folic Acid on the Central Nervous System of Normal Subjects With Anemias Other Than Pernicious Anemia, Blood 5: 148-154, 1950.
9. Chodos, R. B., and Ross, J. F.: The Effects of Combined Folic Acid and Liver Extract Therapy, Blood 6: 1213-1233, 1951.
10. Conley, C. L., Green, T. W., Hartmann, R. C., and Krevans, J. R.: Prolonged Treatment of Pernicious Anemia With Vitamin B_{12}, Am. J. Med. 13: 284-290, 1952.
11. Evelyn, K. A.: A Stabilized Photoelectric Colorimeter With Light Filters, J. Biol. Chem. 115: 63-75, 1936.
12. Wintrobe, M. M.: Clinical Hematology, Philadelphia, 1951, Lea & Febiger.
13. Lear, A. A., Harris, J. W., Castle, W. B., and Fleming, E. M.: The Serum Vitamin B_{12} Concentration in Pernicious Anemia, J. Lab. & Clin. Med. 44: 715-722, 1954.
14. Moncher, G. C., and Heinle, R. W.: Maintenance Therapy of Pernicious Anemia With Vitamin B_{12}, J. Lab. & Clin. Med. 41: 68-77, 1953.
15. Spray, C. R., and Wills, L. J.: The Utilization of Folic Acid Given by Mouth, Clin. Sc. 11: 273-281, 1952.
16. Girdwood, R. H.: A Folic Acid Excretion Test in the Investigation of Intestinal Malabsorption, Lancet 2: 53-60, 1953.
17. Ungley, C. C.: The Pathogenesis of Megaloblastic Anaemia and the Value of Vitamin B_{12}, Brit. J. Nutrition 6: 296-315, 1952.
18. Ritz, N. D., Meyer, L. M., Brahin, C., and Sawitsky, A.: Further Observations on the Oral Treatment of Pernicious Anemia With Subminimal Doses of Folic Acid and Vitamin B_{12}, Acta Haematologica 5: 324-338, 1951.
19. Reisner, E. H., Jr., and Weiner, L.: Studies on the Mutual Effect of Suboptimal Oral Doses of Vitamin B_{12} and Folic Acid in Pernicious Anemia, New England J. Med. 247: 15-17, 1952.
20. Mollin, D. L., and Ross, G. I. M.: The Vitamin B_{12} Concentrations of Serum and Urine of Normals and of Patients With Megaloblastic Anaemias and Other Diseases, J. Clin. Path. 5: 129-139, 1952.
21. Wallerstein, R. O., Schilling, R. S., Harris, J. W., Lear, A. A., and Castle, W. B.: Observations to be published.
22. Lear, A. A., Earle, C., Denny Brown, D. E., and Castle, W. B.: Unpublished observations.
23. Spies, T. D., Vilter, R. W., and Aska, W. P.: Pellagra, Beri Beri and Riboflavin Deficiencies in Human Beings, J. A. M. A. 113: 931-937, 1939.
24. Davidson, L. S. P., and Girdwood, R. H.: The Imbalance of Vitamins With Particular Reference to Folic Acid, Lancet 1: 360-363, 1948.

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STRUCTURE OF THE 5,6-DIMETHYLBENZIMIDAZOLYLCOBAMIDE COENZYME

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RECENTLY, H. A. Barker *et al.* reported the isolation of a crystalline 5,6-dimethylbenzimidazolylcobamide (DBC) coenzyme[1]. This is one of a series of coenzymes which were first recognized as catalysts in the conversion of glutamate to β-methylaspartate in certain bacteria. The coenzyme was shown to be active in systems requiring vitamin B_{12}, but it differed from cyanocobalamin in having an adenine nucleoside in place of the cyanide group. We have now carried out an X-ray analysis of the coenzyme structure, having for our use crystalline preparations kindly given to us by Dr. H. A. Barker, Dr. Karl Folkers and Prof. A. W. Johnson.

Part of Dr. Barker's preparation was recrystallized by slowly diffusing acetone into an aqueous coenzyme solution. Two freshly grown crystals prepared in thin-walled glass capillary tubes in dim light and kept in contact with their mother liquor were used to obtain the X-ray diffraction data for the structure analysis. Copper $K\alpha$ radiation was used throughout. The intensity measurements were made visually from multi-film equi-inclination Weissenberg photographs taken about the *b* and *c* axes. The Lorentz-polarization correction and a spot shape correction were applied. The scale factor for each layer was calculated from reflexions common to other layers and the absolute scale was obtained by Wilson's method. 3,145 independent reflexions extending out to a spacing limit of 0·9 Å. were observed. These included about 85 per cent of the reflexions with a spacing greater than 1·2 Å.

The unit cell dimensions characteristic of the wet crystal lattice, as measured from precession photographs, are: $a = 27{\cdot}9_3$, $b = 21{\cdot}7_2$ and $c = 15{\cdot}3_1$ Å. The space group is $P2_12_12_1$ and the measured density of the fully solvated crystals, $1{\cdot}35_4 \pm 0{\cdot}01$ gm./cm.^{3}. The molecular weight, coenzyme plus solvent, calculated from this information is 1,899. This is larger, as would be expected, than the weight measured by Mattern on air-dried crystals[2]; the difference corresponds to the weight of about 8 molecules of water of crystallization.

The structure analysis began with the calculation of a sharpened three-dimensional Patterson synthesis which clearly showed the Co—Co vectors. The first electron-density synthesis was computed using phases calculated from the cobalt positions and the observed P^2 values weighted according to a method suggested by Sim[3]. The resulting electron-density map contained peaks representing most of the atoms in the structure, and in addition, spurious peaks, many of which were larger than those corresponding to atoms. The position of the cobalt atom ($z = 0{\cdot}02$) produced a pseudo-mirror plane which distorted the electron-density pattern representing the corrin nucleus and increased the number of spurious peaks. In spite of this, reasonably accurate positions were worked out for nearly all the atoms in the corrin nucleus, the 5,6-dimethylbenzimidazole, the ribose and the phosphate group.

A second electron-density synthesis, again using Sim's weighting scheme, was computed with phases calculated from the 54 atoms with known positions. This synthesis, containing less false symmetry and fewer spurious peaks, enabled us to place nearly all the remaining atoms of the coenzyme molecule. The electron-density in the region above the corrin nucleus, on the side occupied by cyanide in cyanocobalamin, is of particular interest (Fig. 1). The adenine moiety can be clearly seen among the spurious peaks. It is evident as a planar set of peaks with the 5- and 6-membered rings and the amino-group correctly related. Further, the five nitrogen atoms, with one exception, correspond to the peaks of greater electron-density. The sugar part of the nucleoside is not so easily seen, but on careful study it is no less convincing. This arrangement of peaks represents a *d* ribose unit linked directly to the cobalt atom at C 5′. No peak can be found which would correspond to the hydroxyl oxygen of C 5′. Most of the spurious peaks are in chemically inadmissible positions and have disappeared from subsequent electron-density syntheses.

Any residual doubt about the correctness of the above interpretation was completely dispelled by the results of the next structure factor calculation and the appearance of the corresponding difference synthesis. All the atoms in the coenzyme molecule (except six in the vitamin B_{12} part) and three water molecules were included in the third structure factor calculation. The agreement factor, *R*, was thereby reduced from 0·44 to 0·28.

Agreement between the observed and calculated structure factors has been further improved by adding the remaining six atoms of the vitamin B_{12} part, by including additional solvent molecules and by making adjustments in the positional and thermal parameters

Fig. 1.—Peaks in the second electron-density synthesis in the region of the adenine and sugar groups. The contours are at levels corresponding approximately to [illegible] Å^{-1}. The distribution is shown viewed along *a* and the contours are drawn only near the level of the peak maxima.

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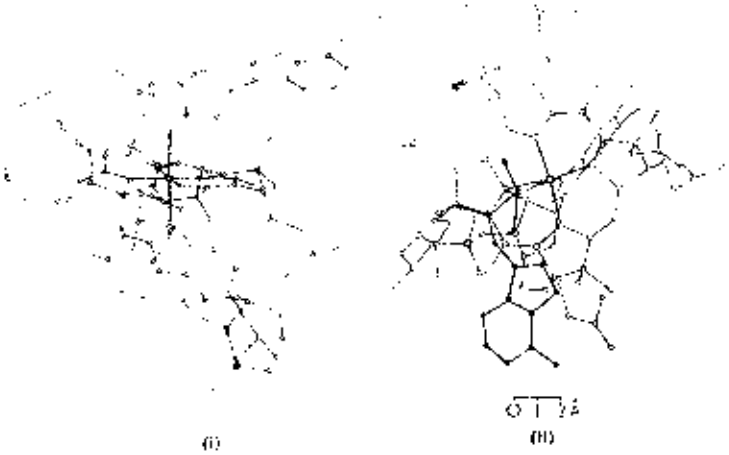
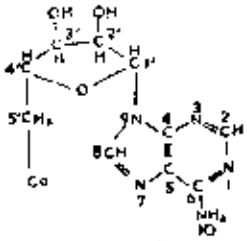


(i) (ii)

Fig. 2. The atomic positions in one molecule of the 5,6-dimethylbenzimidazolylcobamide coenzyme as found in the wet crystals and viewed along (i) the *b* axis, (ii) the *a* axis. In (ii) the atoms of the nucleoside unit are marked by filled circles.

of the other atoms. All the atoms of the coenzyme molecule (excluding hydrogen) and contributions from 11 water molecules have been included in the latest structure factor calculation. The agreement factor is 0·173.

The structural formula of the nucleoside as it occurs in the intact coenzyme should almost certainly be written as in I, which represents it as an adenosine derivative.



(I)

The *d* ribose unit and the adenine are linked at C 1′ (β) and N 9 in accordance with the observations of Ladd *et al.*[4] The unsaturated sugar, CH_2 = CH.CHOH.CHOH.CHO, which these authors obtained by the action of cyanide or mild acid hydrolysis, must be formed on degradation of the coenzyme following the rupture of the Co—C 5′ bond. The observed tetrahedral character of the atomic centres C 2′, C 3′ and C 4′ makes it unlikely that a double bond involving any of these atoms is initially present in the molecule; and very recently, adenosine derivatives in which the pentose ring is intact and the 5′ CH_2OH is replaced by COOH and CHO have been isolated from among the photolysis products of the coenzyme by Johnson and Shaw and by Barker *et al.*[5].

Fig. 2 shows the arrangement of the atoms in the coenzyme molecule as found in this crystal structure. All the atoms of the vitamin B_{12} molecule appear to be present except the cyanide group which has been replaced in the coenzyme by the adenosyl residue. The cobalamin nucleus and the nucleoside are connected by the C 5′—Co bond of 2·05 ± 0·05 Å.; the Co—C 5′—C 4′ angle is rather large, 130°, which suggests that this bond may have partial ionic character. No other direct covalent or hydrogen bonds connect the corrin nucleus and the nucleoside. The closest non-bonded contact is 3·1 Å.

While several of the amide side chains have adopted conformations different from those found in B_{12}, there is no evidence of any change in the corrin nucleus itself. Within the accuracy of the present partly refined co-ordinates, the corrin nucleus appears to be identical in the vitamin and coenzyme molecules. In the circumstances we could most easily formulate the cobalt atom as trivalent in the coenzyme molecule and write the structure in a similar way to that of vitamin B_{12}. The coenzyme molecule would then have a molecular formula of $C_{22}H_{100}O_{12}N_{12}PCo$ and a molecular weight of 1,580. The uncertainties here are in the number of hydrogen atoms, the valency of cobalt and the exact character of the bonding in the corrin nucleus; they may possibly be resolved by further refinement of the crystal structure, particularly if we can observe the hydrogen atom positions. Other evidence, notably on the magnetic character of the crystals, is very conflicting at present[6,7] and it seems likely that these transformations occur within the molecule which involve the valency of cobalt and the corrin nucleus. These, combined with the unusual character of the carbon–cobalt bond, are presumably responsible for the catalytic activity of the coenzyme.

The crystal structure of the DBC coenzyme is, in detail, quite different from that found in the vitamin B_{12} crystal structures. Although the orientation of the corrin nucleus with respect to the crystal axes is similar, the relationship between the four molecules which make up the translational unit of the crystal has altered. In the new arrangement, very interesting intermolecular contacts arise which suggest methods of approach by different reagents in the neighbourhood of the Co—C 5′ bond. For example, the adenosyl 3′ OH is hydrogen bonded to the phosphate group of one neighbouring molecule while the NH_2 group of the adenine unit is in contact with the phosphate group of another. The geometrical relations strongly suggest easy pathways both for the formation of the coenzyme from adenosine triphosphate and for its possible intervention in nucleic acid synthesis.

We wish to thank Dr. J. S. Rollett for the use of his structure factor programme and for many helpful discussions. The Fourier series programme written by Dr. O. S. Mills and other programmes written by various members of this Laboratory have greatly facilitated the work. All the calculations were done on the Ferranti *Mercury* computer with the co-operation of the staff of the Computing Laboratory, Oxford. This investigation was carried out during the tenure by one of us (P. G. L.) of a postdoctoral fellowship from the Division of General Medical Sciences, United States Public Health Service.

[1] Barker, H. A., Smyth, R. D., Weissbach, H., Toohey, J. I., Ladd, J. N., and Volcani, B. E., *J. Biol. Chem.*, 235, 480 (1960).
[2] Mattern, C. F. T., *J. Biol. Chem.*, 235, 489 (1960).
[3] Sim, G. A., *Acta Cryst.*, 12, 813 (1959).
[4] Ladd, J. N., Gogenkamp, H. P. C., and Barker, H. A., *Biochem. Biophys. Res. Comm.*, 2, 143 (1960).
[5] Disc. at the Second European Symp. on Vitamin B_{12} and the Intrinsic Factor, Hamburg (August 2, 1961).
[6] Bernhauer, K., Galser, P., Müller, O., Müller, E., and Gonter, F., *Biochem. Z.*, 333, 560 (1961).
[7] Johnson, A. W., and Shaw, N., *Proc. Chem. Soc.*, 420 (1960).

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VITAMIN B_{12} AND THE PRODUCTION OF POLYCYTHEMIA BY COBALT

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Numerous investigations have established the fact that the daily oral or parenteral administration of a small amount of cobalt will produce a striking polycythemia in a number of species of animals. Thus far the mechanism involved in the stimulation of hemopoiesis is unknown. There does not appear to be a "passive accumulation" of erythrocytes (Orten, '36), nor does cobalt affect the nature of hemoglobin or its oxygen-carrying capacity (Bucciero and Orten, '49). Cobalt may increase the utilization of iron (Wintrobe et al., '47); however, current work (Berlin et al., '51) employing radioactive iron does not support this suggestion. There is some evidence that cobalt may block sulfhydryl groups of enzymes active in cellular respiration (Burk et al., '46; Michaelis, '47; Orten and Bucciero, '48) and thus produce a compensatory polycythemia. However, neither cytochrome (Levey, '50) nor ascorbic acid (Levey, '49), also alleged to be involved in cellular respiration, appears to be involved in the polycythemic action of cobalt, at least in the rat.

Another way in which cobalt might conceivably produce a polycythemia is by increasing the formation of vitamin B_{12}, a cobalt-containing substance now known to exert a profound effect on hemopoiesis. The present investigation was therefore designed to obtain information on this point by comparing the hemopoietic effect of the daily parenteral administration of relatively large amounts of vitamin B_{12}

with that obtained from cobalt alone. For additional evidence, the vitamin B_{12} contents of the feces and of the liver of the vitamin B_{12}- and the cobalt-treated groups of rats were also determined.

EXPERIMENTAL

Young adult, male albino rats of the Sprague-Dawley strain weighing between 150 to 210 gm were used. They were fed a commercial dog pelled ration and were housed individually in the usual screen mesh cages. Three groups were studied: (1) untreated controls, 6 rats; (2) cobalt-injected, 11 rats; and (3) vitamin B_{12}-injected, 11 rats. Both the cobalt and vitamin B_{12} solutions were administered subcutaneously for 5 days a week, with double dosages on Mondays and Fridays. Cobalt was given at a level of 0.5 mg cobalt per day as repeatedly crystallized cobaltous sulfate ($COSO_4 \cdot 5H_2O$) in a 1.0% aqueous solution. Vitamin B_{12} was administered at a dosage level of 3 μg per rat per day for 6 weeks and then the dosage was increased to 10 μg per day for the last two weeks of the study. A sterile saline solution of crystalline vitamin B_{12} containing 10 μg per milliliter was used.[1]

The hemoglobin levels of the blood of the various groups of animals were determined biweekly during the 8-week period of observation by an acid-hematin procedure, calibrated by the oxygen-capacity method. Body weights were followed weekly. During the final three days of the study all feces were collected and were pooled groupwise for vitamin B_{12} assay [2] by the method of Skeggs, Huff, Wright and Bosshardt ('48). Three separate determinations were run on each pooled sample. The samples were stored at −15°C. until the assays were run. At autopsy, the livers were removed, weighed, immediately frozen in dry ice and stored frozen until the vitamin B_{12} assays were made.

[1] Appreciation is expressed to Dr. R. A. Peterman of Merck and Company, Rahway, New Jersey, for a generous supply of ampoules of "Cobione."

[2] We wish to thank Dr. O. D. Bird of the Research Laboratory, Parke, Davis and Company, for determining the vitamin B_{12} content of the samples of feces and liver.

RESULTS

The average hemoglobin values obtained for the various groups of animals, together with the standard deviations, are given in table 1. It is evident that whereas the rats injected with cobalt developed the expected characteristic polycythemia, as manifested by a marked progressive increase in the hemoglobin concentration of the blood, the animals to whom vitamin B_{12} was administered showed little or no evidence of increased hemopoiesis. The hemoglobin content of their blood was slightly greater at each biweekly period than that of the untreated controls, which perhaps might be expected because

TABLE 1

Hemoglobin values for control and cobalt- and vitamin B_{12}-injected rats

GROUP	NUMBER OF RATS	HEMOGLOBIN — GM PER 100 ML. Weeks of observation 0	2	4	6	8
Untreated controls	6	11.0 ± 0.3[1]	12.9 ± 0.3	14.0 ± 0.1	14.4 ± 0.1	14.7 ± 0.3
Cobalt-injected	11	12.2 ± 0.4	14.9 ± 0.3	16.9 ± 0.2	18.4 ± 0.2	20.4 ± 0.5
Vitamin B_{12}-injected	11	13.0 ± 0.3	13.6 ± 0.3	14.0 ± 0.4	14.5 ± 0.2	15.3 ± 0.3

[1] Standard deviation $\sqrt{\frac{\Sigma d^2}{n-1}}$.

of the added supply of vitamin B_{12} to supplement possible suboptimum amounts in the stock ration, but in no way approached that of the cobalt-injected group.

The data obtained from the assays of the pooled samples of liver and feces for vitamin B_{12} are given in table 2. It is evident that the livers of the vitamin B_{12}-injected animals contained somewhat more vitamin B_{12} than those of either the untreated or cobalt-injected groups, as might be expected. The livers of the cobalt-injected rats, indeed, appeared to contain even less vitamin B_{12} than those of the untreated control group. The differences among the three groups were somewhat more pronounced when the average

weights of the livers were taken into account and the average total hepatic vitamin B_{12} content was calculated. The total liver content of vitamin B_{12} for the three groups was 1.23, 0.878, and 1.66 μg for the untreated, cobalt- and vitamin B_{12}-injected groups, respectively.

The vitamin B_{12} content of the feces of the three groups is of interest. It is evident from the data in table 2 that the vitamin B_{12} content of the feces of the cobalt-injected group was greatest, whereas that of the vitamin B_{12}-injected group was least. It was not possible to calculate whether or not these differences were statistically significant, since the determinations were made on pooled samples; however, the range of the individual assays for each group showed no overlapping.

TABLE 2

Vitamin B_{12} content of the pooled livers and feces of the various groups of animals

Group	Vitamin B_{12} content — μg per gm			
	Liver		Feces	
	Ave.	Range	Ave.	Range
Untreated controls	0.121	0.116–0.126	3.3	3.2–3.3
Cobalt-injected	0.113	0.107–0.120	5.2	5.12–5.2
Vitamin B_{12}-injected	0.155	0.144–0.162	1.65	1.54–1.80

DISCUSSION

The present data thus indicate that the daily administration of relatively large doses of vitamin B_{12} to the rat does not produce a polycythemia, as does cobalt under similar conditions. It is possible, of course, that the dosage of vitamin B_{12} employed in the present investigation was too small to elicit a striking increase in erythropoiesis. However, it should be pointed out that the initial dosage used (3 μg per day) is the average therapeutic maintenance dose given to patients with pernicious anemia, an amount which should be several hundred times the daily requirement for the rat on a weight-to-weight basis. Furthermore, the increased daily dosage (10 μg) during the final two weeks would further exaggerate

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the relative magnitude of the dose. If the dosage of vitamin B_{12} were increased much further than this, the cobalt content of vitamin B_{12} (4%) could conceivably itself then become a complicating factor in interpreting results. Any increase in hemopoiesis might then be attributable to cobalt per se.

The data obtained from the assays for vitamin B_{12} in the livers of the three groups of rats likewise indicate that vitamin B_{12} does not play a causative role in the production of polycythemia by cobalt. Were this the case, one might logically expect an increased or at least an equal amount of vitamin B_{12} in the livers of the cobalt-treated, polycythemic rats, rather than a smaller amount, as was found. The smaller amount of vitamin B_{12} in the livers of the cobalt-injected rats may also be interpreted in another way; that is, as indicating a greater utilization of the vitamin, thus decreasing its hepatic storage. Further studies will obviously be required in order to answer this question.

The increased amount of vitamin B_{12} in the feces of the cobalt-injected rats is of considerable interest. Probably this is a reflection of an increased synthesis of vitamin B_{12} by the intestinal flora stimulated, in turn, by the excretion of some of the injected cobalt into the intestinal tract, probably by way of the bile. The fact that no more vitamin B_{12} appeared in the feces of the B_{12}-injected rats than in those of the control animals may be taken as an indication that this vitamin is not excreted by way of the bile or the intestinal tract.

SUMMARY

The daily subcutaneous administration of vitamin B_{12} in a dosage of 3 μg per day for 6 weeks, then at a dosage of 10 μg per day for two weeks longer, did not produce a significant increase in hemopoiesis in the normal rat as measured by the concentration of hemoglobin in the blood.

The daily parenteral administration of cobalt produced the characteristic polycythemia without increasing the vitamin

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B_{12} content of the liver, although there was some increase in the vitamin B_{12} content of the feces.

These results are interpreted as evidence that cobalt does not produce a polycythemia by way of the intermediary formation of vitamin B_{12}.

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The authors wish to express their sincere appreciation to Sarah Sheinfeld for technical assistance throughout the course of this investigation.

LITERATURE CITED

BERLIN, N. I., R. L. HUFF AND T. G. HENNESSEY 1951 Iron metabolism in the cobalt-polycythemic rat. J. Biol. Chem., *188:* 445–450.

BUCCIERO, M. C., AND J. M. ORTEN 1949 The effect of cobalt on the oxygen capacity and the methemoglobin content of the blood. Blood, *4:* 395–397.

BURK, D., J. HEARON, L. CAROLINE AND A. L. SCHADE 1946 Reversible complexes of cobalt, histidine, and oxygen gas. J. Biol. Chem., *165:* 723–724.

LEVEY, S. 1949 Effect of ascorbic acid, *d*-isoascorbic acid and gluconascorbic acid on cobalt polycythemia in the rat and rabbit. Am. J. Physiol., *158:* 315–318.

——— 1950 Cobalt polycythemia and cytochrome C. Science, *111:* 13.

MICHAELIS, L. 1947 The magnetic properties of the cobalt-histidine complex before and after oxygenation. Arch. Biochem., *14:* 17–22.

ORTEN, J. M. 1936 On mechanism of hemopoietic action of cobalt. Am. J. Physiol., *114:* 414–422.

ORTEN, J. M., AND M. C. BUCCIERO 1948 The effect of cysteine, histidine, and methionine on the production of polycythemia by cobalt. J. Biol. Chem., *176:* 961–968.

SKEGGS, H. R., J. W. HUFF, L. D. WRIGHT AND D. K. DOSSHARDT 1948 The use of *Lactobacillus leichmannii* in the microbiological assay of the "animal protein factor." Ibid., *176:* 1459–1460.

WINTROBE, M. M., M. GRINSTEIN, J. J. DUBASH, S. R. HUMPHREYS, H. ABUENBRUCKER AND W. WORTH 1947 Anemia of infection; influence of cobalt on anemia associated with inflammation. Blood, *2:* 323–331.

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17240. Vitamin B_{12} Content of Various Organs and Tissues of the Rat.*

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The glandular tissues of various domestic animals have long been known to be effective in pernicious anemia when administered orally.[1] Beef liver and kidney have proved to be the richest source of the active principle, kidney, however, possessing only one-half to two-

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[1] Subbarow, Y., Hastings, A. B., and Elkin, M., *Vitamins and Hormones*, 1943, 3, 237.

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TABLE 1.
Vitamin B_{12} Content of Rat Tissues.

Daily supplement	No. of animals per assay group	Series 1 Total avg gain (g) 2 wk	Series 2 Total avg gain (g) 2 wk	Min. B_{12} (γ) per g of sample
None	5	48	37	—
.1 γ crystalline B_{12}	5	73	60	
1 g kidney	3	65	51	0.04
1 g " (B_{12})*	3	89	87	0.17
1 g liver	5	47	31	†
1 g " (B_{12})	5	75	66	0.08
1 g intestine	5	38	—	†
1 g " (B_{12})	5	63	—	0.04
1 g heart	1	—	36	†
1 g " (B_{12})	1	—	62	0.07
1 g spleen	1	—	32	†
1 g " (B_{12})	1	—	30	†
2.5 g muscle	5	57	43	†
2.5 g " (B_{12})	6	71	66	Trace

* (B_{12}) From rats that received B_{12} concentrate.
† No measurable quantity.

thirds the activity of liver. Spleen, brain, heart, and pancreas are effective to a lesser extent. These same organs from the pig and lamb have likewise been used beneficially in the dietary treatment of pernicious anemia.

An assay method has been reported[2] which can be used to measure fairly quantitatively the activity of anti-pernicious anemia preparations, and all subsequent work with the assay has indicated that vitamin B_{12} is the active component being determined. The quantitative response obtained with crystalline vitamin B_{12} affords a basis for the estimation of the amount of B_{12} present in the material tested. Application of this was made in the testing of various beef and pork samples for B_{12} content.[3] The work to be reported was undertaken to determine the relative concentration of vitamin B_{12} in various organs and tissues of the rat in an effort to locate the main storage site of the vitamin within the body.

Experimental. Animals. The rats used as sources of the organ and tissue samples were obtained as weanlings. They were housed in stock colony cages and given food and water *ad libitum*. A total of 30 rats were used and all received the corn-soybean ration described previously.[4] Fifteen of the animals were given in addition to this diet a vitamin B_{12} concentrate (Merck and Co.) which was mixed directly into the basal ration to provide 3 γ of B_{12} per 100 g of food. The rats were kept on these diets for 6 weeks before sacrificing.

Preparation of samples. The rats were decapitated, bled, and the tissues and organs removed. The liver, kidney, heart, spleen, small intestine, and the femoral muscles were taken. The livers, kidneys, and intestines were weighed, combined with an equal weight of distilled water, and homogenized in a Waring blendor. The skeletal muscle was treated in the same manner except that a 3 to 1 dilution was necessary to obtain a smooth homogenate. All samples were stored at ordinary refrigeration temperature.

Assay. The assay method used has been described in a previous paper.[2] It consists of placing weanling rats on a basal ration for a 2-week depletion period and following the growth response during another 2-week period when the material to be tested is given. The samples were administered on alternate days in separate food containers. Four ml of the liver and kidney homogenates were given

2 Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 129.

3 Register, U. D., Lewis, U. J., Thompson, H. T., and Elvehjem, C. A., Proc. Soc. Exp. Biol. and Med., 1949, **70**, 167.

4 Jaffé, W. G., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **169**, 287.

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every other day, thus supplying the animals with an equivalent of 1 g of the sample per day. Because of the higher dilution of the muscle homogenate and its probable lower B_{12} content, 15 ml were administered which provided 2.5 g of muscle tissue per day.

The number of rats used for each assay group is recorded in Table I. A smaller number of rats was necessary in the cases of the kidney, spleen and heart because of an insufficient amount of sample. The assay was run in two series, a different group of rats being used as a source of the organs and tissues in each case.

Results and discussion. The data on the vitamin B_{12} content of all the rat tissues and organs tested are presented in Table I. The values obtained for the heart and spleen are based on results from one assay animal and, therefore, are to be regarded only as indications of the actual.

The greater weight gain in the first series can be explained by the fact that the basal gain, that is, without supplement, was higher throughout all the groups. However, the relative values for B_{12} content of the samples in the two individual series were similar.

The data show that in all instances, except possibly with the spleen, the inclusion of the B_{12} concentrate in the animal's diet caused an increase in the quantity of B_{12} in the organs and tissues.

The kidneys were found to be the site of greatest B_{12} concentration. Also the results indicate that the vitamin is retained in larger quantity for a longer period of time in the kidneys than in the other organs. The liver, heart, and intestine contained no appreciable amount of vitamin B_{12} after the animals had been kept on the corn-soybean ration for 6 weeks. However, the increase was very marked in these organs, as with the kidney, upon the addition of the B_{12} concentrate to the rat's diet. The skeletal muscle was not only lowest in B_{12} but also remained most nearly constant in content.

Summary. The kidney, liver, heart, small intestine and femoral muscles of rats raised on a corn-soybean diet were assayed for vitamin B_{12} content.

The kidney was found to be the site of greatest B_{12} concentration. The liver, heart, small intestine, and muscle contained no appreciable amount of the vitamin after the animals had been kept on the basal ration for 6 weeks. The amount of B_{12} increased in the organs and tissues when vitamin B_{12} was added to the diet.

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THE INFLUENCE OF VITAMIN B_{12} ON CARBOHYDRATE AND LIPIDE METABOLISM*

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In previous communications (1, 2) evidence has been presented to show that vitamin B_{12} deficiency causes derangement in carbohydrate and lipide metabolism in the animal body, and a marked diminution of glutathione concentration in the blood. The present report describes further studies on the effect of vitamin B_{12} on carbohydrate and lipide metabolism, and a possible relationship between these metabolic changes and the glutathione concentrations in the blood and tissues.

EXPERIMENTAL

Experimental Animals and Composition of Diets—The vitamin B_{12}-deficient rats used in this investigation were raised in our laboratory as described before (1). Diets A and B, the composition of which has been reported previously, were used in the early part of this work. However, observations in this (3) and other laboratories (4) indicate that a high carbohydrate and low fat diet accelerates the development of vitamin B_{12} deficiency; hence, in the later experiments, one such ration, Diet HCLF-7, was used. This diet consists of 70 per cent of a commercial soy bean product,[1] 26 per cent sucrose, 4 per cent Salts IV (5), and vitamin supplements (6), and, by analysis, it contains approximately 25 per cent soy protein, 1 per cent fat, and 68 per cent carbohydrate.

Determination of Reducing Sugars in Blood and Urine—Reducing sugar in samples of whole blood or filtered urine was determined by Somogyi's method (7). Both iodometric titration and Nelson's photometric analysis (8) were used according to the estimated amounts of sugar in the samples.

Isolation and Estimation of Phospholipides—Total lipides from body tissues were isolated and estimated as described previously (1). The ex-

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[1] Mead Johnson and Company, laboratory product No. 376, which contains soy bean flour, arrowroot starch, Dextri-Maltose, calcium phosphate (dibasic), and sodium chloride.

Federation Proc. 1954 13, 253

838. Effect of vitamin B_{12} on ribose formation in erythrocytes. CHUN T. LING* AND BACON F. CHOW. *Depts. of Biochemistry, Jefferson Med. College, Philadelphia, Pa., Johns Hopkins School of Hygiene and Public Health, Baltimore, Md.*

Our previous studies indicate that 1) vitamin B_{12} deficiency results in impaired carbohydrate metabolism and decreased blood glutathione concentration; and 2) an interrelationship exists between glucose utilization and glutathione concentration. Several investigators have presented evidence implicating vitamin B_{12} in the synthesis of nucleosides or nucleic acids. It has been found that whereas thymidine and other desoxyribosides or desoxyribonucleic acid may replace vitamin B_{12} for the growth of certain microorganisms thymine cannot. Since thymine differs from thymidine in that it lacks desoxyribose, vitamin B_{12} may be concerned with the sugar moiety of the nucleosides or nucleic acids. The influence of this vitamin on carbohydrate metabolism and nucleic acid synthesis could be readily comprehensible on this basis. In this connection, it should be interesting to ascertain whether vitamin B_{12} influences the formation of ribose in tissue cells. The first phase of this study concerns the *in vitro* formation of ribose in erythrocytes with glucose as substrate. Incubation of erythrocytes in isotonic phosphate buffer containing glucose, under aerobic conditions for 2-3 hr. resulted in a marked increase of ribose in the erythrocytes. These results suggest that ribose formation may be an important pathway of glucose utilization in mammalian erythrocytes. Red cells from B_{12}-deficient rats showed significant depression of ribose formation and oxygen consumption as compared with erythrocytes from B_{12}-repleted or normal animals. Addition of reduced glutathione to the incubation mixture restored the normal rate of ribose synthesis and oxygen consumption.

traction of lipides from whole blood was carried out according to the method of Boyd (9), and the extract was rectified with purified petroleum ether. Total phospholipides were isolated by precipitation with acetone and magnesium chloride as described by the same author. Estimation of phospholipides in both ether soluble and ether-insoluble fractions was made by phosphorus determination after hydrolysis with 10 N H_2SO_4 and oxidation with hydrogen peroxide. The phosphate was then estimated by the method of Fiske and Subbarow (10) with Elon as the reducing reagent.

Studies on Possible Relationship between Carbohydrate Metabolism and Glutathione Concentration in Blood and Tissues. The results of our earlier studies indicate that both derangement in carbohydrate metabolism and diminution in blood glutathione content occur in vitamin B_{12} deficiency. Since many of the enzymes involved in carbohydrate metabolism are sulfhydryl enzymes, it appears probable that there may be an interrelationship between the impairment in carbohydrate metabolism and changes in concentration of glutathione which occur simultaneously in a deficiency of this vitamin. If vitamin B_{12} is required for the utilization of carbohydrate as well as for the formation of glutathione in the animal body, an increased burden of carbohydrate metabolism would accelerate the depletion of stores of this vitamin in the tissues, and this might be reflected by changes in the blood sugar level and glutathione content. To test this hypothesis, six normal male rats, approximately 2.5 months old and with an average weight of 202 (197 to 207) gm., were placed on a soy bean diet (HCLF-7) and given additional glucose by injection. The dosage of glucose and frequency of injection were so adjusted that the food intake of the experimental animals was not appreciably decreased. Alternating intraperitoneal and subcutaneous injections of 5 to 10 ml. of a 25 per cent sterile glucose solution to each rat fulfilled this purpose. The level of blood glutathione was determined as described elsewhere (2). The results of these studies are presented in Figs. 2 and 3.

Results

Blood Sugar Levels and Glucose Tolerance Curves.—In the preliminary experiments it was observed that the blood sugar levels of growing young rats after a short period of fasting (12 to 24 hours) were lower in the vitamin B_{12}-deficient group than in the vitamin B_{12}-treated controls. But when the fasting period was extended to 48 hours, several of the deficient rats had abnormally high blood sugars (170 to 498 mg. per cent), showing a tendency toward "hunger diabetes," whereas none of the vitamin B_{12}-treated controls had such an abnormality. The urinary excretion of reducing sugar during a fasting period declined much more rapidly among the vitamin B_{12}-deficient rats than among the normal animals. One possible explanation for the above findings is that the vitamin B_{12}-deficient animals have less carbohydrate reserve in their body. In Fig. 1 are shown the glucose tolerance curves of vitamin B_{12}-deficient rats and controls receiving the vitamin. After fasting overnight, the rats received glucose

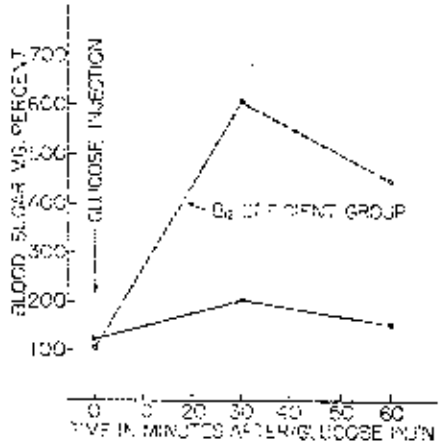

FIG. 1. Glucose tolerance curve of vitamin B_{12}-deficient and vitamin B_{12} injected rats. The dash line represents the average value of four vitamin B_{12}-deficient rats, while the solid line represents the average value of three normal rats.

TABLE I

Phospholipide Content of Body Tissues of Rats

	Vitamin B_{12}-deficient (3 rats)	Vitamin B_{12}-deficient, vitamin B_{12}-treated (3 rats)
Initial body weight (average), *gm.*	36	36
Final body weight (average), *gm.*	60	147
Weight of tissues analyzed,* *gm.*	33.6	76.4
Total phospholipides per rat (average), *mg.*	190 ± 17	564 ± 68
Phospholipide per unit tissue weight, %	0.56 ± 0.03	0.74 ± 0.09

* Vitamin B_{12}-deficient weanling rats fed Diet B for a period of 6 weeks before sacrifice. The sampling of tissues assayed is described in the text.

by intravenous injection in a dose of 750 mg. per kilo of body weight. It will be noted that the average blood sugar level of the deficient rats remained unusually high at the end of 60 minutes after injection, while that of the vitamin B_{12}-treated rats had returned to normal.

Phospholipide Content of Blood and Tissues in Vitamin B_{12} Deficiency.—In Table I are presented data on the phospholipide content of tissues in vitamin B_{12}-deficient and B_{12}-treated young rats. The fat was isolated

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from the lean tissues of the carcass and did not include skin, subcutaneous adipose layer, or viscera, and, therefore, the values represent chiefly the intercellular and intracellular lipides, a major portion of which constitutes an integral part of the cellular constituents. Even though these rats were litter mates and had approximately the same average initial body weights and were raised under the same dietary regimen, the injection of vitamin B_{12} resulted in a 3-fold increase of the total phospholipides in the lean tissues of the carcass as well as in a higher phospholipide content per unit weight of tissues.

TABLE II

Phospholipide Content of Blood in Pernicious Anemia

Date, 1951	Vitamin B_{12}, orally	Hematocrit	Total phospholipides
	F. G., male; age 56 yrs.		
	mg.	*per cent*	*mg. per 100 ml. blood*
Sept. 1		17	57
" 1	3.0		
" 8		24	138
" 17		33	253
	H. J., male; age 63 yrs.		
Aug. 29		34	185
" 29	3.0		
Sept. 5		37	180
" 17		38	236
" 24		37	245

Pernicious anemia is considered a state of vitamin B_{12} deficiency in man, and such patients are known to have an abnormally low blood phospholipide content when in relapse (11, 12). Table II contains the results of the determination of the blood phospholipide levels of two patients with pernicious anemia before and after vitamin B_{12} therapy. Both patients had significantly low blood phospholipide before treatment, in proportion to the severity of the anemia, but the levels rapidly returned to normal after treatment with the vitamin.

Effect of High Carbohydrate Intake on Blood Sugar and Glutathione Levels and Influence of Glutathione or Vitamin B_{12} Administration on Hyperglycemia—When a group of six normal, young, male adult rats was fed Diet HCLF-7 and given injections of a 25 per cent glucose solution at the intervals indicated in Fig. 2, hyperglycemia and a concomitant decrease of glutathione content of the blood resulted. The average fasting blood sugar

level was elevated from its initial value of 106 mg. per cent to 152 mg. per cent 10 days after the start of glucose administration and reached 249 mg. per cent on the 21th day, *i.e.* 2 days after the termination of glucose injection. The fasting blood sugar level remained at 151 mg. per cent 11 days after the last parenteral dose of glucose. The average blood glutathione was 410 μM per 100 ml. of blood cells at the beginning, but the value decreased to 328 μM 6 days after the cessation of glucose injection, a 20 per cent reduction from its initial level, with a P value of less than 0.02.

To study the effect of glutathione on the blood sugar levels of these hyperglycemic rats, 25 mg. of reduced glutathione were given to each rat

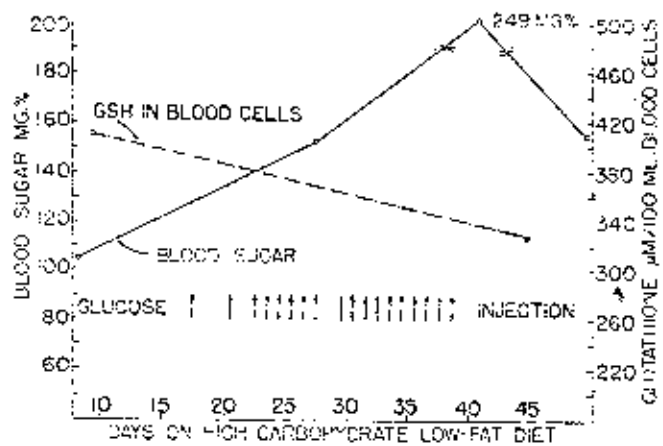

FIG. 2. Effects of diet and glucose injection on the levels of blood sugar and blood glutathione. The values represent the average of six male rats. See the text.

by subcutaneous injection, and the blood sugar levels were followed for 3 hours. Administration of glutathione produced a transient, but significant, drop in the blood sugar in these animals, from an average of 134 to 112 mg. per cent in 3 hours. Blood sugar levels of these rats measured at the same hours (2 and 5 p.m.) on the following day showed that it had risen from 143 to 153 mg. per cent without injection of glutathione.

The effect of vitamin B_{12} on induced hyperglycemia was next studied with the same group of rats. The results are illustrated in Fig. 3. A few days after the termination of the above experiment, injection of glucose was resumed while the animals were maintained on the same diet. This treatment again resulted in a rise in blood sugar levels from an average of 117 to 181 mg. per cent in 9 days. At this point, the animals were divided randomly into two groups of approximately the same average blood sugar levels. To one group was given vitamin B_{12} (1.0 γ per rat per day) by

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subcutaneous injection, while the glucose injection to both groups of animals was continued. 1 week after the administration of vitamin B_{12} was begun, the treated group gained an average of 12 gm. in body weight, and the blood sugar levels decreased to an average of 171 mg. per cent, while the untreated group lost an average of 2 gm. in body weight and had an average blood sugar of 408 mg. per cent. Attempts were made to produce an even greater difference in blood sugar levels between the two groups of rats by increasing the frequency of glucose injection (daily instead of every other day) to both groups while the injection of vitamin B_{12} to one group was maintained on the same daily dosage. With the increased dose of glucose, both groups of rats lost weight, the loss averaging 5 gm. for the

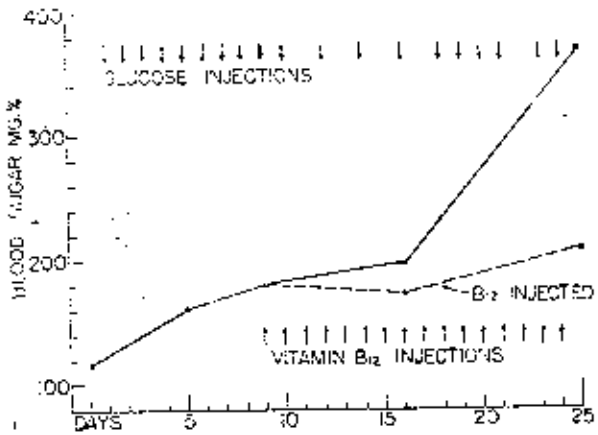

FIG. 3. Effect of vitamin B_{12} on induced hyperglycemia. Changes in body weight are described in the text.

vitamin B_{12}-treated group and 14 gm. for the untreated group during the ensuing 8 day period, but the average blood sugar for the vitamin B_{12}-treated group remained at 209 mg. per cent, while that of the latter group had risen to 367 mg. per cent. The blood sugar determination was repeated 4 days later, and a difference of the same order of magnitude was observed.

DISCUSSION

In this communication are presented data which provide two additional points of evidence demonstrating the importance of vitamin B_{12} in carbohydrate and lipide metabolism. In the first place, the abnormally high blood sugar levels of vitamin B_{12} deficient rats in the glucose tolerance test (Fig. 1) indicate that they suffer from derangement in their capacity to utilize carbohydrate. As a result the deficient animals have a smaller carbohydrate reserve and consequently are more sensitive to fasting. This

is further evident from the fact that they had consistently lower blood sugar levels after a short period of fasting, but developed "hunger diabetes" sooner than the control animals. The "hunger diabetes" is considered as a sign of alarm reaction due to increased gluconeogenesis from tissue protein. The sharp drop in the urinary excretion of reducing sugar by the deficient rats during fasting may be taken as another sign of a poor carbohydrate reserve.

In the second place, data presented in Tables I and II demonstrate that vitamin B_{12} deficiency entails disturbance in lipide metabolism, which can be corrected by administration of the vitamin. It is well known that a major part of the carbohydrates utilized is converted into lipides and, according to Stetter and Boxer (13, 14), this conversion would amount to approximately 30 per cent of the available body glucose in normal rats placed on a high carbohydrate diet. The rapidity with which our vitamin B_{12}-deficient rats lost body weight and developed hyperglycemia under the influence on a high carbohydrate-low fat diet, and the reported increased requirement of vitamin B_{12}-deficient rats for this vitamin when raised on such a diet (3, 4), further suggest that the deficient animals have lost part of their ability to transform carbohydrate into lipides. In harmony with this hypothesis is the fact that abnormally small amounts of phospholipides were found in the tissues of vitamin B_{12}-deficient rats and in the blood of patients with pernicious anemia in relapse. Administration of this vitamin to the above resulted in marked increases in phospholipide content of blood and tissues. Muller (11) and Kirk (12) have observed that in pernicious anemia in relapse the blood phospholipide and cholesterol were abnormally low, and that both of these substances increased after an effective liver therapy, owing presumably to the action of vitamin B_{12}.

In the latter part of this investigation, a possible relationship between blood glutathione concentration and carbohydrate utilization was brought to light. It was found that the blood glutathione levels in normal rats could be lowered by feeding a high carbohydrate-low fat diet coupled with glucose injection for a relatively short period of time (Fig. 2), with a concomitant rise in blood sugar levels which persisted long after the cessation of glucose injection. Since vitamin B_{12} deficiency likewise causes the lowering of blood glutathione, these results are suggestive that a high carbohydrate intake brought about a rather rapid depletion of vitamin B_{12} stores in the animal body, owing to increased requirement for the vitamin in carbohydrate metabolism. Furthermore, injection of glutathione resulted in a transient, but significant, drop in blood sugar levels of these rats within 2 to 3 hours, indicating the existence of a close relationship between blood glutathione concentration and carbohydrate utilization. However, considering the extremely rapid turnover of glutathione in the animal body,

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as shown by the studies of Waelsch and Rittenberg (15, 16), one would not expect that the derangement in carbohydrate metabolism in vitamin B_{12} deficiency can be fully corrected by the injection of glutathione, owing to the impossibility of maintaining an adequate supply from temporary exogenous sources. Therefore, an adequate supply of the peptide to meet the physiological requirement can be maintained only when the glutathione synthesis in the animal body is restored to normalcy by the administration of vitamin B_{12}. In such a situation, it would be expected that hyperglycemia would be corrected also owing to the increase in glutathione synthesis. This belief is substantiated by our data, presented in Fig. 3, which demonstrate that injection of vitamin B_{12} to these deficient animals effectively prevented the hyperglycemia and loss of body weight induced by such a diet and glucose injection.

Although the mechanism by which vitamin B_{12} aids in the utilization of carbohydrate is still unknown, experimental evidence from these studies indicates that the maintenance of an adequate concentration of glutathione in the blood and tissues may be at least one of several important factors in the functioning of this vitamin. There are several plausible explanations of the mechanism of action by which glutathione could improve the utilization of carbohydrates, such as the activation of sulfhydryl enzymes and the protection of the β-cells in the pancreas (17–19) from the deleterious effect of certain metabolic products, alloxan and dehydroascorbic acid for instance. Although these substances are usually found only in minute quantities in the animal body, their concentration can increase to a considerable extent under certain conditions (20, 21). For example, Banerjee *et al.* (22) found that dehydroascorbic acid was present in considerable amounts in scorbutic guinea pig tissues and that the glutathione content was markedly diminished in these tissues, and particularly so in the pancreas. However, the rapidity with which glutathione acts to lower the blood sugar after its injection suggests that this substance may be directly involved in the utilization of carbohydrate. This supposition is substantiated by the observations of Cavallini (23) on the rôle of glutathione in the coupled oxidative decarboxylation of pyruvate and of Racker (24) on the mechanism of glyoxalase function and by the discovery of Krimsky and Racker (25, 26) that glutathione is the prosthetic group of 3-phosphoglyceraldehyde dehydrogenase.

Yacowitz *et al.* (27) reported that vitamin B_{12} exerted pantothenic acid-sparing action for the growth of chicks, and that this vitamin seems to increase the conversion of pantothenic acid to coenzyme A in the liver. This finding further establishes a relationship between vitamin B_{12} and another soluble sulfhydryl compound, coenzyme A. Since coenzyme A is an important participant in the formation of fatty acids, phospholipides, cholesterol, and acetylcholine, it furnishes additional evidence that vitamin B_{12} also plays an essential rôle in lipogenesis and the metabolism of choline.

SUMMARY

The effect of vitamin B_{12} on carbohydrate and lipide metabolism was studied by measurement of blood sugar levels, by glucose tolerance tests, and by estimation of the phospholipide content of blood and tissues. Its possible relationship to the changes in blood glutathione content was also investigated.

Experimental results indicate that vitamin B_{12} deficiency entails a derangement in carbohydrate utilization and decreases the phospholipide content of blood and tissues.

Administration of glutathione or vitamin B_{12} lowered the blood sugar levels of rats with hyperglycemia induced by a high carbohydrate-low fat diet and by glucose injections.

It is concluded that vitamin B_{12} plays an important rôle in carbohydrate and lipide metabolism and that the effect of this vitamin on blood glutathione concentration may be of significance in its rôle in metabolism.

BIBLIOGRAPHY

1. Ling, C. T., and Chow, B. F., *J. Biol. Chem.*, **198**, 439 (1952).
2. Ling, C. T., and Chow, B. F., *J. Biol. Chem.*, **202**, 445 (1953).
3. McCollum, E. B., and Chow, B. F., *Proc. Soc. Exp. Biol. and Med.*, **75**, 20 (1950).
4. Bosshardt, D. K., Winifred, J. P., and Barnes, R. H., *J. Nutr.*, **40**, 505 (1950).
5. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **138**, 459 (1941).
6. Davis, R. L., and Chow, B. F., *Proc. Soc. Exp. Biol. and Med.*, **77**, 218 (1951).
7. Somogyi, M., *J. Biol. Chem.*, **160**, 61 (1945).
8. Nelson, N., *J. Biol. Chem.*, **153**, 375 (1944).
9. Boyd, E. M., *Am. J. Clin. Path., Tech. Suppl.*, **2**, 77 (1938).
10. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
11. Muller, G. L., *Am. J. Med. Sc.*, **179**, 316 (1930).
12. Kirk, E., *Am. J. Med. Sc.*, **196**, 648 (1938).
13. Boxer, G. E., and Stetten, D., Jr., *J. Biol. Chem.*, **156**, 237 (1944).
14. Stetten, D., Jr., in Handbook of nutrition, Chicago, 2nd edition, 63 (1951).
15. Waelsch, H., and Rittenberg, D., *J. Biol. Chem.*, **139**, 761 (1941).
16. Waelsch, H., and Rittenberg, D., *J. Biol. Chem.*, **144**, 53 (1942).
17. Lazarow, A., *Proc. Soc. Exp. Biol. and Med.*, **61**, 441 (1946).
18. Patterson, J. W., and Lazarow, A., *J. Biol. Chem.*, **186**, 141 (1950).
19. Sen, P. B., and Bhattacharya, G., *Science*, **115**, 41 (1952).
20. Loubatières, A., and Bouyard, P., *Compt. rend. Acad.*, **232**, 257 (1951).
21. Patterson, J. W., *J. Biol. Chem.*, **183**, 81 (1950).
22. Banerjee, S., Deb, C., and Belavady, B., *J. Biol. Chem.*, **195**, 271 (1952).
23. Cavallini, D., *Biochem. J.*, **49**, 1 (1951).
24. Racker, E., *J. Biol. Chem.*, **190**, 685 (1951).
25. Krimsky, I., and Racker, E., *J. Biol. Chem.*, **198**, 721 (1952).
26. Racker, E., and Krimsky, I., *J. Biol. Chem.*, **198**, 731 (1952).
27. Yacowitz, H., Norris, L. C., and Heuser, G. F., *J. Biol. Chem.*, **192**, 141 (1951).

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Clinical Science (1971) 40, 1–16.

CHROMATOGRAPHIC AND BIOAUTOGRAPHIC ESTIMATION OF PLASMA COBALAMINS IN VARIOUS DISTURBANCES OF VITAMIN B_{12} METABOLISM

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SUMMARY

1. This paper reports a survey of values for individual plasma cobalamins in normal subjects, hospital controls, and patients with vitamin B_{12} deficiency and diseases in which there are disturbances of B_{12} metabolism. The values were obtained by thin-layer chromatography and bioautography followed by photometric scanning or visual assessment.

2. Normal plasma contains methylcobalamin (the predominant component), deoxyadenosylcobalamin and hydroxocobalamin. The latter two compounds are not separable in the solvent system routinely used. In some samples there were traces of cyanocobalamin. The ratio of methylcobalamin to deoxyadenosylcobalamin plus hydroxocobalamin, normally greater than 1·0, was reduced in B_{12} deficiency. Many cases of untreated pernicious anaemia showed a high proportion (up to 40%) of cyanocobalamin. In folate deficiency there was no consistent change in individual cobalamins. Patients with leukaemias and liver disease showed a variety of changes, some attributable to alterations in plasma binding capacity and loss of deoxyadenosylcobalamin from the liver.

3. After oral or parenteral administration of cyanocobalamin, plasma cyanocobalamin increased and there was evidence of conversion to methylcobalamin. After parenteral administration of methylcobalamin, this compound increased in the plasma, but deoxyadenosylcobalamin plus hydroxocobalamin did not change.

4. Possible reasons for pathological changes in individual plasma cobalamins are discussed.

Very little is known about the distribution of individual forms of vitamin B_{12} in the body, or about how this distribution may be altered in disease (Chanarin, 1969). Deoxyadenosylcobalamin ('coenzyme B_{12}') has been identified and estimated in human liver (Toohey & Barker, 1961; Morrow, Barness, Cardinale, Abeles & Flaks, 1969) and is apparently the main storage

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form. Lindstrand & Ståhlberg (1963) were the first to show that there were at least two different cobalamins in human plasma, the major components being (1) methylcobalamin and (2) a second compound, probably either deoxyadenosylcobalamin or hydroxocobalamin (Lindstrand, 1964; Ståhlberg, 1967). The use of thin-layer chromatography has made it possible to apply chromatographic and bioautographic methods for separation of plasma cobalamins to relatively small quantities of blood (5 ml) (Linnell, Mackenzie, Wilson & Matthews, 1969c) instead of the very large volumes previously required. A technique for approximate quantitation of these cobalamins by photometric scanning of the stained bioautogram has been developed, and results obtained in normal subjects briefly reported (Linnell, Mackenzie & Matthews, 1969b). These methods have made it feasible to make a survey of values for individual plasma cobalamins in a large number of healthy people, hospital controls, and patients suffering from a variety of disturbances of vitamin B_{12} metabolism including deficiency states of differing origin. The desirability of this survey was suggested by the following (1) the normal ratio of methylcobalamin to the other plasma cobalamin components is disturbed in pernicious anaemia (Ståhlberg, 1967; Linnell *et al.*, 1969c), (2) in certain cases, this ratio may be disturbed even in the presence of a normal total plasma B_{12} (Linnell, Hoffbrand, Peters & Matthews, 1969a), (3) an excessive proportion of cyanocobalamin may appear in the plasma in Leber's optic atrophy and certain other optic neuropathies, possibly owing to abnormalities of cyanide metabolism (Linnell, Wilson & Matthews, 1969d), and (4) the recent demonstration of inborn errors in the metabolism of the coenzyme forms of vitamin B_{12} (Mudd, Levy & Abeles, 1969; Morrow *et al.*, 1969; Levy, Mudd, Schulman, Dreyfus & Abeles, 1970).

METHODS

Chromatography and bioautography of plasma cobalamins

The method of estimation of individual cobalamins was based on thin-layer chromatography of ethanol extracts of plasma followed by bioautography to locate the separated cobalamins. The amounts of each cobalamin represented by individual growth zones were then estimated by photometric scanning and comparison with growth zones obtained by chromatography and bioautography of appropriate cobalamin standards.

Heparinized blood samples were taken into foil-wrapped syringes with strict precautions against exposure to light. Extraction of cobalamins, chromatography and bioautography were carried out as described by Linnell *et al.* (1969c). Standard cobalamin solutions (methylcobalamin, deoxyadenosylcobalamin and cyanocobalamin) were subjected to chromatography and bioautography under the same conditions, being run together with 'unknowns' on the same chromatography plate. The growth areas on the bioautograms, which are stained crimson with a tetrazolium salt, were scanned by reflected light with the Joyce-Loebl 'Chromoscan' (Model J-313, filter 5040, transmission band 500–600 mμ). The amount of cobalamin represented by each growth area in the 'unknown' was estimated by reference to the relevant standard curve (Fig. 1). This gave the proportion of each cobalamin in the extract. The concentrations of individual cobalamins in the original sample were estimated from these proportions and the total B_{12} concentration in the sample as determined by radioisotopic assay (Matthews, Gunasegaram & Linnell, 1967). In some cases, including many of the earlier ones, approximate estimates of cobalamin concentrations were made by visual assessment of the intensity and area of the growth zones, with appropriate proportionation of the total B_{12}. With experience, such estimates correspond well with values obtained by scanning.

Estimation of liver cobalamins

Portions of liver biopsies obtained for diagnostic purposes were extracted by heating with acetate-cyanide buffer (Matthews *et al.*, 1967) and total vitamin B_{12} measured by radioisotopic assay. For estimation of individual cobalamins, the tissue was homogenized in water by grinding with sand and the homogenate extracted with hot ethanol as for plasma.

Other estimations

Serum folate was estimated microbiologically by the method of Waters & Mollin (1961). The normal range in completely healthy people is from 5·9 to 21·0 ng/ml. *Excretion of methylmalonic acid* was estimated in 24-h urine specimens collected before and after L-valine (10 g) loading by the method of Dreyfus & Dubé (1967).

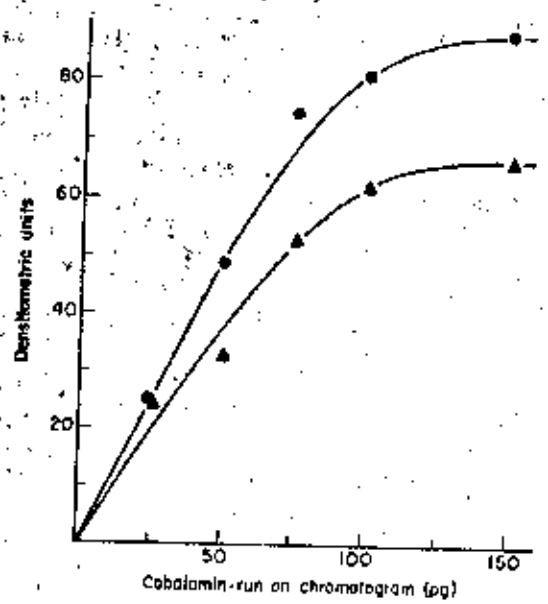

FIG. 1. Standard curves for deoxyadenosylcobalamin (●) and methylcobalamin (▲). The growth responses to cyanocobalamin and hydroxocobalamin, which are not shown, are usually indistinguishable from that to deoxyadenosylcobalamin.

Control subjects and patients

Normal subjects were healthy adult volunteers, mainly medical and laboratory staff. *Hospital controls* were inpatients suffering from conditions in which there was no reason to expect vitamin B_{12} deficiency or disturbance of vitamin B_{12} metabolism.

The diagnosis of pernicious anaemia was made on the basis of a megaloblastic marrow, subnormal serum vitamin B_{12} level, histamine- or pentagastrin-fast achlorhydria and malabsorption of radioactive B_{12} corrected by intrinsic factor. In each case the anaemia responded satisfactorily to vitamin B_{12} therapy.

The diagnosis of folate deficiency was made in patients with megaloblastic anaemia and a low serum folate who showed complete haematological remission when given folic acid, 200 μg daily.

TABLE 1. Plasma cobalamins in normal subjects and hospital controls: results obtained by photometric scanning of bioautograms

Subject	Smoking habits	Age	Sex	Total B_{12} (pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	CN B_{12} percentage of total
Normal subjects									
1	0	35	M	625	437	0	188	2·3	0
2	0	35	M	350	294	0	56	5·2	0
3	0	27	M	345	190	0	155	1·2	0
4	0	38	M	1085	597	0	488	1·2	0
5	40 Cigarettes per day	43	M	595	355	0	240	1·5	0
6	15 Cigarettes per day	52	F	490	314	3	173	1·8	0·6
7	30 Cigarettes per day	46	M	765	594	3	168	3·5	0·4
8	25–30 Cigarettes per day	55	M	385	292	0	93	3·1	0
9	25 Cigarettes per day	31	M	510	398	0	112	3·5	0
10	10 Cigarettes per day	46	F	570	467	0	103	4·5	0
11	20–25 Cigarettes per day	38	M	560	465	0	95	4·9	0
12	20–25 Cigarettes per day	48	M	400	270	0	130	2·1	0
13	3½ oz Tobacco per week	52	M	350	294	0	56	5·4	0
14	3 oz Tobacco per week	55	M	250	157	3	90	1·8	1·2
15	6 oz Tobacco per week	64	M	575	282	46	247	1·1	8
16	3½ oz Tobacco per week	63	M	335	171	3	161	1·1	0·9
17	10–15 Cigarettes per day	57	F	755	460	0	295	1·6	0
18	20 Cigarettes per day	43	M	555	480	0	75	6·4	0
19	25 Cigarettes per day	19	F	660	505	0	155	3·3	0
20	25 Cigarettes per day	22	M	275	170	0	105	1·6	0
21	3½ oz Tobacco per week	52	M	550	443	5	102	4·3	0·9
22	25–35 Cigarettes per day	62	M	575	418	3	154	2·7	0·5
23	20–25 Cigarettes per day	43	M	800	616	24	100	3·8	3·0
Hospital controls									
24	0	16	F	550	435	0	115	3·8	0
25	20 Cigarettes per day	31	F	405	247	0	158	1·6	0
26	10 Cigarettes per day	55	F	370	230	0	140	1·6	0
27	15–20 Cigarettes per day	55	M	425	342	0	83	3·0	0
28	15–20 Cigarettes per day	55	M	510	270	0	240	1·1	0
	Mean			522	364	3·2	155	2·9	0·5
	SEM			±33·8	±24·7	±1·8	±16·4	±0·3	±0·3
	Range			(250–1085)	(157–616)	(0–46)	(56–488)	(1·1–6·4)	(0–8)

The *vegans* all ate a diet completely devoid of meat, fish or animal produce (except milk in three cases). They showed vitamin B_{12} deficiency but no malabsorption of radioactive B_{12}.

The patients with *atrophic gastritis* all showed atrophic changes on gastric biopsy, but were free from megaloblastic anaemia or neurological damage and showed only mild reduction in vitamin B_{12} absorption, corrected by intrinsic factor.

RESULTS

When applied to plasma, the technique normally shows two main growth zones. Zone 1, which is predominant in healthy subjects, has an R_f corresponding to that of methylcobalamin. Zone 2 has an R_f corresponding to that of deoxyadenosylcobalamin ('coenzyme B_{12}'). In the solvent system routinely used, low concentrations of deoxyadenosylcobalamin are not adequately separated from hydroxocobalamin. Chromatography in a solvent system (water saturated with benzyl alcohol) which gives complete separation of hydroxocobalamin from the other cobalamins shows that Zone 2 represents a mixture of deoxyadenosylcobalamin and hydroxocobalamin. In ten healthy subjects, the mean proportion of deoxyadenosylcobalamin in this zone was 51 %. In this paper the mixture of deoxyadenosylcobalamin ('coenzyme B_{12}') and hydroxocobalamin is referred to as 'Co-OH B_{12}'. In a minority of samples a third zone appears with the R_f of cyanocobalamin.

Normal subjects and hospital controls

Results obtained by photometric scanning of bioautograms of plasma in a series of twenty-eight normal subjects and hospital controls, some of them heavy smokers, are shown in Table 1. Methylcobalamin was the predominant cobalamin in all subjects, the mean concentration being about three times that of Co-OH B_{12}. The ratio of methylcobalamin to Co-OH B_{12} varied widely, but was greater than 1·0 in all cases. Cyanocobalamin appeared in eight of the samples (28 %) always in small amounts and usually in traces. The possible relationship between the presence of cyanocobalamin and smoking has previously been described and it has been shown that this compound is not confined to smokers (Lindstrand *et al.*, 1966; Linnell *et al.*, 1969c).

The results of visual assessment of cobalamins in seventy-three plasma samples from normal subjects and hospital controls are summarized in Table 2, together with the results obtained by photometric scanning. The mean figures obtained by visual assessment are very similar to those obtained by scanning. Results obtained by visual assessment are indicated in subsequent Tables by an asterisk. In interpreting pathological values, the following values have been taken as normal: total plasma B_{12}, 185–935 pg/ml; methylcobalamin (Me B_{12}), 130–600 pg/ml; deoxyadenosylcobalamin plus hydroxocobalamin (Co-OH B_{12}), 60–310 pg/ml; cyanocobalamin (CN B_{12}), 0–50 pg/ml; ratio Me B_{12}/Co-OH B_{12}, 1–5·5 (Table 2).

Untreated pernicious anaemia

The findings in untreated pernicious anaemia (Table 3) confirm and extend those in the smaller series of cases previously published (Linnell *et al.*, 1969c). In all cases methylcobalamin was disproportionately reduced in relation to deoxyadenosylcobalamin plus hydroxocobalamin, so that the ratio Me B_{12}/Co-OH B_{12} was less than 1·0. In approximately half of the cases methylcobalamin was present only in traces or not detectable at all. Whereas methylcobalamin

was well below the lower limit of normal in all cases, Co-OH B_{12}, though frequently subnormal, was just within normal limits in a minority of cases. An interesting feature of the chromatographic pattern in pernicious anaemia was that twelve of the twenty-nine cases (41 %) showed amounts of cyanocobalamin which, though low in absolute terms, made up a substantial proportion (up to 40 %) of the total plasma vitamin B_{12}. No relationship was observed between

TABLE 2. Plasma cobalamins in normal subjects and hospital controls: summary of results obtained by scanning and by visual assessment of bioautograms

Method of estimation of individual cobalamins	No. of subjects	Total B_{12} (pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	CN B_{12} as percentage of total
Scanning	28 (23 N, 5H*)	522 ± 34† (250–1085)	364 ± 25 (157–616)	3·2 ± 1·8 (0–46)	155 ± 16 (56–488)	2·9 ± 0·3 (1·1–6·4)	0–8
Visual – same samples as above	28 (23 N, 5H)	522 ± 34 (250–1085)	343 ± 24 (150–600)	(0–45)	178 ± 14 (100–434)	2·1 ± 0·2 (1·0–3·0)	0–8
Visual	73 (39 N, 34H)	474 ± 25 (220–1085)	298 ± 17 (125–651)	(0–50)	174 ± 6 (66–434)	1·6 ± 0·1 (0·7–3·0)	0–8
Control range adopted‡		185–935	130–600	0–50	60–310	1–5·5	0–8

* N = normal, H = hospital control. † Mean ± S.E. Range in parenthesis.

‡ Values for individual cobalamins approximate to the 95% range of all estimations. The range for total B_{12} is derived from the present data and that of Matthews, Gunasegaram & Linnell (1967), and approximates to a 98% range from 173 subjects.

this phenomenon and the occurrence of subacute combined degeneration of the cord. There was no correlation between plasma methylcobalamin and serum folate. In several cases (Nos. 4, 21, 22, 24) increased methylmalonate excretion occurred with a plasma level of Co-OH B_{12} which was just within normal limits.

Other causes of vitamin B_{12} deficiency

Tables 4 and 5 show results in a number of conditions capable of causing B_{12} deficiency, though not all patients were actually deficient.

Of five untreated *vegans* (Table 4) all had subnormal total B_{12} levels. Three showed a pattern of plasma cobalamins indistinguishable from that of pernicious anaemia, but in the other two, methylcobalamin was only slightly subnormal and the ratio Me B_{12}/Co-OH B_{12} was within normal limits. In three B_{12}-supplemented vegans, all cobalamins were within normal limits.

Of the patients with *partial gastrectomy* or *atrophic gastritis* (Table 5), and subnormal total plasma B_{12} levels, five out of nine had a subnormal Me B_{12}/Co-OH B_{12} ratio, the pattern being similar to that of untreated pernicious anaemia. An interesting feature was the occurrence of a subnormal Me B_{12}/Co-OH B_{12} ratio in three patients (Nos. 43, 44 and 45) with total plasma B_{12} levels which were within normal limits. All three patients with subnormal or borderline total B_{12} in the group with *intestinal disease* (Table 5) had a subnormal Me B_{12}/Co-OH B_{12} ratio, and one (No. 55) had a subnormal ratio in the presence of a normal total B_{12} level.

TABLE 3: Plasma cobalamins in untreated pernicious anaemia: uncomplicated and with subacute combined degeneration of the cord

Patient	Age	Sex	Total B_{12} (pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	CN B_{12} percentage of total	Haemoglobin (g/100 ml)	Serum folate (ng/ml)	Methylmalonate excretion test	
											Pre-valine	Post-valine
Uncomplicated pernicious anaemia												
1	60	M	50	0	6	44	0	12	12·0	13·0	+	+
2	61	M	50	5	0	45	0·2	0	11·2	28·0		
3	66	M	80	29	0	51	0·6	0	11·3	19·0	+	+
4	60	F	100	0	25	75*	0	25	10·0	9·6	+	+
5	77	F	10	0	3	7	0	30	6·0	11·8	+	+
6	71	F	75	0	0	75*	0	0	13·8	21·0		
7	77	F	68	0	0	68*	0	0	8·7	8·0		
8	85	F	64	16	0	48*	0·3	0	10·4	3·0		
9	67	F	86	17	17	52*	0·3	20	6·2	1·2		
10	75	F	50	13	0	37*	0·4	0	7·3	4·2		
11	65	F	30	0	0	30*	0	0	4·9	6·4		
12	65	M	65	26	0	39*	0·7	0	7·8	—		
13	71	F	35	14	0	21*	0·7	0	7·4	—		
14	63	F	75	trace	0	75*	0	0	7·7	4·7		
15	46	F	100	29	29	42*	0·7	29	11·5	8·4		
16	62	F	80	13	27	40*	0·3	34	5·0	2·9		
17	71	F	80	16	16	48*	0·3	20	7·7	>25		
18	34	M	60	0	24	36*	0	40	15·1	—		
19	43	M	35	0	14	21*	0	40	3·8	1·7		
20	74	F	77	trace	trace	75*	0	*ca* 5	11·6	32·0	−	−
21	63	F	130	33	0	97*	0·3	0	18·1	7·6	+	+
22	63	F	86	22	0	64*	0·3	0	7·5	24·0	+	+
23	68	F	49	0	20	29*	0	41	5·9	5·4	+	+
24	49	M	72	0	trace	70*	0	*ca* 5	7·8	28·0	+	+
25	53	F	100	trace	0	100*	0	0	6·1	7·6		
Cases with subacute combined degeneration of the cord												
26	66	M	30	8	2	20	0·4	7	10·0	—		
27	69	F	30	9	9	12*	0·8	30	12·5	—		
28	68	F	50	trace	12	38*	0	24	7·8	11·0		
29	72	M	55	trace	0	55*	0	0	14·9	20·0		
Mean ± SE			64·6 ± 4·8	9·0 ± 1·9	7·2 ± 1·8	48·8 ± 4·3	0·22 ± 0·05	12·5 ± 2·8				
Range			(10–130)	(0–33)	(0–29)	(7–100)	(0–0·8)	(0–41)				

Note: In calculation of statistics 'trace' has been assigned a value of 2.
* Derived from visual score.

TABLE 4. Plasma cobalamins in veganism

Subject	Diet	Age	Sex	Total B_{12} (pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	Haemoglobin (g/100 ml)	Serum folate (ng/ml)	Methylmalonate excretion test	
											Pre-valine	Post-valine
31	B_{12} unsupplemented	38	F	160	107	0	53*	2·0	11·5	8·0		
32	B_{12} unsupplemented	31	F	135	62	0	73	0·9	7·5	3·2	+	+
33	B_{12} unsupplemented	24	F	155	118	0	37	3·2	11·7	0·8	−	+
34	B_{12} unsupplemented	44	F	55	0	0	55	0	13·2	16·6	−	+
35	B_{12} unsupplemented	46	M	100	0	0	100	0	11·0	>25		
36	B_{12} supplemented	33	M	380	190	0	190*	1·0	14·0	>32		
37	B_{12} supplemented	31	F	300	150	trace	150*	1·0	13·6	>16		
38	B_{12} supplemented	64	M	455	273	0	182*	1·5	13·6	14·0		

* Derived from visual score.

TABLE 5. Plasma cobalamins in gastrointestinal disease

Patient	Diagnosis	Age	Sex	Total B_{12} (pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	Haemoglobin (g/100 ml)	Serum folate (ng/ml)	Methylmalonate excretion test	
											Pre-valine	Post-valine
30	Partial gastrectomy: SACD	61	M	49	12	0	37*	0·3	7·2	19·2	+	+
39	Partial gastrectomy	67	M	112	44	0	68	0·7	10·1	2·4	–	+
40	Partial gastrectomy	54	M	135	81	3	53	1·5	13·5	8·2	–	–
41	Partial gastrectomy	29	M	135	56	0	81	0·7	9·3	7·0	–	–
42	Partial gastrectomy	50	M	155	83	0	72	1·2	11·5	11·0	–	–
43	Partial gastrectomy	56	M	205	84	0	121	0·7	13·3	8·4	–	–
44	Partial gastrectomy	68	F	425	170	trace	255	0·7	11·6	0·7		
45	Partial gastrectomy	63	M	255	102	0	153*	0·7	12·3	>32		
47	Partial gastrectomy	63	M	535	289	0	246	1·2	10·0	14·4		
48	Partial gastrectomy	66	F	265	159	0	106*	1·5	10·0	4·6		
49	Partial gastrectomy	59	F	625	375	0	250	1·5	11·6	17·4		
50	Partial gastrectomy	58	M	150	75	0	75	1·0	4·6	0·8	–	–
51	Atrophic gastritis	49	M	170	82	0	86	0·9	12·8	13·0	–	–
52	Atrophic gastritis	73	M	130	52	0	78	0·7	12·8	5·1	–	–
60	Atrophic gastritis	66	F	190	142	0	48*	3·0	10·4	8·0		
61	Atrophic gastritis	74	F	140	101	0	39	2·6	9·0	6·4		
53	Jejunal diverticulosis	38	M	275	170	22	83	2·0	12·0	30		
54	Jejunal diverticulosis	62	M	310	223	0	87	2·6	13·5	3·8		
55	Jejunal diverticulosis	72	F	265	116	0	149	0·8	14·4	13·1		
56	Jejunal diverticulosis	84	F	50	14	0	36	0·4	8·1	15·0	+	+
57	Jejunal diverticulosis: post-gastrectomy	74	M	185	74	0	112*	0·7	14·2	10·2		
58	Crohn's disease	42	M	150	40	0	110*	0·3	12·6	3·0		
59	Adult coeliac disease	53	M	420	252	0	168	1·5	9·9	0·6		

* Derived from visual score.

Leukaemias and liver disease

Table 6 shows results in patients with leukaemias or liver disease, in some of whom the total plasma B_{12} was pathologically high. Two cases of chronic myeloid leukaemia with elevated total B_{12} (Nos. 63 and 64) retained an approximately normal Me B_{12}/Co-OH B_{12} ratio; in Case 63 the level of cyanocobalamin was raised although the *proportion* of this compound was within normal limits. Case 66 (myelomonocytic leukaemia) and Case 67 (erythroleukaemia) had 'borderline' total plasma B_{12} levels and the pattern of individual cobalamins was similar to that in untreated pernicious anaemia. In liver disease, several patients had normal plasma cobalamins, though one case (No. 71) had a subnormal Me B_{12}/Co-OH B_{12} ratio in the presence of a normal total B_{12}. Two cases (Nos. 69 and 76) had a high total B_{12} with a disproportionate increase in Co-OH B_{12}, so that the Me B_{12}/Co-OH B_{12} ratio was subnormal.

In five liver biopsy samples (two alcoholic fatty liver, two cirrhotic and one found to be normal) the mean total vitamin B_{12} content was 520 ng/g wet wt, range 177–1530. In all samples nearly all the B_{12} was in the form of Co-OH B_{12}. Two showed traces (<10 ng/g) of methylcobalamin. No cyanocobalamin was detected.

Folate deficiency

Table 7 shows results and clinical diagnoses in patients suffering from folate deficiency. Some of these patients have also appeared in previous Tables. A variety of patterns of plasma cobalamins occurred. Some patients (Nos. 44 and 71) had a subnormal Me B_{12}/Co-OH B_{12} ratio in the presence of a normal total plasma B_{12}, while in others the proportions of the two major cobalamins were normal. In folate deficiency, in contrast to B_{12} deficiency, there was no consistent change in the proportions of individual cobalamins.

Effects of oral and parenteral administration of cobalamins

Table 8 shows that an increase in plasma cyanocobalamin occurs after administration of this compound by oral or parenteral routes. This increase was detectable after a few days' 'physiological' dosage (1 μg) by mouth (Subjects 32 and 34), showing that such doses are absorbed at least partly unchanged. In Subject 21 the sample may have been taken a little too early for cyanocobalamin to have entered the plasma in appreciable amounts. In Subject 34, a vegan, there was evidence of conversion to methylcobalamin, which was initially undetectable but appeared in the plasma at increasing concentrations at 5 and 8 days; Co-OH B_{12} showed little change. In two cases of vitamin B_{12} deficiency given small doses of methylcobalamin intramuscularly (Nos. 23 and 30), there was a large increase in this compound in the plasma and the Me B_{12}/Co-OH B_{12} ratio was restored to normal.

DISCUSSION

The results obtained with plasma from normal subjects and hospital controls extend those previously reported (Linnell *et al.*, 1969b, c). They show that there may be as many as four different cobalamins in extracts of normal plasma: (1) methylcobalamin, the predominant component, (2) deoxyadenosylcobalamin, (3) hydroxocobalamin, and (4) cyanocobalamin, never present in more than small amounts and often undetectable. The finding that the main plasma cobalamin is methylcobalamin agrees with that of Lindstrand & Ståhlberg (1963). Our finding

TABLE 6. Plasma cobalamins in leukaemias and cases of liver disease

Patient	Diagnosis	Age	Sex	Total B_{12} (pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	Haemoglobin (g/100 ml)	Serum folate (ng/ml)
63	Chronic myeloid leukaemia	50	M	*ca* 2000	*ca* 1600	*ca* 140	*ca* 260	6·1	11·0	—
64	Chronic myeloid leukaemia	58	M	> 3000	> 1800	trace	> 1200*	1·5	7·7	3·6
65	Myelomonocytic leukaemia	57	M	870	678	17	175	3·9	7·0	8·0
66	Myelomonocytic leukaemia	60	F	210	84	0	126*	0·7	5·9	2·1
67	Erythroleukaemia	48	F	185	66	39	80	0·8	6·1	4·4
68	Erythroleukaemia	47	M	385	192	0	193*	1·0	7·9	> 32
69	Active chronic hepatitis	46	F	*ca* 2000	*ca* 960	0	*ca* 1040	0·9	11·4	7·5
70	Cirrhosis, idiopathic	74	M	1140	900	0	240	2·7	13·4	2·6
71	Cirrhosis, alcoholic	38	M	480	120	trace	360*	0·3	18·1	2·3
72	Cirrhosis, alcoholic	70	M	510	255	0	255	1·0	13·2	14·5
73	Cirrhosis, idiopathic	44	F	515	386	0	129*	3·0	13·1	2·4
74	Cirrhosis, alcoholic	58	M	1185	890	0	295	3·0	11·4	6·2
75	Cirrhosis, idiopathic	18	M	> 2000	> 1200	0	> 800*	1·5	11·5	8·8
76	Active chronic hepatitis	26	F	995	458	0	537	0·9	13·3	—

* Derived from visual score.

TABLE 7. Plasma cobalamins in folate deficiency

Patient	Diagnosis	Age	Sex	Total B_{12} pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	Haemoglobin (g/100 ml)	Serum folate (ng/ml)
33	Vegan	24	F	155	118	0	37	3·2	10·8	0·8
78	Nutritional deficiency	56	F	285	196	0	89	2·2	8·3	0·9
79	Nutritional deficiency	65	F	205	110	0	95	1·2	6·9	2·4
80	Nutritional deficiency	77	F	130	65	0	65*	1·0	9·2	0·4
81	Nutritional deficiency	75	F	230	92	0	138	0·7	7·2	0·6
84	Nutritional deficiency	24	M	495	370	0	125	3·0	8·2	0·4
85	Nutritional deficiency	55	F	250	125	0	125*	1·0	6·9	0·6
46	Post-partum megaloblastic anaemia	26	F	500	360	0	140	2·6	12·8	0·8
44	Post-gastrectomy megaloblastic anaemia	68	F	425	170	trace	255*	0·7	16·0	0·7
50	Post-gastrectomy megaloblastic anaemia	58	M	150	105	0	45	2·3	6·5	0·8
59	Adult coeliac disease	53	M	420	252	0	168	1·5	9·9	0·6
82	Carcinoma of stomach	62	M	240	185	0	55	3·4	8·9	0·6
83	Anticonvulsant megaloblastic anaemia	27	M	165	92	0	73	1·3	7·8	1·5
71	Alcoholic hepatic cirrhosis	38	M	480	120	trace	360*	0·3	17·3	2·3

* Derived from visual score.

TABLE 8. Changes in plasma cobalamins after cobalamin administration by oral and parenteral routes

Subject	Diagnosis	Age	Sex	Compound and dose	Route	Time	Total B_{12} (pg/ml)	Me B_{12} (pg/ml)	CN B_{12} (pg/ml)	Co-OH B_{12} (pg/ml)	Ratio Me/Co-OH B_{12}	CN B_{12} percentage of total
N 29	Normal	18	F	Cyanocobalamin 100 μg	i.m.	Pre-dose	510	306	0	204*	1·5	0
						8 h	1165	260	775	130	2·0	67
N 5	Normal	42	M	Cyanocobalamin 100 μg	i.m.	Pre-dose	595	355	0	240	1·5	0
						24 h	895	546	107	242	2·3	12
N 6	Normal	52	F	Cyanocobalamin 100 μg	oral	Pre-dose	490	314	3	173	1·8	<1
						5 h	475	270	43	162	1·7	9
						24 h	470	268	47	155	1·7	19
P 19	Pernicious anaemia	43	M	Cyanocobalamin 1000 μg	i.m.	Pre-dose	35	trace	14	21*	<0·1	40
						3 h	>2000	trace	>1800	>200*	<0·1	ca 90
P 86	Cirrhosis of liver	28	F	Cyanocobalamin 1000 μg	i.m.	7 days	>2000	>200	>1300	>500*	0·4	ca 65
P 87	Pernicious anaemia	67	F	Cyanocobalamin 4 μg	i.m.	6 days	92	18	56	18*	1·0	61
P 21	Pernicious anaemia	63	F	Cyanocobalamin 1 μg+IF	oral	Pre-dose	77	31	0	46*	0·7	0
						6 h	84	34	0	50*	0·7	0
P 34	Vegan	44	F	Cyanocobalamin 1 μg daily	oral	Pre-dose	55	0	0	55	0	0
						5 days	110	25	3	82	0·3	3
						8 days	115	40	7	68	0·6	6
P 32	Vegan	31	F	Cyanocobalamin 1 μg daily	oral	Pre-dose	135	62	0	73	0·9	0
						7 days	205	82	68	55	1·5	33
P 23	Pernicious anaemia	63	F	Methylcobalamin 1 μg daily	i.m.	Pre-dose	49	0	20	29	0	41
						10 days	175	117	0	58*	2·0	0
P 30	Post-gastrectomy with subacute combined degeneration of the cord	61	M	Methylcobalamin 1 μg daily	i.m.	Pre-dose	49	12	0	37*	0·7	0
						14 days	285	250	0	35*	7·2	0
						21 days	320	274	0	46	6·4	0

* Derived from visual score.

that there are appreciable amounts of deoxyadenosylcobalamin in plasma conflicts with Ståhlberg's recent work, since he has now decided (as a result of zone elution and re-chromatography) that the component previously identified as deoxyadenosylcobalamin is in fact entirely hydroxocobalamin (Ståhlberg, 1967). It is extremely difficult to achieve unequivocal identification of the minute quantities of individual cobalamins in the plasma. Ideally the two coenzyme forms should be identified by demonstration of activity in appropriate enzyme systems.

The pathological results show that several kinds of disturbance may occur in the pattern of individual plasma cobalamins, though few of them can yet be adequately explained. A prominent feature of the results is that the fall in the ratio Me B_{12}/Co-OH B_{12} previously described in untreated pernicious anemia (Linnell *et al.*, 1969c) is frequent in any state of B_{12} deficiency, whatever the cause. The same is true of the related finding that whereas methylcobalamin is usually greatly reduced in patients with low total plasma B_{12} levels, Co-OH B_{12} is low but not infrequently within normal limits. The absence of correlation between subnormal plasma methylcobalamin and elevated serum folate in pernicious anaemia, and the fact that increased methylmalonate excretion occurs in patients with plasma levels of deoxyadenosylcobalamin plus hydroxocobalamin which are within normal limits, suggests that there may be little relationship between plasma and tissue levels of the two coenzyme forms. The cause of the fall in the Me B_{12}/Co-OH B_{12} ratio in B_{12} deficiency remains a matter for speculation. Possibly methylcobalamin is the main transport form of the vitamin, as suggested by Hall (1969), or a shift of methylcobalamin from plasma to tissues occurs in B_{12} deficiency.

Apart from the disturbed Me B_{12}/Co-OH B_{12} ratio, nearly half the cases of untreated pernicious anaemia showed a substantial proportion of cyanocobalamin in the plasma, though in absolute terms this compound was within normal limits. This finding, which implies substantial inactivation of plasma B_{12} coenzymes (though not necessarily of tissue B_{12} coenzymes) is also of uncertain significance. Other conditions in which a high proportion of plasma cyanocobalamin has been observed include tobacco amblyopia, Leber's optic atrophy, dominantly inherited optic atrophy and tropical neuropathy associated with consumption of cassava (Linnell *et al.*, 1969d; Linnell & Matthews, 1970; Osuntokun, 1970). In these cases, which are believed to result from increased cyanide exposure or errors in cyanide metabolism, the total plasma vitamin B_{12} is usually normal and the cyanocobalamin content high in absolute terms. Only one of the five patients with intestinal diverticula (Table 5) had an appreciable proportion of plasma cyanocobalamin. This is interesting since it has been suggested (Ellis & Smith, 1967) that large quantities of cyanide may be produced by the abnormal bacterial flora in the blind-loop syndrome.

The occurrence of low Me B_{12}/Co-OH B_{12} ratios in some cases of folate deficiency might be explained by the hypothesis that methylcobalamin is depressed owing to lack of 5-methyltetrahydrofolate as methyl donor in the reaction leading to methylation of homocysteine, in which methylcobalamin is apparently an intermediary (Weissbach & Taylor, 1968). Lindstrand, Anderson, Cowan, Coates & Hoffbrand (1967) found evidence of impaired methylcobalamin synthesis in folate-deficient chicks. However, the low Me B_{12}/Co-OH B_{12} ratios did not occur consistently, and when they were found, methylcobalamin levels were only moderately depressed.

The occurrence of high total plasma vitamin B_{12} levels with normal proportions of methylcobalamin and Co-OH B_{12} in cases of chronic myeloid leukaemia is consistent with increased B_{12}-binding capacity (Herbert, 1968; Chanarin, 1969). The disproportionate increase in Co-

OH B_{12} in active hepatitis (Table 6) may be attributable to loss of deoxyadenosylcobalamin from the liver, in which it is agreed to be the predominant form. Ståhlberg (1964) previously described changes in plasma cobalamins in myeloid leukaemia and liver disease, stressing the intensity of growth in the area of 'fourth factor' (methylcobalamin).

One unusual but particularly interesting pattern occurs in several types of case: this is a reduced ratio of Me B_{12}/Co-OH B_{12} in the presence of normal total plasma vitamin B_{12} levels, sometimes with subnormal levels of methylcobalamin (Cases 43, 44, 45 and 55, Table 5; Case 71, Table 6; Case 81, Table 7). In some of these cases it may indicate incipient B_{12} deficiency.

The preliminary investigations of plasma cobalamins after administration of cyanocobalamin and methylcobalamin show that there are measurable increases in the plasma level of the cobalamin given and, in the case of cyanocobalamin, there is evidence of conversion to methylcobalamin in the body. It has recently been shown that during absorption of cyanocobalamin in the guinea-pig, some enters the blood unchanged but some is converted to deoxyadenosylcobalamin in the intestinal mucosa (Hoffbrand, Linnell, Matthews & Peters, 1970). The present results suggest that in man appreciable amounts of cyanocobalamin are absorbed unchanged over a wide dose range including 'physiological' doses.

The results of this survey help to show that there are many unsolved problems connected with the bodily handling and plasma and membrane transport of individual cobalamins, and it is hoped that further application of the technique described will contribute to their solution.

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REFERENCES

Chanarin, I. (1969) *The Megaloblastic Anaemias*, Blackwell Scientific Publications, Oxford.

Dreyfus, P.M. & Dubé, V.E. (1967) The rapid detection of methylmalonic acid in urine—a sensitive index of vitamin B_{12} deficiency. *Clinica Chimica Acta*, 15, 525–528.

Ellis, F.R. & Smith, A.D.M. (1967) The blind-loop syndrome. *Monographs in the Surgical Sciences*, 4, 193–215.

Hall, C.A. (1969) Transport of vitamin B_{12} in man. *British Journal of Haematology*, 16, 429–433.

Herbert, V. (1968) Diagnostic and prognostic values of measurement of serum vitamin B_{12}-binding proteins. *Blood*, 32, 305–312.

Hoffbrand, A.V., Linnell, J.C., Matthews, D.M. & Peters, T.J. (1970) The forms of vitamin B_{12} in the ileal enterocyte of the guinea-pig during fasting and absorption of cyanocobalamin. *Journal of Physiology*, 208, 66–67P.

Levy, H.L., Mudd, S.H., Schulman, M.D., Dreyfus, P.M. & Abeles, R.H. (1970) A derangement in B_{12} metabolism associated with homocystinemia, cystathioninemia, hypomethioninemia and methylmalonic aciduria. *American Journal of Medicine*, 48, 390–397.

Lindstrand, K. (1964) Isolation of methylcobalamin from natural source material. *Nature (London)*, 204, 188–189.

Lindstrand, K., Andersson, B.B., Cowan, J.D., Coates, M.E. & Hoffbrand, A.V. (1967) The effect of dietary folate deficiency on the synthesis of methylcobalamin in the chick. *Scandinavian Journal of Haematology*, 4, 181–186.

Lindstrand, K. & Ståhlberg, K.-G. (1963) On vitamin B_{12} forms in human plasma. *Acta Medica Scandinavica*, 174, 665–669.

LINDSTRAND, K., WILSON, J. & MATTHEWS, D.M. (1966) Chromatography and microbiological assay of vitamin B_{12} in smokers. *British Medical Journal*, ii, 988–990.

LINNELL, J.C., HOFFBRAND, A.V., PETERS, T.J. & MATTHEWS, D.M. (1969a) Estimation of individual plasma B_{12} compounds in normal subjects and patients with deranged B_{12} metabolism. *Journal of Clinical Pathology*, 22, 742.

LINNELL, J.C., MACKENZIE, H.M. & MATTHEWS, D.M. (1969b) Normal values for individual plasma cobalamins. *Journal of Clinical Pathology*, 22, 506.

LINNELL, J.C., MACKENZIE, H.M., WILSON, J. & MATTHEWS, D.M. (1969c) Patterns of plasma cobalamins in control subjects and in cases of vitamin B_{12} deficiency. *Journal of Clinical Pathology*, 22, 545–550.

LINNELL, J.C. & MATTHEWS, D.M. (1970) Laboratory studies of interrelations between cyanide and vitamin B_{12}. *Proceedings of Wellcome Symposium on Cassava, Cyanide, and Nutritional Neuropathy, October 1969, London* (In press).

LINNELL, J.C., WILSON, J. & MATTHEWS, D.M. (1969d) Estimation of cyanocobalamin and other plasma cobalamins in hereditary optic atrophies and other ophthalmological disorders. *Clinical Science*, 37, 878.

MATTHEWS, D.M., GUNASEGARAM, R. & LINNELL, J.C. (1967) Results with radioisotopic assay of serum B_{12} using serum binding agent. *Journal of Clinical Pathology*, 20, 683–686.

MORROW, G. III, BARNESS, L.A., CARDINALE, G.J., ABELES, R.H. & FLAKS, J.G. (1969) Congenital methylmalonic aciduria: enzymatic evidence for two forms of the disease. *Proceedings of the National Academy of Sciences of the United States of America*, 63, 191–197.

MUDD, S.H., LEVY, H.L. & ABELES, R.H. (1969) A derangement in B_{12} metabolism leading to homocystinemia, cystathioninemia and methylmalonic aciduria. *Biochemical and Biophysical Research Communications*, 35, 121–126.

OSUNTOKUN, B.O. (1970) Chronic cyanide neurotoxicity and neuropathy in Nigerians. *Proceedings of Wellcome Symposium on Cassava, Cyanide and Nutritional Neuropathy, October 1969, London* (In press).

STÅHLBERG, K-G. (1964) Forms of plasma B_{12} in health and in pernicious anaemia; chronic myeloid leukaemia and acute hepatitis. A preliminary report. *Scandinavian Journal of Haematology*, 1, 220–222.

STÅHLBERG, K-G. (1967) Studies on methyl-B_{12} in man. *Scandinavian Journal of Haematology*, Supplementum No. 1.

TOOHEY, J.I. & BARKER, H.A. (1961) Isolation of coenzyme B_{12} from liver. *Journal of Biological Chemistry*, 236, 560–563.

WATERS, A.H. & MOLLIN, D.L. (1961) Studies on the folic acid activity of human serum. *Journal of Clinical Pathology*, 14, 335–344.

WEISSBACH, H. & TAYLOR, R.T. (1968) Metabolic role of vitamin B_{12}. *Vitamins and Hormones*, 26, 395.

Technical methods

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A two-dimensional chromato-bioautographic method for complete separation of individual plasma cobalamins

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In concentrated aqueous solution methylcobalamin, cyanocobalamin, deoxyadenosylcobalamin, and hydroxocobalamin are readily separable by unidirectional chromatography on paper or thin layers of cellulose (Suomela, 1967). No location reagent is required since the cobalamins are visible as red or orange spots.

The total cobalamin concentration of a plasma extract is between 10^{-6} and 10^{-7} that of an aqueous solution used for visible loading of chromatograms. These small amounts lie far below the detection limit of normal chemical methods and necessitate the use of bioautography for location. Lindstrand and Ståhlberg (1963) were the first to carry out paper chromatography and bioautography of plasma cobalamins and more recently a method has been described (Linnell, Mackenzie, and Matthews, 1969b; Linnell, Mackenzie, Wilson, and Matthews, 1969c) which uses thin-layer chromatography and is applicable to small quantities (2 ml of plasma). Neither of these methods will achieve clear separation of the four cobalamins apparently existing in human plasma (methylcobalamin, cyanocobalamin, deoxyadenosylcobalamin, and hydroxocobalamin). Though our method gives good separation of methylcobalamin, cyanocobalamin, and deoxyadenosylcobalamin plus hydroxocobalamin, the latter two compounds do not separate adequately at the low concentrations present in plasma.

A method has now been developed giving complete separation of these four compounds.

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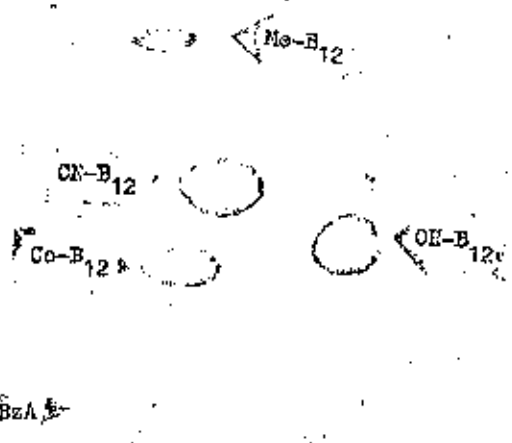

Fig. 1 *Separation and location of cobalamin markers in aqueous solution by two-dimensional chromatography and bioautography. 5-Deoxyadenosylcobalamin is labelled as 'Co-B_{12}'.*

Method

The method of extracting and concentrating plasma cobalamins is as previously described (Linnell *et al.* 1969c). Of the plasma extract, 4 to 1 µl, is applied to a point 2·5 cm from the corner of a 20 cm square plate previously coated with a 3:1 mixture of Whatman CC 41 cellulose and Merck silica gel G. One µl of an aqueous solution containing 50 pg each of the four cobalamins per µl is applied in like manner to a second plate. Cobalamins are separated by ascending chromatography in sec. butanol/ammonia/water (75:2:25) for three hr at 20-23°C. The plates are then removed from this solvent, dried, and re-developed at right angles to the direction of the first separation for 30 min in water saturated with benzyl alcohol at 20-23°. Up to this point, all steps are carried out in darkness or by red photographic 'safelight' in order to prevent the photolytic conversion of other cobalamins to hydroxocobalamin. The plates are finally air-dried in daylight. Bioautograms are prepared using B_{12}-deficient agar medium inoculated with a B_{12}-requiring mutant of *E. coli* and containing a tetrazolium indicator (Linnell *et al.* 1969c).

Results

Figure 1 shows the separation of aqueous cobalamin standards (50 pg of each) by the system described. The first solvent separates methylcobalamin (R_f 0·35) from cyanocobalamin (R_f 0·20) and from 5-deoxyadenosylcobalamin plus hydroxocobalamin (R_f 0·13). In the second solvent a clear separation is achieved between hydroxocobalamin (R_f 0·85) and 5-deoxyadenosylcobalamin (R_f 0·60).

Figure 2 shows a bioautogram obtained from the plasma of a normal healthy subject. An estimate of each cobalamin obtained by densito-

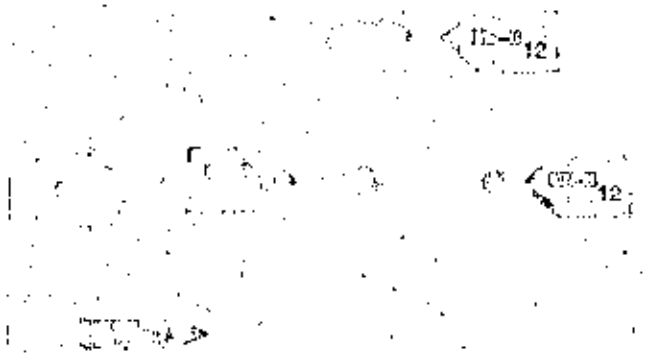

Fig. 2 *Separation and location of cobalamins in extract of plasma from healthy subject. Cyanocobalamin, which is never present in more than trace amounts in normal plasma, and often undetectable, did not appear in this sample.*

metric scanning and comparison with standards similarly run (Linnell, Hoffbrand, Peters, and Matthews, 1971) gave the following result: methylcobalamin 418 pg/ml; cyanocobalamin none detected; deoxyadenosylcobalamin 163 pg/ml; hydroxocobalamin 129 pg/ml. The result in a further normal subject was as follows: methylcobalamin 307 pg/ml; cyanocobalamin ≦ 5 pg/ml; deoxyadenosylcobalamin 162 pg/ml; hydroxocobalamin 51 pg/ml.

Comment

The ability to achieve complete separation of small amounts of the various cobalamins is of obvious utility in the study of B_{12} metabolism. Qualitative and quantitative results obtained by one-dimensional chromatography and bioautography of plasma cobalamins in patients with B_{12} deficiency, known derangements of B_{12} metabolism, and various amblyopias have already been reported (Linnell, Hoffbrand, Peters, and Matthews, 1969a; Linnell, Wilson, and Matthews, 1969d). The technique might be expected to be particularly useful in the study of inborn errors of metabolism involving the coenzyme forms of B_{12} (Morrow, Barness, Cardinale, Abeles, and Flaks, 1969; Levy, Mudd, Schulman, Dreyfus, and Abeles, 1970).

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References

Levy, H. L., Mudd, S. H., Schulman, M. D., Dreyfus, P. M., and Abeles, R. H. (1970). A derangement in B_{12} metabolism associated with homocystinemia, cystathioninemia, hypomethioninemia and methylmalonic aciduria. *Amer. J. Med.*, **48**, 390-397.

Lindstrand, K., and Stahlberg, K.-G. (1963). On vitamin B_{12} forms in human plasma. *Acta med. scand.*, **174**, 605-609.

Linnell, J. C., Hoffbrand, A. V., Peters, T. J., and Matthews, D. M. (1969a). Estimation of individual plasma B_{12} compounds in normal subjects and patients with deranged B_{12} metabolism. *J. clin. Path.*, **22**, 742.

Linnell, J. C., Hoffbrand, A. V., Peters, T. J., and Matthews, D. M. (1971). Chromatographic and bioautographic estimation of plasma cobalamins in various disturbances of vitamin B_{12} metabolism. *Clin. Sci.* In press.

Linnell, J. C., Mackenzie, H. M., and Matthews, D. M. (1969b). Normal values for individual plasma cobalamins. *J. clin. Path.*, **22**, 506.

Linnell, J. C., Mackenzie, H. M., Wilson, J., and Matthews, D. M. (1969c). Patterns of plasma cobalamins in control subjects and in cases of vitamin B_{12} deficiency. *J. clin. Path.*, **22**, 545-550.

Linnell, J. C., Wilson, J., and Matthews, D. M. (1969d). Estimation of cyanocobalamin and other plasma cobalamins in hereditary optic atrophies and other ophthalmological disorders. *Clin. Sci.*, **37**, 878.

Morrow, G. III, Barness, L. A., Cardinale, G. J., Abeles, R. H., and Flaks, J. G. (1969). Congenital methylmalonic aciduria: enzymatic evidence for two forms of the disease. *Proc. nat. Acad. Sci. (Wash.)*, **63**, 191-197.

Suomela, H. (1967). A thin layer chromatographic method for separating Vit. B_{12} analogues. *Scand. J. clin. Lab. Invest.*, Suppl. 95, 35.

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Patterns of plasma cobalamins in control subjects and in cases of vitamin B_{12} deficiency

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SYNOPSIS A method for thin-layer chromatography and bioautography of plasma cobalamins is described. It requires only 5 ml of blood.

Results are reported in 35 healthy people and hospital controls, and in 14 cases of untreated pernicious anaemia. In the control group there were two major components, methylcobalamin and a component which is probably a mixture of hydroxocobalamin and deoxyadenosyl cobalamin. Many cases of untreated pernicious anaemia showed a characteristic pattern in which methylcobalamin was reduced in relation to hydroxocobalamin. This pattern was also seen in one vegan who was taking a diet without B_{12} supplement. A minority of subjects showed traces of cyanocobalamin; this compound was not confined to smokers.

The significance of the results and possible diagnostic utility of the technique are discussed.

For some years after the isolation of vitamin B_{12} as cyanocobalamin in 1948, it was assumed that the 'serum vitamin B_{12}', estimated by the usual microbiological methods, was either cyanocobalamin or a mixture of cyanocobalamin and hydroxocobalamin (Wokes and Picard, 1955; Matthews, 1961) though the demonstration of a coenzyme form of vitamin B_{12} in human and animal liver (Toohey and Barker, 1961) raised the possibility that this might also occur in the blood. In 1963, Lindstrand and Ståhlberg showed for the first time, by chromatography and bioautography, that human plasma contained several different microbiologically active cobalamins. These were provisionally identified as methylcobalamin, hydroxocobalamin, cyanocobalamin, and deoxyadenosyl cobalamin (Lindstrand and Ståhlberg, 1963; Lindstrand, 1964; Ståhlberg, 1964). This and more recent work has identified the major component as methylcobalamin with a high degree of certainty, but suggests that the component originally thought to be deoxyadenosyl cobalamin (coenzyme B_{12}) may in fact be largely or entirely hydroxocobalamin (Ståhlberg, 1967).

It is clearly desirable to know as much as possible about the normal proportions of the compounds making up 'serum B_{12}', and how they vary in disease. It is quite possible that a normal figure for total serum B_{12} might conceal some imbalance in individual plasma cobalamins. It might also mask a deficiency of physiologically active cobalamins, owing to their conversion to cyanocobalamin, which is without coenzyme function and is readily excreted—a state of affairs which might lead to haematological and neurological disorders. The hypothesis that an excessive load of cyanide, which may result from heavy smoking, the consumption of cassava, certain infections, and possibly from an error in cyanide metabolism, may inactivate vitamin B_{12} in this way is now suggested by more than one line of evidence (Wilson and Matthews, 1966; Linnell, Smith, Smith, Wilson, and Matthews, 1968). It has recently been referred to by Lester Smith (1968) as the hypothesis of 'trapped B_{12}'.

The ability to determine patterns of plasma cobalamins reasonably readily might lead to significant advances in understanding of B_{12} metabolism and in haematological and neurological diagnosis. The reason why such determinations have not become generally possible is basically technical, and the difficulties are contributed to by the very large volume of blood (at least 40 ml) required for the method of Lindstrand and Ståhlberg. This paper describes a chromatographic and bioautographic technique using thin-layer chromatography and requiring only 5 ml of blood. While we do not claim that the method is easy, it is more convenient than

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that of Lindstrand and Stahlberg (1963) and enables up to 20 analyses to be carried out per week. The results obtained in normal subjects, hospital control patients, and patients with B_{12} deficiency are reported.

MATERIALS AND METHODS

Control blood samples were taken from healthy laboratory staff and hospital inpatients without anaemia.

Total plasma B_{12} was estimated by radioisotopic assay (Matthews, Gunasegaram, and Linnell, 1967).

CHROMATOGRAPHIC PROCEDURE Venous blood (5 to 10 ml) is taken in a foil-wrapped heparinized container. Plasma is separated within 30 min by red darkroom light, and stored deep-frozen until analysis.

The procedures to be described are carried out by darkroom light or in total darkness when possible.

Plasma (2 ml) is mixed with absolute ethanol (8 ml) and heated at 80°C for 20 min, cooled in ice, and filtered on a sintered glass filter (grade 3). The protein precipitate is washed with cold ethanol and the filtrate transferred to a rotary evaporator (Buchi Rotavapor-R). The temperature of the water bath is slowly raised from 25°C to 40°C as ethanol distils. The aqueous residue is thrice washed with ether and evaporated to dryness. The residue is redissolved in water (2 ml) and phenol containing 25% water (8 ml) and placed in a stoppered tube. The aqueous phase is removed and the lower phenol layer washed with water (three 2 ml aliquots). Acetone (7 ml) and ether (21 ml) are added to the phenol and shaken with water (1 ml) to extract the cobalamins. The aqueous extract is then washed with ether and desalted for 10 min in an ion-exchange cell (Baird and Tatlock chromatographic desalting apparatus). Finally, the desalted extract is evaporated to dryness.

Thin-layer plates are prepared by mixing powdered cellulose (Whatman Chromedia CC 41) (15 g) with silica gel (Merck silica gel G) (5 g) and slurried in water (45 ml). A layer 0·25 mm thick is then applied to glass plates and the adsorbent dried in the oven at 100°C for 30 minutes.

The plasma extract is redissolved in water (25 μl) and applied to a thin-layer plate (1 and 4 μl loadings normally, 2 and 6 μl if plasma B_{12} is low) together with markers in aqueous solution (hydroxocobalamin, cyanocobalamin, deoxyadenosyl cobalamin, and methylcobalamin; 50 μμg of each in 1 μl). The chromatogram is developed for three hours at 21°C in sec-butanol/0·880 ammonia/water (75:2:25), removed from the solvent and dried. Subsequent steps are carried out by daylight.

BIOAUTOGRAPHIC PROCEDURE A 20 cm square of Whatman no. 1 filter paper is laid on the previously prepared bioautography plate and a 20 cm square of agar cut out. Agar and paper are lifted together and placed on the chromatogram, agar side uppermost. Air bubbles are displaced and the 'sandwich' is incubated at 37°C overnight.

A trace amount of cobalamin promotes growth of an *E. coli* mutant, which is indicated by conversion of a tetrazolium salt to a coloured form. Red zones appear on the bioautograph plate, in positions corresponding to the zones separated by chromatography.

Preparation of seeded agar for bioautography A standard blood agar plate is inoculated with *E. coli* NCIB-9270 and incubated at 37°C overnight. The growth is subcultured in peptone water (2 ml), incubated for six to eight hours, and used as the inoculum.

The agar medium is made up in two parts and stored separately:

Agar Davis New Zealand Agar (3 g) in water (100 ml) is autoclaved and stored at room temperature.

Medium	Dipotassium hydrogen phosphate ..	1·4 g
	Potassium dihydrogen phosphate ..	0·6 g
	Sodium citrate	0·1 g
	Magnesium sulphate heptahydrate	0·02 g
	Ammonium sulphate	0·2 g
	Sodium chloride	0·01 g
	Distilled water to	100 ml

The medium is filtered, the *p*H adjusted to 7·0, then autoclaved and stored at room temperature.

The agar is melted on a steam bath while glucose (0·6 g) and 2, 3, 5 triphenyltetrazolium chloride (0·03 g) are dissolved in the medium. A bioautography plate (constructed from two plates of glass 25 cm square separated by a Perspex frame 1 cm thick) is cleaned by wiping with ethanol and flaming. The melted agar is poured into the medium, mixed, and cooled to 43°C. The inoculum is added and the seeded agar poured onto the bioautography plate set on a level bench.

SPECIAL PRECAUTIONS Methylcobalamin, cyanocobalamin, and deoxyadenosyl cobalamin are all converted to hydroxocobalamin on exposure to light. For this reason, samples must be protected from light both before and during analysis to prevent alteration of the chromatographic pattern. Syringes and blood containers are wrapped in aluminium foil, while all steps in the extraction and chromatography procedures are carried out either in darkness or by the light of a darkroom lamp (Paterson Safelight).

To prevent contamination of bioautographs, all concentrated cobalamin solutions are prepared in a laboratory remote from that used for the extraction of plasma and chromatography.

RECORDING RESULTS A 'plus' system has been used to record chromatographic results, each growth area being assigned from 0 to 3 — signs. Scoring is carried out according to the area and intensity of each spot *in relation to the total growth on the chromatogram*, so that the score given to an individual spot is related to the *proportion* of the total it represents, not to absolute amount. Where possible, chromatograms were scored 'blind', without knowledge of whether the sample was normal or otherwise.

RESULTS AND DISCUSSION

Though cobalamins are readily separated by paper or thin-layer chromatography, appearing when

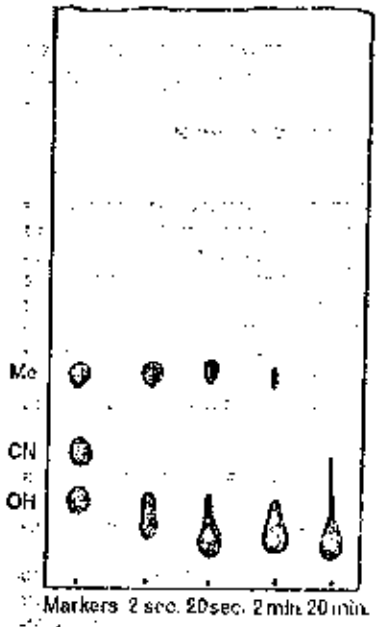

FIG. 1.

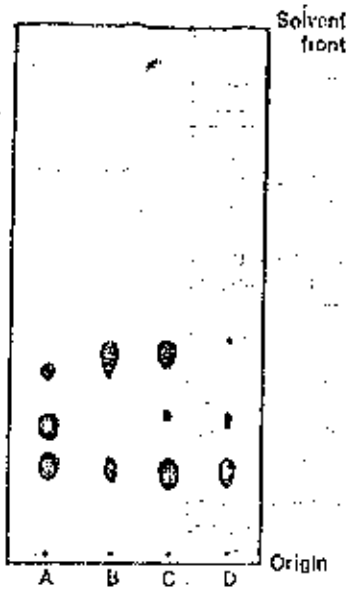

FIG. 2.

FIG. 1. *Photolysis of methylcobalamin in normal plasma exposed to daylight for 20 minutes. At the end of the period all the methylcobalamin has been converted to hydroxocobalamin.*

FIG. 2. *Results of chromatography and bioautography of plasma cobalamins. A Markers (methylcobalamin, cyanocobalamin and hydroxocobalamin); B normal subject; C normal subject, showing trace of cyanocobalamin; D case of untreated pernicious anaemia, showing great reduction in methylcobalamin and a small amount of cyanocobalamin*

present in high concentrations as red or orange spots, their concentrations in plasma are so low that the resulting spots can only be made visible by the technique of bioautography. The present method has certain advantages over the paper chromatographic technique (Lindstrand and Ståhlberg, 1963; Ståhlberg, 1967) apart from the much smaller quantity of blood required. It is simpler to manipulate thin-layer plates, and layer them evenly with bioautographic media than to deal with large (22 × 18 in.) paper sheets. The use of staining to make growth zones more clearly visible makes it easy to identify very small amounts, and to make a photographic record. The definition of growth zones is good provided loading is not excessively heavy, and 'streaking' is minimal or absent. The important cyanocobalamin spot is well separated and can be clearly seen without prior removal of hydroxocobalamin, which was necessary with the paper technique (Lindstrand, Wilson, and Matthews, 1966).

Though the present chromatographic system separates methylcobalamin, cyanocobalamin, hydroxocobalamin, and deoxyadenosyl cobalamin satisfactorily from aqueous solutions containing high concentrations of these compounds, adequate separation of deoxyadenosyl cobalamin and hydroxocobalamin is not usually achieved when these two compounds are added to plasma in low concentrations (1,000 $\mu\mu$g per ml or less) both compounds tending to be represented by a single zone of growth. The work of Ståhlberg (1967) suggests that there is little or no deoxyadenosyl cobalamin in plasma, in which case this growth area will normally represent hydroxocobalamin. We are not, however, satisfied that this is so, and for this reason the area is referred to below as 'hydroxocobalamin' in inverted commas.

The great importance of avoiding even short periods of exposure to light is illustrated in Figure 1. This shows that two minutes' exposure of plasma to daylight will convert nearly all its methylcobalamin, normally a major component, to hydroxocobalamin. Brief exposure of whole blood to light, on the other hand, has little effect.

The results obtained in healthy people and in hospital controls (Fig. 2, Tables I and III) were reasonably similar, and suggest that in the majority of cases, at least half the plasma B_{12} is in the form of methylcobalamin. In about half the individuals in the pooled control group (normals and hospital patients) the methylcobalamin growth zone was approximately equal to that of 'hydroxocobalamin'. In about one third, the methylcobalamin zone was definitely predominant. In a minority, 'hydroxocobalamin' was the predominant zone. A zone with the R_F of cyanocobalamin appeared inconstantly, and when present was always small. The presence of this component was *not* confined to smokers. It tended to appear more frequently in them (50%) than in non-smokers (24%) but the difference was not statistically significant ($\chi^2 = 2{\cdot}56$; $P > 0{\cdot}083$).

TABLE I

RESULTS IN NORMAL SUBJECTS AND HOSPITAL CONTROLS

	Age	Sex	Total Plasma B_{12} (μg/ml)	Smoking Habits (cigarettes/day)	Plasma Cobalamins OH-B_{12}	CN-B_{12}	Me-B_{12}
Normal Subjects							
1	34	M	470	Nil	+++	0	+++
2	32	M	505	Nil	+++	0	+++
3	22	F	380	Nil	+++	0	+++
4	35	M	625	Nil	+++	0	++
5	21	F	250	Nil	+++	±	+++
6	21	M	270	Nil	++	+	+++
7	25	F	450	Nil	++	0	+++
8	24	F	375	Nil	++	0	+++
9	27	M	345	Nil	++	0	+++
10	30	M	415	Nil	+	0	+++
11	30	F	375	Nil	++	+	+++
12	23	F	—	Nil	++	0	+++
13	26	F	490	Nil	+++	0	++
14	30	M	510	Nil	+++	0	+++
15	40	M	550	40/day	+++	0	+++
16	26	F	415	10/day	+++	+	++
17	47	M	350	10/day	+++	+	++
18	32	M	—	15/day	++	0	+++
19	52	M	380	20/day	+++	+	++
20	26	F	265	10/day	+++	0	+++
Hospital Controls							
1	48	F	—	Nil	+++	0	+++
2	55	F	415	Nil	+++	0	+++
3	62	M	365	Nil	++	0	+++
4	78	M	—	Nil	+++	+	++
5	60	F	540	Nil	+++	+	+++
6	43	M	265	Nil	+++	0	+++
7	40	F	320	Nil	+++	0	+++
8	63	M	575	10/day	+++	±	++
9	65	F	525	5/day	+++	0	+++
10	42	M	430	5/day	++	0	+++
11	58	F	650	10/day	+++	+	+++
12	44	M	455	15/day	+++	±	+++
13	65	M	615	5/day	+++	±	+++
14	67	M	530	30/day	+++	0	+++
15	59	M	285	5/day	+	0	+++

TABLE II

RESULTS IN PATIENTS WITH PERNICIOUS ANAEMIA AND IN VEGANS

	Diagnosis	Age	Sex	Total Plasma B_{12} (μg/ml)	Hb (g/100 ml)	Serum Folate (mg/ml)	Smoking Habits (cigarettes/day)	Plasma Cobalamins OH-B_{12}	CN-B_{12}	Me-B_{12}
Patients										
1	Untreated pernicious anaemia	71	F	75	13·8	21	Nil	+++	0	0
2	Untreated pernicious anaemia	77	F	68	8·7	8·0	—	+++	0	0
3	Untreated pernicious anaemia	85	F	65	10·4	3·0	Nil	+++	0	+
4	Untreated pernicious anaemia	67	F	150	6·2	1·2	Nil	+++	+	+
5	Untreated pernicious anaemia	75	F	50	7·3	4·2	10/day	+++	0	+
6	Untreated pernicious anaemia	65	F	30	4·9	6·4	Nil	+++	0	0
7	Subacute combined degeneration of the cord	69	F	30	12·5	—	Nil	+++	++	++
8	Untreated pernicious anaemia	80	F	65	7·8	—	—	+++	0	++
9	Untreated pernicious anaemia	71	F	35	7·4	—	Nil	+++	0	++
10	Untreated pernicious anaemia	63	F	75	7·7	4·7	—	+++	0	±
11	Subacute combined degeneration of the cord	68	F	50	7·8	11·0	Nil	+++	+	±
12	Untreated pernicious anaemia	46	F	100	11·5	8·4	8/day	+++	++	++
13	Untreated pernicious anaemia	62	F	80	5·0	2·9	Nil	+++	++	+
14	Untreated pernicious anaemia	71	F	80	7·7	>25	Nil	+++	+	+
Pernicious Anaemia Cases										
15	One hr after 1 mg CN-B_{12}	—	F	>1,000	8·0	2·0	—	++	+++	±
16	Six days after 4 mg CN-B_{12}	67	F	>1,000	11·4	6·8	—	+	+++	+
Vegans										
17	B_{12}-supplemented diet	33	M	380	16·0	>32	Nil	+++	0	+++
18	B_{12}-supplemented diet	31	F	300	13·6	>16	Nil	++	±	+++
19	B_{12}-supplemented diet	64	M	455	13·6	14	Nil	++	0	+++
20	Diet not B_{12}-supplemented	46	M	100	11·0	>25	Nil	+++	0	0

TABLE III

SUMMARY OF PATTERNS OF PLASMA COBALAMINS IN CONTROLS AND UNTREATED PERNICIOUS ANAEMIA

	Normals and Hospital Controls ($n = 35$)	(%)	*Untreated Pernicious Anaemia* ($n = 14$)	(%)
Me-B_{12} > OH-B_{12}	11	32	0	0
Me-B_{12} = OH-B_{12}	17	48	0	0
Me-B_{12} < OH-B_{12}	7	20	14	100
CN-B_{12} present	12	34	6	43

Preliminary experiments on quantitation of the individual cobalamins, by elution of areas located with adjacent markers, gave the following results in three healthy people: subject 1, total plasma B_{12} 470 $\mu\mu$g per ml, methylcobalamin 57%, 'hydroxocobalamin' 43%; subject 2, total B_{12} 620 $\mu\mu$g per ml, methylcobalamin 70%, 'hydroxocobalamin' 30%; subject 3, total B_{12} 350 $\mu\mu$g per ml, methylcobalamin 55%, 'hydroxocobalamin' 45%. No cyanocobalamin was present in any of these samples. The quantitative results were in general agreement with visual impressions.[1]

The pattern of plasma cobalamins in most cases of untreated pernicious anaemia (Fig. 2, Tables II and III) was quite abnormal, so much so that such cases could usually be picked out without any knowledge of the total plasma B_{12} or reference to the absolute intensity of the zones of growth. In the majority of cases, the approximate equality between methylcobalamin and 'hydroxocobalamin' found in controls was lost, and 'hydroxocobalamin' was clearly the predominant component. In three of 14 cases (21%) methylcobalamin was undetectable, and in most of the others present only in traces. Only four cases (29%) had a pattern approximating to normal, and in all these, methylcobalamin was weaker than 'hydroxocobalamin'—a pattern which occurs in only a small proportion of control subjects. As in the control group, the cyanocobalamin growth zone was present in some cases (six, or 43%) of untreated pernicious anaemia. In all of these cases, this zone was as strong as, or stronger than, that of methylcobalamin. It is interesting to compare two subjects in the 'low normal' range of total serum B_{12}. Subject 5 (Table I), a normal subject with a total serum B_{12} concentration of 250 $\mu\mu$g per ml, had roughly equal plasma concentrations of methylcobalamin and 'hydroxocobalamin'. Subject 4 (Table II), a case of pernicious anaemia with a total serum B_{12} concentration of 150 $\mu\mu$g per ml, which falls at the lower limit of the normal range, had a clearly abnormal pattern of plasma cobalamins in which the proportion of methylcobalamin was unusually small. Thus estimation of individual plasma cobalamins might be useful in the differentiation of healthy subjects with relatively low serum vitamin B_{12} from cases of incipient B_{12} deficiency.

Table II shows two cases of pernicious anaemia after treatment with intramuscular cyanocobalamin. In patient 15, one hour after treatment, the bulk of the plasma B_{12} was in the form of cyanocobalamin, large amounts of this component being superimposed on the characteristic pernicious anaemia pattern. In patient 16, six days after treatment, much of the plasma B_{12} still remained in this form. This is in keeping with other observations showing that conversion of cyanocobalamin to physiologically active forms is a relatively slow process (Reizenstein, 1967; Yagiri, 1967).

The pattern of plasma cobalamins in one untreated vegan, subject 20, who had avoided B_{12} supplementation of his diet but remained in reasonable health, was similar to that in untreated pernicious anaemia. In three vegans taking a B_{12}-supplemented diet (nos. 17 to 19) the pattern was normal.

In some important respects, the present results agree with those of Lindstrand and Ståhlberg. They confirm the finding that in control subjects a major plasma B_{12} component appears to be methylcobalamin, and that the second major component has an R_F similar to that of hydroxocobalamin, though we are not yet certain whether or not this growth zone represents deoxyadenosyl cobalamin (coenzyme B_{12}) in addition. The observation that at least half the plasma B_{12} is usually in the form of methylcobalamin is borne out by the preliminary quantitative estimations. With respect to cyanocobalamin, the results differ. In their earliest report, Lindstrand and Ståhlberg (1963) found a component with the R_F of cyanocobalamin in all eight people studied. In a second paper reporting results in 20 normal samples, the presence of this compound was not mentioned (Ståhlberg, 1964). In a more recent, and very detailed, report (Ståhlberg, 1967) plasma from 18 healthy people was examined and cyanocobalamin found in none; this paper refers to cyanocobalamin as an artefact with no metabolic function. On the other hand, we have found small amounts of a component with the R_F of cyanocobalamin in a substantial proportion (34%) of 35 control subjects, including some non-smokers. It is known that cyanide participates in normal metabolic processes (Boxer and Rickards, 1952; Wilson and Matthews, 1966), and the cyanide pool is in equilibrium with thiocyanate, much of which, in non-smokers, is derived from foodstuffs. The formation of cyanocobalamin from other forms of vitamin B_{12} may be

[1] A larger series of quantitative values has recently been reported (Linnell, J. C., Mackenzie, H. M., and Matthews, D. M., 1969), *J. clin. Path.*, 22, 506.

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part of one of the metabolic pathways normally available to the cyanide radicle.

In pernicious anaemia, the two sets of results are totally different. Ståhlberg (1964) found that in untreated pernicious anaemia the plasma 'fourth factor' (methylcobalamin) tended to persist while the other component or components tended to disappear and were sometimes absent. A similar disturbance in the ratio of cobalamins was subsequently found in some samples of B_{12}-deficient liver, and led to the suggestion that in B_{12} deficiency, B_{12} might be blocked in the methyl form owing to a disturbance in demethylation (Ståhlberg, Radner, and Nordén, 1967). In contrast, we find that plasma methylcobalamin is severely reduced while the other major component 'hydroxocobalamin' tends to persist. We have at present no explanation for this discrepancy.

We are indebted to Dr L. Mervyn of Glaxo Laboratories for gifts of methylcobalamin, deoxyadenosyl cobalamin, and hydroxocobalamin, specially purified for use as chromatographic markers, and are also grateful to Mr S. Vincent of Glaxo Laboratories for advice concerning the bioautographic method and for the gift of a culture of *E. coli* NCIB 9270. Samples of blood from vegans were kindly supplied by Dr F. R. Ellis and Mr A. Kurtha. The work was supported by a grant from the Wellcome Trust.

REFERENCES

Boxer, G. E., and Rickards, J. C. (1952). *Arch. Biochem.*, **39**, 7.
Lindstrand, K. (1964). *Nature (Lond.)*, **204**, 188.
——, and Ståhlberg, K-G. (1963). *Acta med. scand.*, **174**, 665.
——, Wilson, J., and Matthews, D. M. (1966). *Brit. med. J.*, **2**, 988.
Linnell, J. C., Smith, A. D. M., Smith, C. L., Wilson, J., and Matthews, D. M. (1968). *Brit. med. J.*, **2**, 215.
——, Mackenzie, H. M., and Matthews, D. M. (1969). *J. clin. Path.*, **22**, 506.
Matthews, D. M. (1961). *Lancet*, **1**, 1289.
——, Gunasegaram, R., and Linnell, J. C. (1967). *J. clin. Path.*, **20**, 683.
Reizenstein, P. (1967). *Blood*, **29**, 454.
Smith, E. Lester (1968). *Plant Fdn. hum. Nutr.*, **1**, 7.
Ståhlberg, K-G. (1964). *Scand. J. haemat.*, **1**, 220.
——, (1961). *Ibid*, Suppl. 1.
——, Radner, S., and Nordén, A. (1967). *Ibid*, **4**, 312.
Tomkey, J. I. and Barker, H. A. (1961). *J. biol. Chem.*, **236**, 560.
Wilson, J. and Matthews, D. M. (1966). *Clin. Sci.*, **31**, 1.
Wokes, F. and Picard, C. W. (1955). *Amer. J. clin. Nutr.*, **3**, 383.
Yagiri, Y. (1967). *J. Vitaminol.*, **13**, 228.

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Vitamin B_{12} excretion by the rat small intestine

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SUMMARY Two phases of intestinal excretion are demonstrated after a parenteral dose of ^{60}Co vitamin B_{12}. The first phase was maximal immediately after injection, and was related to plasma radioactivity. The second phase was delayed 36 to 48 hours and was related to labelled cells reaching the tips of the villi. The loss of vitamin B_{12} through increased exudation and rapid cell exfoliation together with failure of reabsorption may play a part in the deficiency state seen in the [illegible] syndrome.

It has been established that a proportion of a parenteral dose of vitamin B_{12} is excreted in the faeces both in experimental animals (Okuda, Gräsbeck, and Chow, 1958; Willigan, Cronkite, Meyer, and Noto, 1958; Gräsbeck, Rinneberg, and Simons, 1959; Booth and Spray, 1962) and in man (Gräsbeck, Nyberg, and Reizenstein, 1958; Adams, 1963). Although part of this excretion takes place in the bile, animals still excrete a proportion of an injected dose after ligation of the bile duct (Okuda *et al*, 1958) suggesting that the small intestinal mucosa itself plays a part in vitamin B_{12} elimination. In a previous series of experiments (Loehry, Croft, Singh, and Creamer, 1968) we have demonstrated how iron injected parenterally may be excreted into the small intestine by two pathways. Plasma iron appears to pass directly into the lumen while iron is also taken up by the newly formed crypt epithelial cells and lost with them into the intestinal lumen after migration up to the tips of the villi. It seemed possible that the rapid cell turnover in the small intestine might be responsible for the faecal loss of a number of substances, and in the present study we have investigated the role of the small intestinal mucosa in the excretion of vitamin B_{12}.

METHODS

Male albino rats, weighing 380 to 450 g, were used for the experiments; 3 μc (1 μg) of ^{60}Co vitamin B_{12} was injected intravenously or intraperitoneally and small intestinal perfusion was performed at varying time intervals after injection. A method of perfusion was used that has been previously described (Loehry *et al*, 1968) whereby a constant number of naturally desquamated epithelial cells was collected without contamination from gastric contents or bile. Perfusions were performed at the following time intervals after injection of the isotope: 10 hours, 24 hours, 48 hours, 72 hours, 96 hours, and [illegible] hours. Three rats were perfused at each of these times and the perfusion was maintained for three to four hours for each rat. After each experiment the rat was killed, the small intestine removed, washed, and fixed in 10% formalin. The radioactivity of the hourly perfusates and of the intestines was counted in a large well-type scintillation counter.

In the experiments where radioactivity was related to epithelial cells, the number of cells collected was varied by altering the rate of perfusion for each hour; this has been shown to produce a varying number of cells in the collected fluid proportional directly to the rate of perfusion (Loehry *et al*, 1968). The number of cells collected was estimated by the chemical DNA content of the hourly specimens (Croft and Lubran, 1966).

RESULTS

In the perfusions immediately after intravenous injection of ^{60}Co vitamin B_{12} a peak radioactivity appeared in intestinal perfusions in the first hour which fell rapidly over the next three hours; a second peak was seen at the 36- to 48-hour period after injection (Fig. 1). The difference between the radioactivity in all specimens at the 36- and 48-hour period and that at the 12- and 24-hour period was statistically highly significant ($P < 0.001$). Radioactivity in the intestinal wall rose to a maximum at 12 hours and then gradually fell until at 120 hours less than 0.5% of the administered dose was present in the small intestinal wall (Fig. 2).

In order to assess whether the radioactivity present in the perfusions was related to desquamated epithelial cells a number of perfusions were performed immediately after injection and at the 36- to 48-hour period, with a varied hourly perfusion rate thus collecting a different number of cells at each

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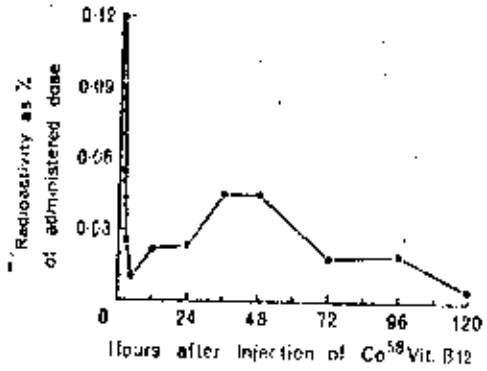

FIG. 1. *A graph showing the radioactivity in perfusion specimens at various times after parenteral administration of ^{58}Co vitamin B_{12}.*

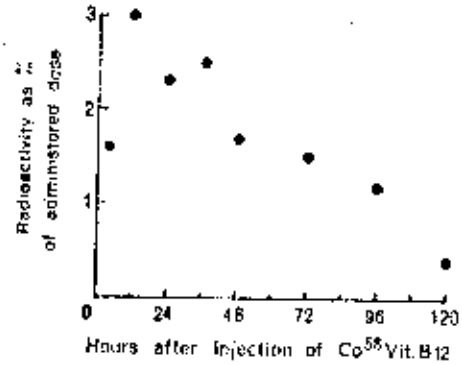

FIG. 2. *A graph showing the radioactivity in the small intestine removed at various times after the parenteral administration of ^{58}Co vitamin B_{12}.*

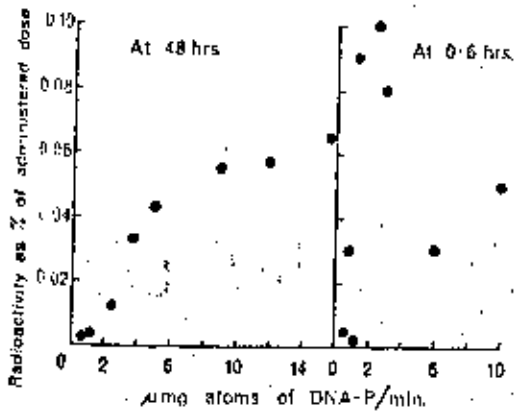

FIG. 3. *The cell in perfusion specimens, measured as DNA, plotted against radioactivity at 48 hours after injection of ^{58}Co vitamin B_{12}, on the right at 0 to 6 hours after injection and on the left at 48 hours. Note the absence of any relationship at 0 to 6 hours as opposed to 48 hours where there is a clear relationship.*

hour. At the 36- to 48-hour period there was a direct relationship between DNA and radioactivity implying that the radioactivity came from intracellular ^{58}Co vitamin B_{12} (Fig. 3). At the early period immediately after injection it was clear that the radioactivity was not related to desquamated cells and was probably due to plasma exudation (Fig. 3).

DISCUSSION

The experiments with ^{58}Co vitamin B_{12} demonstrate two phases in the excretion of the isotope into the intestinal lumen. The first phase was maximal soon after injection, was unrelated to cell loss, and fell rapidly to a baseline after three to four hours. This period represented the time that ^{58}Co vitamin B_{12} was present in the plasma in large amounts (Mollin, Pitney, Baker, and Bradley, 1956) and the intestinal loss was probably a reflection of plasma vitamin B_{12} exuding into the lumen. The second peak at 36 to 48 hours after injection correlated with cell desquamation and this suggested that the newly formed crypt cells took up the isotope maximally in the period shortly after injection, and these labelled cells then migrated up the tips of the villi where they were shed into the lumen. A similar pattern has been demonstrated after parenteral administration of ^{59}Fe (Loehry *et al*, 1968) and it seems possible that several substances may be taken up into the newly formed crypt cells with their high mitotic activity, remain in them as they mature and migrate up the villi, and are finally lost with them from the extrusion zones into the intestinal lumen.

It has been estimated that in man approximately 60,000 × 10^6 small intestinal epithelial cells are extruded each day (Croft, unpublished observations). Iron and vitamin B_{12} have now been shown to be lost with them and it is possible that other substances such as protein, fats, and folic acid are similarly affected. Clearly in health the normal mucosa recovers much of this loss by reabsorption, and it has been demonstrated that the net faecal loss of vitamin B_{12} in man, after exclusion of bile is small (Adams, 1963). There are, however, situations where this loss may be of clinical significance. If the mucosa of the small intestine is in an increased turnover state a greater volume of cells will be lost and there is evidence that this situation occurs in the coeliac syndrome (Croft, Loehry, and Cremer, 1968). Here also the mucosa is abnormal and may be unable to absorb not only ingested substances but also those desquamated into the intestinal lumen. Perhaps in this state of 'exfoliative enteropathy' the small intestinal loss of vitamin B_{12} may reach significant proportions.

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REFERENCES

Adams, J. F. (1963). The biliary and faecal loss after parenteral ^{60}Co vitamin B_{12} in man. *Scot. med. J.*, 8, 118-121.

Booth, M. A., and Spray, G. H. (1962). The excretion and distribution in the tissues of parenterally administered ^{60}Co vitamin B_{12} in normal and gastrectomized rats. *Brit. J. Haemat.*, 8, 114-119.

Croft, D. N., and Lubran, M. (1965). The estimation of deoxyribonucleic acid in the presence of sialic acid: application to analysis of human gastric washings. *Biochem. J.*, 95, 612-620.

——, Lochry, C. A., and Creamer, B. (1968). Small-bowel cell loss and weight-loss in the coeliac syndrome. *Lancet*, 2, 68-70.

Grasbeck, R., Nyberg, W., and Reizenstein, P. (1958). Biliary and fecal vitamin B_{12} excretion in man. An isotope study. *Proc. Soc. exp. Biol. (N.Y.)*, 99, 780-784.

——, Runeberg, L., and Simons, K. (1959). Intrinsic factor and radio-vitamin B_{12} excretion in rats. *Acta physiol. scand.*, 43, 370-374.

Lochry, C. A., Croft, D. N., Singh, A. K., and Creamer, B. (1968). Cell turnover in the rat small intestinal mucosa: An appreciation of cell loss. *Gut*, 10, 13-18.

Mollin, D. L., Pitney, W. R., Baker, S. J., and Bradley, J. E. (1958). The plasma clearance and urinary excretion of parenterally administered ^{60}Co B_{12}. *Blood*, 11, 31-43.

Okuda, K., Grasbeck, R., and Chow, B. F. (1958). Bile and vitamin B_{12} absorption. *J. Lab. clin. Med.*, 51, 17-23.

Wolfgan, D. A., Cronkite, E. P., Meyer, L. M., and Soto, S. F. (1958). Biliary excretion of Co^{60} labeled vitamin B_{12} in man. *Proc. Soc. exp. Biol. (N.Y.)*, 97, 81-84.

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Effect of Feeding "Carbohydrate-free" Diets on the Chick's Requirement for Vitamin B-12 [1,2,3]

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ABSTRACT Experiments were conducted to study the effect of fat on the chick's requirement for vitamin B-12 when fat was substituted isocalorically for carbohydrate in a semipurified diet in which protein was supplied by isolated soybean protein and in which methionine was supplied in limited amounts. Using growth as the criterion, the chick's requirement for vitamin B-12 was shown to increase when hydrogenated fat was substituted isocalorically for glucose. Pair-feeding studies showed that vitamin B-12 stimulated growth of chicks fed either carbohydrate-containing or "carbohydrate-free" diets by stimulating appetite and not by increasing utilization of either protein or energy. J. Nutr. *104*: 394–399, 1974.

INDEXING KEY WORDS vitamin B-12 · fat · nutrient requirement · "carbohydrate-free" · chick

Recent studies have shown that the chick has the ability to utilize large quantities of fat when nutrient balance is maintained (1–4). In these studies excesses of the B vitamins were added in order to avoid the possibility of deficiencies since little information was available on the effect on nutrient requirements of substituting fat isocalorically for carbohydrate. Fox et al. (5), March and Biely (6, 7) and Siedler and Schweigert (8) conducted studies to determine the effect of fat on nutrient requirements when fat was substituted weight for weight for carbohydrate; however, these studies are difficult to interpret since chicks automatically reduce their consumption of these high fat diets due to their greater caloric density, thus reducing the intake of not only the nutrient under study but also others which may affect the requirement.

The following experiments were conducted to determine the effect of fat on the chick's requirement for vitamin B-12 when fat was substituted isocalorically for carbohydrate in a semipurified diet formulated to be low in one-carbon metabolites by supplying methionine in limited amounts. Concurrently, studies were conducted to determine whether vitamin B-12 increased growth by stimulating appetite or by increasing the utilization of protein and/or energy.

PROCEDURES

Three experiments were conducted in which duplicate groups of 10 male day-old crossbred (Dominant White × White Plymouth Rock) chicks were fed ad libitum "carbohydrate-free" and carbohydrate-containing diets in which nonprotein energy was supplied by hydrogenated fat or glucose, respectively, and to which 0, 2.5, 5.0, 10.0, 50.0 or 100.0 μg vitamin B-12 per kilogram diet was added. In addition in experiments 1 and 2, the response to vitamin B-12 was determined by two pair-fed comparisons: one compared the carbohydrate-free diets with and without supplemental vitamin B-12; the second was a similar comparison of the carbohydrate-containing

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diet with and without vitamin B-12. The pair-fed chicks received their daily allotment of feed once every 24 hours.

The semipurified diets used in all experiments contained the constant dietary ingredients shown in table 1. Carbohydrate-containing diets were formulated by adding glucose to the constant dietary ingredients. Their "carbohydrate-free" counterparts were formulated by replacing glucose isocaloriically by hydrogenated fat using the values 3.64 and 8.59 kcal/g for the metabolizable energy of glucose and hydrogenated fat, respectively. Cellulose was added to improve the texture of the "carbohydrate-free" diets. Because the "carbohydrate-free" diets do not total to 100 their content of methionine and vitamin B-12 will be referred to as the level present in the carbohydrate-containing diets from which they were derived. In experiments 1 and 2 the level of supplemental methionine was 0.1%, while in experiment 3 the level of supplemental methionine was 0.074%. All diets were formulated on a dry matter basis to contain 15.0 kcal/g protein and to have similar energy: vitamin and energy: mineral ratios.

For the first week, the chicks were fed the "carbohydrate-free" diet containing 0.1% supplemental methionine and no added vitamin B-12. They were then assigned to the experimental groups on the basis of body weight, equalizing both mean body weight and weight distribution among the groups and fed the experimental diets to 29 days of age. The chicks were housed in electrically heated, thermostatically controlled battery brooders with raised wire-screen floors in a temperature-controlled laboratory. Data on growth and feed consumption were obtained weekly and feed wastage was determined daily.

During the last week of experiment 1, excreta were collected at 24-hour intervals on three successive days from chicks fed diets containing no added vitamin B-12 and from those being pair-fed the vitamin B-12-supplemented diet. The methods for processing excreta, conducting chemical analyses for moisture, nitrogen, combustible energy and chromium oxide and computing metabolizable energy from these data have been described previously (9, 10).

At the termination of experiments 1 and 2 selected groups of chicks were killed with chloroform. After cooling, the contents of the gastrointestinal tract were removed, and the entire carcasses from each experimental group were frozen, ground, mixed and an aliquot dried by lyophilization. In order that tissue gains could be determined, a representative group of chicks was killed at the beginning of these experiments and prepared for analysis using the same procedure. Carcass samples were analyzed for protein, fat and moisture as described by Hill and Anderson (9).

TABLE 1

Composition of basal diets[1]

Ingredients	Carbohydrate-containing[2]	"Carbohydrate-free"
	g	g
Variables		
Glucose[3]	64.36	—
Hydrogenated fat[4]	—	27.27
Cellulose[5]	—	6.39
Constants		
Soybean protein[6]	25.00	25.00
DL-Methionine[7]	0.10	0.10
L-Cystine	0.25	0.25
Soybean oil	4.00	4.00
Limestone	1.49	1.49
Dicalcium phosphate	2.15	2.15
Sodium chloride	0.60	0.60
Mineral mixture[8]	1.26	1.26
Vitamin mixture[9]	0.43	0.43
Chromium oxide	0.30	0.30
Antioxidant[10]	0.025	0.025
Total	100.00	69.3

[1] Composition is expressed on a dry matter basis. One hundred grams dry matter of the carbohydrate-containing diet and 69.3 g dry matter of the "carbohydrate-free" diet were calculated to supply 364 kcal, 24.3 g protein (N × 6.25), 0.40 g methionine and 0.35 g cystine. [2] Control. [3] Cerelose. [4] Crisco. Composition in g/100 g: $C_{14:0}$, 0.2; $C_{16:0}$, 13.0; $C_{18:0}$, 12.0; $C_{18:1}$, 44.0; $C_{18:2}$, 27.0; other polyunsaturated fatty acids, 1.8 (Benfield et al., 1962). [5] Solka-Floc S.W.-40-A, Brown Forest Products, Limited, Montreal, Quebec. [6] Prolsoy, Central Soya Chemical Division, Chicago, Ill. 60639. [7] Supplied at 0.1% level in experiments 1 and 2 and 0.074% in experiment 3. [8] Supplied in mg/364 kcal of ration: KH_2PO_4, 900; $MgSO_4$, 275; KI, 0.29; $FeSO_4 \cdot 7H_2O$, 25; $CuSO_4 \cdot 5H_2O$, 1.56; $ZnCO_3$, 11.50; $CoCl_2 \cdot 6H_2O$, 0.17; $NaMoO_4 \cdot 2H_2O$, 0.53; Na_2SeO_3, 0.22; and $MnSO_4 \cdot H_2O$, 34. [9] Supplied per 364 kcal of ration: thiamin, 1.0 mg; riboflavin, 1.0 mg; Ca pantothenate, 3.0 mg; biotin, 0.01 mg; pyridoxine, 2.0 mg; niacin, 8.0 mg; folic acid, 0.3 mg; menadione, 0.3 mg; vitamin palmitate, 1000 IU; cholecalciferol, 450 ICU; α-tocopheryl acetate, 3.5 IU; choline chloride, 1.0 mg and choline chloride, 145 mg. [10] Contents 25% ethoxyquin, Monsanto Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

Data showing average weight gains and energetic efficiencies of chicks fed diets containing graded levels of vitamin B-12 in

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TABLE 2

Weight gain and energetic efficiency of chicks fed either carbohydrate-containing or "carbohydrate-free" diets with graded levels of vitamin B-12

Supp. vit. B-12	Exp. no.	Weight gain: Carbohydrate: Containing	Weight gain: Carbohydrate: "Free"	Energy consumption: Carbohydrate: Containing	Energy consumption: Carbohydrate: "Free"	Energetic efficiency: Carbohydrate: Containing	Energetic efficiency: Carbohydrate: "Free"
μg/kg		*g*	*g*	*kcal*	*kcal*	*kcal/g gain*	
0	1	258[a]	271	1727	1767	6.68	6.52
	2	284	291	1819	1798	6.54	6.47
	3	174	167	1364	1249	7.82	7.45
		248[a]	243[a]	1647[a,b]	1605[a]	7.01[a]	6.71[a]
2.5	1	284	298	1773	1811	6.24	6.09
	2	305	301	1916	1796	6.29	5.90
	3	170	252	1251	1519	7.38	6.84
		253[a,b]	275[b,c,d]	1647[a,b]	1710[a,b,c]	6.64[a]	6.28[a]
5.0	1	284	316	1826	1884	6.42	5.96
	2	291	310	1882	1822	6.47	5.86
	3	191	200	1364	1384	7.15	6.94
		255[a,b,c]	275[b,c,d]	1691[a,b]	1697[a,b]	6.68[a]	6.25[b,c]
10.0	1	313	304	1913	1828	6.14	6.01
	2	318	313	1968	1770	6.18	5.65
	3	213	234	1456	1532	6.84	6.54
		281[b,c,d]	284[c,d]	1770[a,b,c]	1710[a,b,c]	6.30[a]	6.07[b]
50.0	1	306	359	1960	2082	6.22	5.80
	2	340	360	1992	2026	5.87	5.52
	3	233	266	1553	1584	6.80	5.96
		293[d]	331[c,d]	1823[b,c,d]	1897[c,d]	6.30[a]	5.76[a]
100.0	1	332	337	2053	1893	6.18	5.62
	2	371	384	2221	2146	5.98	5.58
	3	215	290	1506	1815	6.99	6.28
		306[d,e]	337[d]	1927[c,d]	1951[d]	6.38[a]	5.83[a]

[1] Values are averages of duplicate groups. Underlined values are averages of triplicate experiments. Values without a common letter in their superscript are significantly different ($P < 0.05$).

which nonprotein energy was supplied by either glucose or hydrogenated fat are summarized in table 2. Analysis of variance and application of Duncan's multiple range test (11) to the growth data obtained in the three experiments showed that chick growth was increased significantly ($P < 0.01$) by the addition of vitamin B-12. The results show that the addition of 10 μg of vitamin B-12 per kilogram of diet caused a significant increase ($P < 0.05$) in growth of chicks fed the carbohydrate-containing diet with no further significant increase being observed on the addition of higher levels of vitamin B-12. In the case of chicks fed the "carbohydrate-free" diet in which nonprotein energy was provided by hydrogenated fat, the addition of 2.5 μg of vitamin B-12 per kilogram of diet caused a significant increase with a still further significant increase being observed on the addition of 50 μg vitamin B-12 per kilogram of diet. Plots of weight gains versus level of supplemental vitamin B-12 expressed as $\log_{10}$ (10 + μg vitamin B-12 per kilogram) using regression analysis showed that linear ascending and plateau lines intersected at 5 μg/kg of carbohydrate-containing diet and at 31 μg/kg of "carbohydrate-free" diet. Thus, in these experiments the vitamin B-12 requirement of chicks fed carbohydrate-containing and "carbohydrate-free" diets was 5 and 31 μg/kg of diet, respectively. The vitamin B-12 requirement of chicks fed the carbohydrate-containing diet is in agreement with the finding of Ott et al. (12) and Fox et al. (5) although somewhat lower than the requirement as stated by NRC (13). The finding that the isocaloric substitution of fat for carbohydrate increased the vitamin B-12 requirement is in agreement with Fox et al. (5). They observed that the vitamin B-12 requirement of chicks increased 10- to 20-fold when 20% lard was substituted for an equal amount of corn in a corn-

TABLE 3

Weight gain, energy consumption and energetic efficiency of chicks fed diets with and without supplemental vitamin B-12 in which nonprotein energy was supplied by glucose or hydrogenated fat

Treatment		Weight gain		Energy consumption		Energetic efficiency	
		Carbohydrate		Carbohydrate		Carbohydrate	
Supplemental vitamin B-12	Feeding regimen	Containing	"Free"	Containing	"Free"	Containing	"Free"
μg/kg		g	g	kcal	kcal	kcal/g gain	
0	ad libitum	258[a]	271	1728	1767	6.68	6.52
		283	291	1849	1798	6.54	6.17
		270[a]	281[a]	1788[a]	1782[a]	6.61[a]	6.34[b,a]
100	pair-fed	265	282	1760	1776	6.63	6.29
		293	299	1872	1795	6.40	6.01
		279[a]	290[a]	1816[a]	1786[a]	6.52[a]	6.15[b]
100	ad libitum	332	337	2053	2146	6.18	5.62
		371	384	2221	1893	5.98	5.58
		352[b]	360[a]	2137[a]	2020[b]	6.08[b]	5.60[a]

[1] Values are averages of duplicate groups. Underlined values are averages of duplicate experiments. Values without a common letter in their superscript are significantly different ($P < 0.05$).

soybean oil meal diet. This they suggested might be due to a possible role of vitamin B-12 in fat metabolism or to an indirect effect mediated through some other nutrient as fat was not substituted isocalorically for corn. Since in the present study the isocaloric substitution of fat for carbohydrate increased the requirement for vitamin B-12 it can be concluded that the increase in vitamin B-12 requirement observed by Fox et al. (5) was due at least in part to fat and not merely to reduced intakes of one-carbon metabolites, their precursors or nutrients involved in their synthesis. These results implicate vitamin B-12 in fat metabolism.

Statistical analysis of the growth data obtained in the three experiments also showed that growth was affected significantly by both experiment ($P < 0.01$) and source of energy ($P < 0.01$); however, the interaction between level of vitamin B-12 and experiment was not significant. The significantly slower growth of chicks in experiment 3 may be due at least in part to the reduction of level of supplemental methionine from 0.1% to 0.074%. The finding that chicks fed the "carbohydrate-free" diets grew faster than chicks receiving the carbohydrate-containing diets without increasing caloric consumption suggests that fat has a methionine-sparing action. Recently Nakano and Ashida (14) have shown that the protein-sparing action of both fat and carbohydrate is closely related to their inhibiting effect on the induction of amino acid-degrading enzymes. They emphasized that the activity of all the hepatic amino acid-degrading enzymes in rats previously adapted to a high fat diet was much less than that in rats previously fed a high carbohydrate diet. In parallel, the amount of urinary nitrogen output was found to be less in rats fed the high fat diet. Their results suggest that dietary fat improves protein retention through its inhibiting effect on the induction of hepatic amino acid-degrading enzymes. In the present study, chicks fed the "carbohydrate-free" diets should have been well adapted to the high fat diet, since the chick embryo develops on a medium containing only 1% carbohydrate, and after hatching, they were fed the "carbohydrate-free" diet until allotted to the experimental groups. Whether the greater growth of chicks receiving the "carbohydrate-free" diets was a reflection of a similar amino acid-sparing action of fat as in the case of rats is unknown.

Similar statistical treatment of the data on energetic efficiency lends support to the finding that the isocaloric substitution of fat for carbohydrate increases the chick's requirement for vitamin B-12. The data (table 2) show that the vitamin B-12 requirement of chicks fed carbohydrate-containing and "carbohydrate-free" diets was

TABLE 4

Protein gained, fat gained, protein retained and energetic efficiency of chicks fed carbohydrate-containing and "carbohydrate-free" diets with and without vitamin B-12

Treatment		Protein gained		Fat gained		Protein retained[1]		Energetic efficiency[2]	
Supplemental vitamin B-12	Feeding regimen	Carbohydrate		Carbohydrate		Carbohydrate		Carbohydrate	
		Containing	"Free"	Containing	"Free"	Containing	"Free"	Containing	"Free"
μg/kg		g	g	g	g	%	%		
0	ad libitum	54.7[a]	50.5	25.3	20.1	38.7	36.6	4.15	4.02
		54.6	51.8	25.1	33.1	41.6	39.6	4.34	3.65
		54.2[a]	51.2[a]	25.1[a]	31.2[a]	39.2[a]	38.1[a]	4.24[a]	3.74[a]
100	pair-fed	54.6	54.0	23.5	34.5	45.4	40.3	4.12	3.50
		55.9	54.8	25.4	31.8	43.3	40.9	4.30	3.30
		54.8[a]	53.9[a]	24.0[a,b]	34.0[a]	40.9[a]	40.6[a]	4.11[a]	3.43[a]

[1] (Gain in excess protein, g/protein consumed, g) × 100. [2] Kilocalories of metabolizable energy consumed/kilocalories gained.
[3] Values are averages of duplicate groups. Underlined values are averages of duplicate experiments. Values without a common letter in their superscript are significantly different ($P < 0.05$).

5.0 to 10 and 10 to 50 μg/kg, respectively, when energetic efficiency was used as the criterion of adequacy.

Summarized in table 3 are data showing weight gains, energy consumption and energetic efficiencies of chicks pair-fed diets with and without supplemental vitamin B-12 in which nonprotein energy was supplied by either glucose or hydrogenated fat. Analysis of variance and application of Duncan's multiple range test (11) to the combined data from the two experiments showed that when chicks were pair-fed, the addition of vitamin B-12 to either the carbohydrate-containing or "carbohydrate-free" diets had no effect on growth or energetic efficiency. In comparison chicks fed ad libitum the vitamin B-12-supplemented diets consumed more feed, grew faster and utilized their feed more efficiently than chicks fed ad libitum the unsupplemented diet, irrespective of source of nonprotein energy.

Data showing protein gained, fat gained, protein retained and energetic efficiency of chicks fed diets with and without supplemental vitamin B-12 are summarized in table 4. Analysis of variance and application of Duncan's multiple range test (11) to the data showed that when chicks were pair-fed, supplementation with vitamin B-12 did not alter body composition. The data show that chicks fed the vitamin B-12-deficient diets utilized both energy and protein as efficiently as chicks fed the vitamin B-12-supplemented diets. Thus, it can be concluded that vitamin B-12 stimulated growth under ad libitum feeding by increasing appetite rather than by improving protein utilization or caloric efficiency. Previously, Chow and Barrows (15) observed that vitamin B-12 stimulated growth of rats only when fed ad libitum. They found that vitamin B-12 failed to stimulate growth when feed intake was restricted to 6 to 8 g per day.

The reason that appetite is depressed when chicks are fed vitamin B-12-deficient diets is unknown. Blaxter (16) generalizes that, in specific dietary deficiencies, animals reduce the load on the enzyme system affected by the deficiency. He states that the primary method animals employ is reducing food intake, but the enzyme system affected by the deficiency also may be avoided by using alternative metabolic pathways of dissimilation and synthesis, some of which are not as efficient in the transfer of energy. Results of the experiments reported herein showed that chicks fed vitamin B-12-deficient diets reduced feed intake. If the chicks used alternative metabolic pathways, then these pathways must have been as efficient since no significant difference in energetic efficiency was observed on supplementation with vitamin B-12 when feed intakes were equalized.

The data summarized in table 4 also show that irrespective of whether the diet was adequate or deficient in vitamin B-12, the substitution of hydrogenated fat isocaloric ally for carbohydrate increased the amount of energy deposited as fat and im-

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proved efficiency of the utilization of energy. These results are in agreement with those reported by Forbes et al. (17) and Carew and Hill (18) which showed that rats and chicks, respectively, utilized energy from fat more efficiently than energy from carbohydrate when fat was incorporated in the diet at a level of 20%. Recently, Baldwin (19) calculated the theoretical efficiency of utilization of energy (100 × net energy/metabolizable energy) from glucose and triglyceride for fattening to be 89 and 96 to 97%, respectively. Blaxter (20) summarized data on the efficiencies of utilization of metabolizable energy provided above maintenance for fattening in adult animals. Values for glucose were about 70% and for fat ranged from 80 to 86%. Although Baldwin's calculated efficiencies are higher than determined efficiencies, they do show that dietary fat can be converted to body fat more efficiently than can dietary carbohydrate. Results of these experiments show that chicks like other animals utilize dietary fat more efficiently than dietary carbohydrate for fattening.

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LITERATURE CITED

1. Renner, R. (1964) Factors affecting the utilization of "carbohydrate-free" diets by the chick. I. Level of protein. J. Nutr. *84*, 322–326.
2. Brambila, S. & Hill, F. W. (1966) Comparison of neutral fat and free fatty acids in high lipid–low carbohydrate diets for the growing chicken. J. Nutr. *89*, 84–92.
3. Bond, N. T., Scott, H. M. & Kummerow, F. A. (1958) Dietary fat in the nutrition of the growing chick. Poultry Sci. *37*, 1075–1085.
4. Donaldson, W. E., Combs, G. F., Romoser, G. L. & Supplee, W. C. (1957) Studies on energy levels in poultry rations. 2. Tolerance of growing chicks to dietary fat. Poultry Sci. *36*, 807–813.
5. Fox, M. R. S., Ortiz, L. O. & Briggs, G. M. (1956) Effects of dietary fat on requirement of vitamin B_{12} by the chick. Proc. Soc. Exp. Biol. Med. *93*, 501–504.
6. March, B. & Biely, J. (1956) Folic acid supplementation of high protein–high fat diets. Poultry Sci. *35*, 550–551.
7. March, B. & Biely, J. (1956) Fat studies in poultry. 5. The effect of dietary fat level on the choline requirement of the chick. Poultry Sci. *35*, 545–549.
8. Siedler, A. J. & Schweigert, B. S. (1953) Effect of feeding graded levels of fat with and without choline and antibiotic on B_{12} supplements to chicks. Poultry Sci. *32*, 449–454.
9. Hill, F. W. & Anderson, D. L. (1958) Comparison of metabolizable energy and productive energy determinations with chicks. J. Nutr. *64*, 587–603.
10. Hill, F. W., Anderson, D. L., Renner, R. & Carew, L. B., Jr. (1960) Studies of the metabolizable energy of grain and grain products for chickens. Poultry Sci. *39*, 573–583.
11. Steel, R. G. D. & Torrie, J. H. (1960) Principles and Procedures of Statistics with Special Reference to the Biological Sciences. McGraw-Hill Book Co., New York.
12. Ott, W. H., Rickes, E. L. & Wood, T. R. (1948) Activity of crystalline vitamin B_{12} for chick growth. J. Biol. Chem. *174*, 1047–1052.
13. National Research Council, Subcommittee on Poultry Nutrition (1971) Nutrient requirements of poultry, publ. 1345, National Academy of Sciences, National Research Council, Washington, D. C. 20025.
14. Nakano, K. & Ashida, K. (1970) Effect of dietary carbohydrate and fat on amino acid-degrading enzymes in relation to their protein-sparing action. J. Nutr. *100*, 208–218.
15. Chow, B. F. & Barrows, L. (1959) Role of B_{12} in nitrogen retention of rats fed on soybean diets at different calorie levels. Federation Proc. *9*, 354–355.
16. Blaxter, K. L. (1964) Dietary factors affecting energy utilization. Proc. Nutr. Soc. *23*, 3–11.
17. Forbes, E. B., Swift, R. W., Elliott, R. F. & James, W. H. (1946) Relation of fat to economy of food utilization. I. By the growing albino rat. J. Nutr. *31*, 203–227.
18. Carew, L. B., Jr. & Hill, F. W. (1958) Studies of the effect of fat on metabolic efficiency of energy utilization. Poultry Sci. *37*, 1191.
19. Baldwin, R. L. (1970) Metabolic function affecting the contribution of adipose tissue to total energy expenditure. Federation Proc. *29*, 1277–1283.
20. Blaxter, K. L. (1962) The Energy Metabolism of Ruminants. Hutchinson, London.

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Placental Transfer and Biological Half-life of Radioactive Vit. B_{12} in the Dog.* (24576)

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Little is known about placental transfer of Vit. B_{12} in humans. In the rat, Chow *et al.* studied placental transfer using radioactive $Co^{60}B_{12}$ as tracer. They found that after subcutaneous injection into pregnant rats, 12% of 200 mμg dose(1) and in later experiments, 60% of 10 μμg dose(2) was transferred to the litter. Rice *et al.*(3) also reported on placental transfer of $Co^{60}B_{12}$ in the rat, showing average of 2.3% of 3 μg orally administered dose given to pregnant rats, was transferred to the fetuses. We investigated the placental transfer and biological half-life of $Co^{60}B_{12}$ in the dog, during studies of the physiology of Vit. B_{12} in human maternal and fetal nutrition. The dog was chosen in preliminary investigation of certain technical problems anticipated in the study of human maternal-fetal Vit. B_{12} metabolism. Some of these results are reported herein.

Methods and materials. A 16 lb. bitch of mixed breed was given subcutaneous dose of 9.6 μc Vit $Co^{60}B_{12}$ equivalent to 9.6 μg Vit. B_{12}, 5 months prior to mating and approximately 7 months prior to delivery of her litter. She nursed the litter of 4 pups until weaned at 4 weeks of age. Uptake and retention of radioactivity was studied by external scanning over the hepatic area. A scintillation probe-type counter employing 1" x 1" NaI thallium-activated crystal was used for external radioactivity scanning. Hepatic surface radioactivity in counts/min. (CPM) was determined 3 times a week on each dog. "Shielded" background radioactivity was determined by scanning the left lower leg. Absolute radioactivity (body cpm – shielded background cpm) were corrected for physical decay of Co^{60}. The mother and pup No. 1 were followed by surface scanning of radioactivity for 6 weeks post partum. At this time they were sacrificed and certain organs obtained. The tissues were cleaned, homogenized in Waring blender with measured amounts of water, and 3 weighed aliquots of each homogenate counted in duplicate for radioactivity in well-type NaI scintillation counter. Radioactivity counts were equated to microcuries of Vit. $Co^{60}B_{12}$ by comparison with known standard $Co^{60}B_{12}$ solution counted under identical conditions. Pups No. 2 and 3 were given small parenteral "calibration" doses of $Co^{60}B_{12}$, 4 weeks after birth. By determining increase in external hepatic area counts due to "calibration" dose, it was possible to estimate(4) the amount of Vit. $Co^{60}B_{12}$ transferred placentally. Pup No. 4 was continued on routine diet until all externally detectable radioactivity disappeared.

Results. Placental transfer of $Co^{60}B_{12}$ estimated from hepatic radioactivity. Data for calculation of amount of Vit. B_{12} transferred placentally as determined by external hepatic area radioactivity scanning technic, are presented in Table I. The 2 small physiologic doses of 0.0385 and 0.116 μc $Co^{60}B_{12}$ (μc = μg) were assumed to be completely retained. From the increment in cpm over the hepatic area after the calibration dose, it was calculated that millicuries/cpm were 0.208 and 0.172 for pup Nos. 2 and 3, respectively. Therefore, counts of 390 and 400 at birth represent 0.081 and 0.069 μg $Co^{60}B_{12}$ transferred placentally. Thus 3-4% of initial subcutaneous dose given to the bitch was transferred to the 4 pups.

Tissue distribution of placentally transferred $Co^{60}B_{12}$. Distribution of $Co^{60}B_{12}$ in certain major organs of the bitch and pup No. 1 are presented in Fig. 1. In tissues studied, concentration (μμg/g) in the bitch was highest in the kidney followed in order by spleen, pancreas, liver and small intestine. However, by far the greatest total amount of radioactivity resided in the liver because of its much greater weight. Similarly, in the pup, the kidney contained the highest concentration of $Co^{60}B_{12}$ although the liver con-

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TABLE I. Placental Transfer of $Co^{60}B_{12}$ in the Dog, Calculated from external hepatic area Radioactivity (EHAR) scanning.

	EHAR at birth (avg cpm)	"Calibration" dose (μc $Co^{60}B_{12}$)	Increment of EHAR (avg cpm)	mμc/cpm	μg $Co^{60}B_{12}$ placentally transferred
Pup 2	300	.0385	155	.208	.081
3	400	.110	670	.172	.069

tained the largest amount of this vitamin. The proportional distribution of $Co^{60}B_{12}$ among liver, kidneys and pancreas for both dogs was similar.

Placental transfer from tissue distribution of $Co^{60}B_{12}$. The above characteristic permits calculation of placentally transferred $Co^{60}B_{12}$ by comparison of individual organ content of $Co^{60}B_{12}$ in the mother dog with that in her pups. Such data are presented in Table II, from which it is evident that an average of 5.1% of radioactive $Co^{60}B_{12}$ *retained* by the mother was placentally transferred to the pup. Since externally detectable radioactivity over the hepatic area at birth was similar for the other 3 pups, it is reasonable to assume that approximately 20% of mothers B_{12} goes to the entire litter.

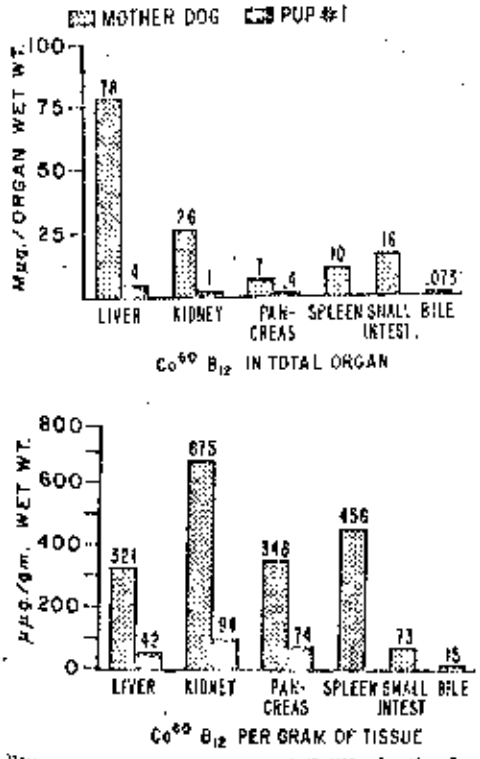

FIG. 1. Tissue distribution of $Co^{60}B_{12}$ in the dog: Comparison of mother dog with that placentally transferred to her pup.

Biological half-life of $Co^{60}B_{12}$. Biological elimination of radioactive $Co^{60}B_{12}$ was studied in all dogs, by following disappearance rate of externally detectable radioactivity over the hepatic area, measured in carefully standardized manner so that the relative geometry was always essentially the same. The percentage of counts over the hepatic area decreased in all pups and the bitch at a similar rate, regardless of number of counts in the particular animal. The time for radioactivity to decrease by half varied from 48 to 70 days (Fig. 2). This indicates a biological half-life for Vit. B_{12} of 1.6 to 2.3 months in the post partum adult dog and pups.

Discussion. Two techniques were employed for study of placental transfer of $Co^{60}B_{12}$ in the dog: (a) calibration of externally detectable hepatic radioactivity in the newborn animal, and (b) comparison of organ content of $Co^{60}B_{12}$ in mother and pup. The dog lends itself well to the first method because of its relatively large size. In this respect, the technical situation is akin to that in human newborns.

Determination of extent of placental transfer by comparison of $Co^{60}B_{12}$ content of similar organs in mother and newborn is a novel method, capable of great accuracy, and is

TABLE II. Placental Transfer of $Co^{60}B_{12}$ in Dog. Calculated from comparison of individual organ tissue content of $Co^{60}B_{12}$ in mother dog and her pup.

Organ	mμg $Co^{60}B_{12}$ Mother	Pup	Total	% of total $Co^{60}B_{12}$ transferred to pup
Liver	77.8	4.3	82.1	5.23
Kidney	96	1.35	97.3	4.6
Pancreas	7.3	.4	7.7	5.2
				5.1 Avg

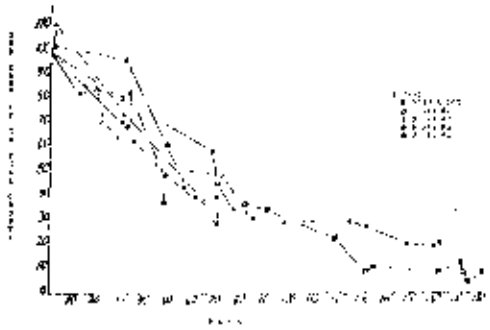

FIG. 2. Biological half-life of Co^{60} B_{12} in post partum dog and puppies.

especially useful in large animal species where there is difficulty in determining B_{12} content of the entire carcass. For this comparison to be a true reflection of physiologic placental transfer, it is essential that Vit. B_{12} be administered and distributed in the mother's organs before gestation begins. From our data in humans(5,6) it appears that administration of B_{12} during pregnancy floods the blood stream and a considerable portion of these newly introduced B_{12} molecules are taken up by the placenta and fetus.

It is significant that concentration of Co^{60} B_{12} in liver, kidney and pancreas of the newborn pup was ⅓ to ¼ that of corresponding maternal tissue (Fig. 1).

From data of Ross and Mollin(7) on total Vit. B_{12} content of liver in human infants, it is apparent that concentration of B_{12} in the liver of human newborns is in the same proportional range to adult liver as we found in the dog. The finding that maternal plasma Vit. B_{12} level falls progressively throughout pregnancy, reaching subnormal levels at term, and plasma Vit. B_{12} level of the newborn is very high, approaching 2 to 5 times that of the corresponding maternal value(8) has been explained by many workers(2,3,9) as indicating that the fetus parasitizes and depletes the maternal stores of Vit. B_{12}. The recently reported increased gastrointestinal absorption of Vit. B_{12} during normal pregnancy(2,3) also supports the latter inference. However, huge unphysiologic doses of Vit. B_{12} were used in some of the latter experiments, and it is questionable whether such data reflect the status of absorption of dietary amounts of B_{12}.

An alternate explanation for the physiology of maternal-fetal Vit. B_{12} interrelationships may be suggested from our data. The progressively decreasing Vit. B_{12} plasma level during pregnancy may be explained by our previously published finding(5,6) that (a) gastrointestinal absorption of dietary amounts of Vit. B_{12} is diminished in the last trimester of pregnancy, and that (b) a large part of Vit. B_{12} introduced into the maternal blood stream during latter part of pregnancy (whether after gastrointestinal absorption or following parenteral administration) is selectively distributed to the placenta and fetus in addition to the maternal tissues. The fetal parasitization of the mother may thus be limited to Vit. B_{12} in the mother's "mobilizable stores" which in turn is possibly highly dependent upon her dietary intake rather than her tissue stores which may be tightly bound.

The high Vit. B_{12} levels in the newborn, on the other hand, do not appear to reflect increased accumulation of Vit. B_{12} in fetal tissues. An explanation of these high fetal levels is not forthcoming from currently available data, but it is probable that this phenomenon is due to a characteristic of placental B_{12} transfer system. Such a mechanism explains the maintenance of a similar maternal fetal plasma gradient for riboflavin(10).

The biological half-life of Vit. B_{12} in the dog is surprisingly short in comparison to that in humans. The dog may thus be a good experimental subject for study of factors involved in biological turnover of Vit. B_{12}.

Summary. Placental transfer of radioactive Vit. B_{12} in the dog following pregestational administration, was found in the entire litter to be 20% of the residual radioactive vitamin in the bitch at time of delivery. Because the proportional distribution of radioactive B_{12} among major organs of the mother and pups was found to be similar, it was possible to calculate placental transfer from this characteristic as well as by postpartum "calibration" of the hepatic radioactivity in pups. Biological half-life of the radioactive Vit. B_{12} averaged 2 months in pups and bitch during postpartum period. A new explanation is suggested for the low maternal and high fetal plasma levels of Vit. B_{12}.

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1. Chow, B. F., Barrows, L., Ling, C. T., *Arch. Biochem. & Biophys.*, 1951, v34, 151.

2. Hellegers, A., Okuda, K., Neshitt, Jr., R. E. L., Smith, D. W., Chow, B. F., *Am. J. Clin. Nutr.*, 1957, v5, 327.

3. Rice, E. G., Herndon, J. F., Van Loon, E. J., Greenberg, S. M., *Am. J. Physiol.*, 1958, v193, 513.

4. Glass, G. B. J., Boyd, L. J., Lubby, A. L., *J. Lab and Clin. Med.*, 1955, v46, 60.

5. Lubby, A. L., Cooperman, J. M., Donnenfeld, A. M., Herrero, J. M., Teller, D. N., Wenig, J. B., *Proc. Soc. Ped. Res.*, May 1958, p140.

6. ——, *A.M.A. J. Dis. Child.*, 1958, v96, 532.

7. Ross, G. I. M., Mollin, D. L., *Vit. B_{12} and Intrinsic Factor*, F. Enke, Stuttgart, 1957, p437.

8. Boger, W. P., Bayne, G. M., Wright, L. D., Beck, G. D., *New England J. Med.*, 1957, v256, 1085.

9. Izak, G., Rachmilewitz, M., Stein, Y., Berkovici, R., Sadowsky, A., Aronovitch, Y., Grossowicz, N., *A.M.A. Arch. Int. Med.*, 1957, v99, 346.

10. Lust, J. E., Hagerman, D. D., Villee, C. A., *J. Clin. Invest.*, 1954, v33, 38.

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Paroxysmal Positional Vertigo in Vitamin B_{12} Deficiency

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A patient with vitamin B_{12} deficiency without anemia developed early neurologic manifestations of pernicious anemia, including a previously unreported complication of paroxysmal positional vertigo. All complications promptly disappeared with cyanocobalamin therapy.

NEUROLOGICAL manifestations of pernicious anemia include the following: numbness, tingling, and other paresthesias involving distal extremities; weakness and heaviness of lower extremities, followed by difficulty in walking (dragging, stiffness, and unsteadiness) with ultimate flaccid paralysis in some cases; exaggeration of deep tendon reflexes when lateral columns of the spinal cord are involved; and hypoactive deep reflexes when the degenerative process is mainly in the peripheral nerves or posterior columns. The sensory changes comprise early diminution of vibration and position senses; and to some extent those of touch, pain, and temperature. The earliest hand involvement is a clumsiness of the fingers when handling fine objects, and is due to involvement of position sense.[1,2] Sphincter disturbances, mental symptoms, cranial nerve palsies, and optic neuritis and atrophy have also been described. These complications usually follow the onset of anemia; however, in certain cases the order of occurrence has been reversed.[2,3] This communication reports the case of a patient in whom vitamin B_{12} deficiency led, among other neurological manifestations, to paroxysmal positional vertigo, which promptly disappeared following treatment with cyanocobalamin.

Report of a Case

A 54-year-old registered nurse, known to have generalized vitiligo and controlled hypothyroidism for several years, sought medical help in May 1969 for dizziness. This symptom first occurred when the patient arose from bed one morning, and continued intermittently for several days, being brought on mainly by upward and leftward movements of the head and eyes, and by turning to the right while in the recumbent position. At times nausea and vomiting accompanied this symptom. However, no tinnitus or deafness developed. There was no history of upper-respiratory tract infection during or immediately preceding this symptomatology. The patient also complained of numbness of toes and of clumsiness of fingers which had caused difficulty in handling fine objects for several months.

Physical examination revealed scattered vitiligo, normal ear, nose, and throat, and normal results from Weber and Rinne tests. No significant neurological abnormalities could be detected. However, on positional testing, subjective vertigo and a nystagmus developed upon lowering the trunk from a sitting to a supine position with the head turned to the right and neck extended. The nystagmus developed im-

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mediately upon assuming this position, its rapid component being towards the right ear. It lasted 15 to 20 seconds, and was fatigable on repeated testing. The results of caloric and audio tests were within normal limits. A diagnosis of benign paroxysmal positional vertigo was made. The patient was given reassurance, and discharged on a regimen of niacin pills.[4] Three months later she was seen again with the same complaints which had failed to improve. At this time, although her hemoglobin level was normal (14.7 gm./100 ml), a peripheral blood smear showed hypersegmentation of the neutrophils. Further work-up revealed a serum vitamin B_{12} level of 75 picograms (pg)/ml and a histamine-fast achlorhydria. A plasma vitamin B_{12} absorption test gave levels of 1.3 pg/ml and 2.9 pg/ml without and with intrinsic factor, respectively, a pattern characteristic of pernicious anemia.[5] The serum contained an antiintrinsic factor antibody of the blocking type. Therapy with cyanocobalamin injections was started at this time with 100 μg daily. Within ten days the patient was completely relieved of vertigo. Clumsiness of the fingers and numbness of the toes also disappeared with continued monthly injections of cyanocobalamin. At the present time, six months after the beginning of therapy, the patient is completely free of neurological symptoms.

Comment

Positional vertigo has been associated with various conditions, such as head injury,[6] trauma during stapes surgery or ultrasonic therapy,[7] and at times with Ménière's disease[8] and otitis media.[8] Vascular confusion involving the vestibular system, which usually causes sudden dizziness and tinnitus with a variable degree of high-tone deafness, may be followed by positional vertigo and nystagmus.[9] Occasionally unilateral deafness and positional vertigo may be due to a virus such as that of mumps, causing partial unilateral destruction of labyrinthine function.[9] Multiple sclerosis,[10] cerebellar tumors, particularly midline lesions, and tumors of the fourth ventricle and temporal lobe[11] are among the other known causes. The rest, which are the majority of the cases of positional vertigo, are those for which no etiology can be determined. These have usually been referred to as cases of benign paroxysmal positional vertigo.

Although the older literature briefly refers to tinnitus and vertigo as symptoms of anemia in vitamin B_{12} deficiency,[12] paroxysmal positional vertigo has not been previously reported as a direct neurological consequence of this deficiency. Our case demonstrates the development of this complication along with the more usual early neurological manifestations of pernicious anemia, and their complete reversal following specific therapy with cyanocobalamin. Since neurological degeneration due to vitamin B_{12} deficiency involves white matter in the spinal cord as well as that in the brain and peripheral and cranial nerves, this complication should have been recognized more often. However, as anemia itself can cause dizziness, and as most cases of pernicious anemia nowadays are recognized and treated before neurological manifestations develop, such recognition could have been difficult. Furthermore it is to be expected that cases of positional vertigo, such as the present case in which the patient had normal hemoglobin levels, would "naturally" have escaped investigation for vitamin B_{12} status, and could have been interpreted as cases of benign paroxysmal positional vertigo.

The "benignity" of paroxysmal positional vertigo and its peripheral or central nervous system localization by postural tests are controversial points. Positional vertigo, which develops upon assumption of a critical head position and which is usually associated with a positional nystagmus, can be tested simply by holding the patient's head slightly to one side and then lowering the trunk from sitting to a supine position with neck extended, the same maneuver being repeated with head turned to the other side.[7] Various characteristics of the nystagmus so elicited have been considered significant by several authors regarding the localization of the underlying pathological process. Cawthorne,[13] for example, has claimed that nonfatigable nystagmus (ie, the type that can be elicited on repeated testing) appearing immediately upon arrival of the "critical" position by the head is related to central lesions, whereas peripheral disease produces a nystagmus which begins after a latent period and can be fatigued by repeated testing. The nystagmus elicited in our patient does not fit either of these descriptions. It started without a latent period and it could be fatigued.

Another classification of positional nys-

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tagmus proposed by Aschan et al[14] includes the following: type 1: direction-changing, nonparoxysmal, persistent, and frequently without vertigo; type 2: direction-fixed, persistent, sometimes without vertigo; and type 3: direction-changing, with latent period, and paroxysmal but transitory. Types 1 and 2 are said to be due to central lesions and type 3 to the usual class of end-organ disease, namely, benign paroxysmal positional nystagmus. However, exceptions to these criteria have been seen too often to put much reliance on them.[15] Paroxysmal positional nystagmus resembling the benign variety has consistently been produced by lesions of the vermis involving the nodules,[16] and the type found in multiple sclerosis also has not conformed to any such distinctions.[17] In our patient, nystagmus was nondirection changing like type 2 of Aschan, and transitory like type 3, and therefore could not be localized according to these criteria.

The relationship of type 3, or benign paroxysmal positional vertigo, to a lesion of the utricle as proposed by Dix and Hallpike[17] has not been borne out by experiments in animals. Fernandez and Lindsay[18] have shown that destruction of the cat utricle and saccule not only fails to produce nystagmus but also abolishes a preexisting nystagmus, and that indeed, sectioning of the utricular nerve results in a positional nystagmus.

In short, the entity of benign paroxysmal positional vertigo and nystagmus is not very well understood. It is not always benign and its underlying pathological process when present is difficult to localize. It may, as in the present case, represent a manifestation of vitamin B_{12} deficiency.

References

1. Zavin F: Neurologic manifestations of addisonian and acquired pernicious anemia. *Med Clin N Amer* 48:177-187, 1964.
2. Kokmen M: Pernicious anemia: Neurological manifestations. *Mind Med* 47:67-79, 1964.
3. Grinker RR, Sachs AL: *Neurology*. Springfield, Ill, Charles C Thomas Publisher, 1966, p 1355-1362.
4. Poke JH: Postural vertigo. *Minn Med* 61:1203, 1968.
5. Deschornol-ner A: The fundamental basis for the suggested right from plasma absorption test in the diagnosis of pernicious anemia. *Proceedings of the Ninth Congress of the European Society of Haematology*. Basel, Switzerland, S Karger AG, 1963, pp 1460-1463.
6. Barbor HO: Positional nystagmus, especially after head injury. *Trans Amer Laryng Rhinol Otol Soc*, 1964, pp 661-714.
7. Lindsay JR: Postural vertigo. *Minn Med* 50:1013-1015, 1967.
8. DeBord BA, McKibben BG: The dizzy patient: Diagnosis and management. *Postgrad Med* 39:446-450, 1966.
9. Lindsay JR, Hemenway WG: Postural vertigo due to unilateral sudden partial loss of vestibular function. *Ann Otol* 65:692-706, 1956.
10. Schumacher GA: Demyelinating diseases as a cause for vertigo. *Arch Otolaryng* 85:587-598, 1967.
11. McCabe BF: Clinical aspects of the differential diagnosis of end-organ vertigo. *Ann Otol* 77:190-198, 1968.
12. Wilson SAK, Bruce AN: *Neurology*. Baltimore: Williams & Wilkins, 1940, pp 1533-1538.
13. Cawthorne T: Positional nystagmus. *Ann Otol* 63:461-470, 1954.
14. Aschan G, Bergedett M, Stahle J: Nystagmus, topic: Recording of nystagmus in clinical neurootological examination. *Acta Otolaryng*, suppl 126, pp 1-108, 1956.
15. Weiss AD: Neurological aspects of the differential diagnosis of vertigo. *Ann Otol* 77:216-221, 1968.
16. Lindsay JR: Paroxysmal postural vertigo and vestibular neuronitis. *Arch Otolaryng* 85:544-547, 1967.
17. Dix MR, Hallpike CS: The pathology, symptomatology, and diagnosis of certain common disorders of the vestibular system. *Proc Roy Soc Med* 45:341-354, 1952.
18. Fernandez C, Lindsay JR: Positional nystagmus in man and animals. *J Nerv Ment Dis* 130:488-495, 1960.

Effects of orotic acid and methionine on acid-soluble nucleotides of vitamin B_{12}-deficient chicks

Orotic acid and methionine, like vitamin B_{12}, increase the amount of RNA and acid-soluble nucleotides in vitamin B_{12}-deficient chicks[1,2]. This was confirmed by observations of an increased incorporation of ^{32}P into these structures[3,4], an expression of a higher turnover of these substances.

The present paper reports the effects of orotic acid, methionine and vitamin B_{12}, respectively, on the level of the individual nucleotides in the acid-soluble fraction of the liver of vitamin B_{12}-deficient chicks. This type of work might provide inform-

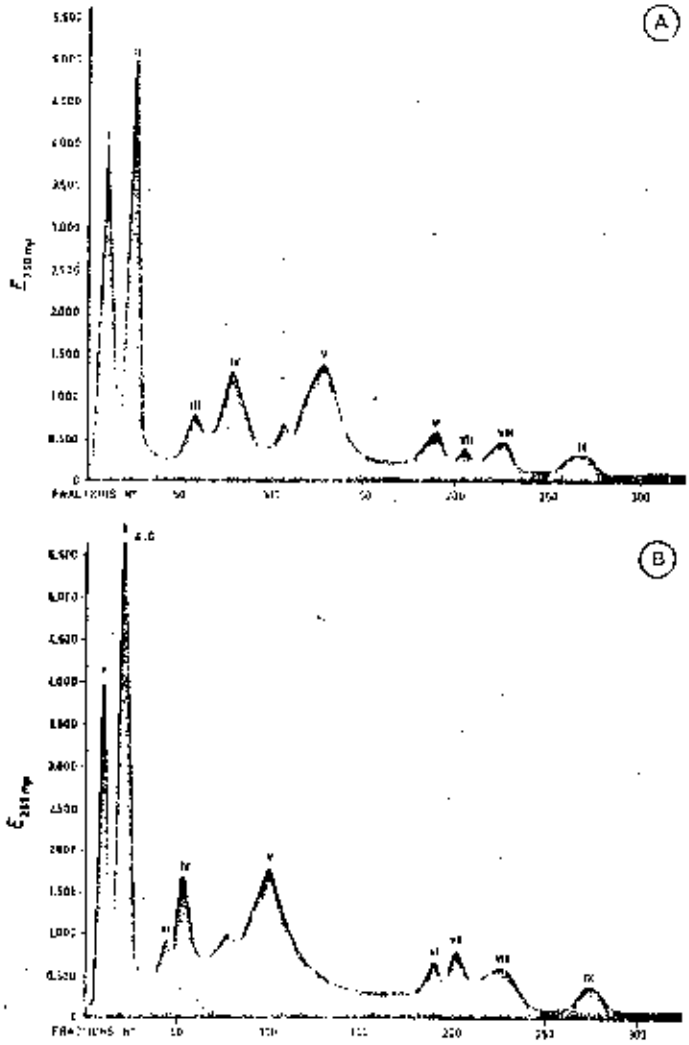

Fig. 1. Chromatograms of the acid-soluble fraction nucleotides: (A) vitamin B_{12}-deficient chicks; (B) vitamin B_{12}-treated chicks; I-CMP, DPN; II-AMP; III-GMP; IV-CDP, IMP, UMP; V-ADP; VI-GDP; VII-UDP; VIII-ATP; IX-UTP, GTP.

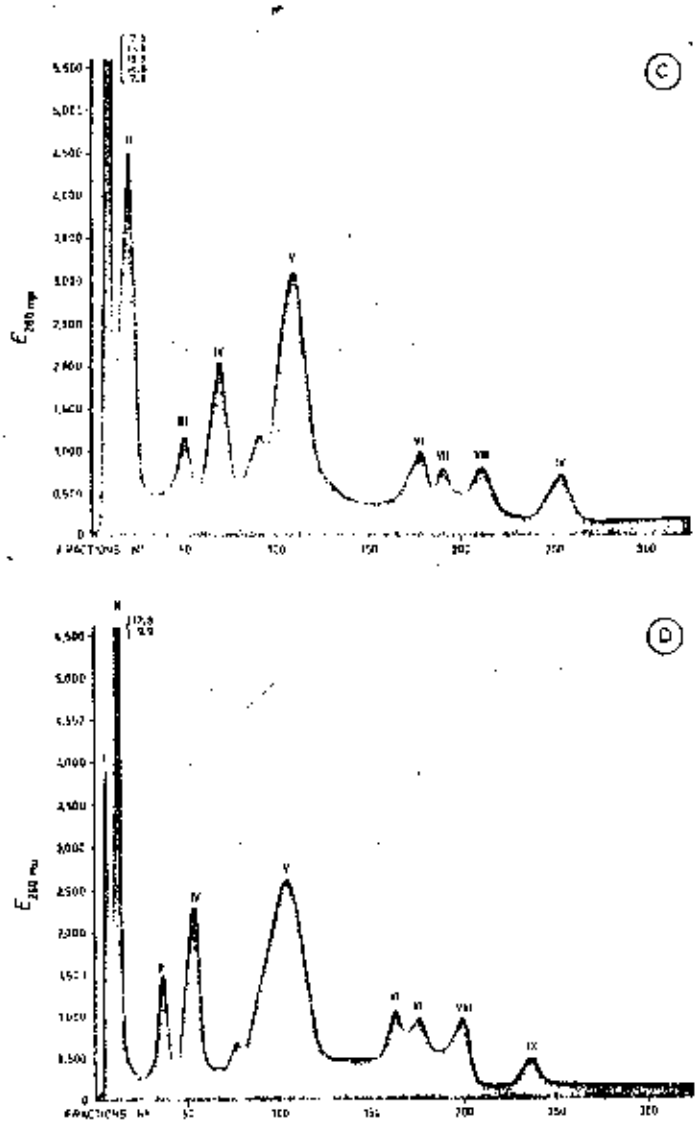

Fig. 1. Chromatograms of the acid soluble fraction nucleotides: (C) orotic acid-treated chicks; (D) methionine-treated chicks. I-CMP, DPN; II-AMP; III-GMP; IV-CDP, IMP, UMP; V-ADP; VI-GDP; VII-UDP; VIII-ATP; IX-UTP, GTP.

tion on the mechanism of action of these three substances in nucleotide metabolism, and improve our knowledge of their intern relationships.

One-day-old New Hampshire x white Leghorn chicks were used. The animals were divided into 4 groups according to their body weight. One group was maintained on the vitamin B_{12}-deficient diet[5] and the other three groups were kept on the same diet supplemented with 20 μg of vitamin B_{12}, 30 mg of orotic acid and 150 mg of DL-methionine, respectively, per 100 g of food. After 5 weeks, 6 animals of each group

were killed by decapitation and their livers were used for the determination of individual nucleotides by chromatography on ion-exchange resin according to HURLBERT *et al.*[5].

The chromatograms of the acid-soluble nucleotides from the livers of the four groups are shown on Fig. 1. The abscissae refer to the number of 5-ml fractions collected. From the area covered by the peaks, it can be seen that the total amount of nucleotides is larger in the treated animals than in the controls[8]. Furthermore, the relative amounts of the individual nucleotides are changed. For instance, the amounts of ADP and GDP are very high in the three groups of treated animals. Orotic acid causes a large increase of CMP. A slight increase is observed for CDP, IMP, UTP and GTP, whereas AMP and GMP do not change significantly. Methionine treatment causes some increase in UDP and in the mononucleotides, and it has no effect on ATP. Vitamin B_{12} exerts some effect on the level of adenylic and uridylic acids.

Many of the actions exerted by vitamin B_{12}, methionine and orotic acid might result from such effects on the level of the free nucleotides, since these play an important role in many metabolic processes in all kind of tissues.

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[1] M. MARCHETTI, C. M. CALDARERA AND G. MORUZZI, *Giorn. Biochim.*, 9 (1960) 378.
[2] M. MARCHETTI, R. VIVIANI AND G. MORUZZI, *Boll. soc. ital. biol. sper.*, 36 (1960) 1002.
[3] M. MARCHETTI, C. M. CALDARERA AND P. PUDDU, *Boll. soc. ital. biol. sper.*, submitted for publication.
[4] M. MARCHETTI, P. PUDDU AND C. M. CALDARERA, *Boll. soc. ital. biol. sper.*, submitted for publication.
[5] M. R. S. FOX, G. M. BRIGG AND L. O. OKITA, *J. Nutrition*, 62 (1957) 539.
[6] R. H. HURLBERT, H. SCHMITZ, A. F. BRUMM AND V. R. POTTER, *J. Biol. Chem.*, 209 (1954) 23.

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Relationships between Biotin and Vitamin B_{12}

EFFECTS OF BIOTIN AND VITAMIN B_{12} ON FOLIC ACID METABOLISM

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1. The effects of dietary biotin compared with vitamin B_{12} on the total content and on the distribution of the various folate derivatives in the liver of rats given a biotin-free diet have been studied. The effect of both vitamins on the conversion *in vitro* of folic acid into citrovorum factor in the same experimental conditions was also examined. 2. In biotin-treated rats as well as in vitamin B_{12}-treated rats the total content of folic acid active substances measured microbiologically by *Pediococcus cerevisiae*, *Streptococcus faecalis* and *Lactobacillus casei* is significantly higher than that in biotin-deficient rats. The liver distribution of various folate derivatives in the three groups of animals is also markedly modified. 3. The amount of citrovorum factor formed in systems with liver homogenate of rats receiving biotin or vitamin B_{12} is higher than that with liver homogenates of deficient rats. 4. The results obtained demonstrate the influence of biotin in the metabolism of folic acid, and the similar actions at this level of both biotin and vitamin B_{12}. These results are discussed in relation to the participation of the two vitamins in the metabolism of C_1 units, as a biochemical interpretation of the relationships between vitamin B_{12} and biotin.

Interrelationships between biotin and vitamin B_{12} have been observed by Marchetti & Testoni (1964). The administration of vitamin B_{12} to rats receiving a biotin-free diet exerts a favourable effect on the growth rate and a delay of the deficiency symptoms. In addition, vitamin B_{12} seems to have an effect on biotin-dependent enzymic activities. On the other hand, the administration of biotin influences methylation *in vivo* and *in vitro* in the same experimental conditions. This last observation led to the hypothesis of the common participation of both vitamins in the metabolism of C_1 units, and this is a biochemical interpretation of the 'sparing action' of vitamin B_{12} in biotin deficiency.

The part played by vitamin B_{12} in the metabolism of C_1 units has already been studied. In particular, the role of vitamin B_{12} in methylation has been demonstrated (Oginsky, 1950; Dietrich, Monson & Elvehjem, 1952; Fatterparker, Marfatia & Sreenivasan, 1955; Marchetti, Viviani & Rabbi, 1956; Ranke, Ranke & Chow, 1962; Moruzzi, Marchetti & Viviani, 1963). According to Arnstein (1955), vitamin B_{12} might be involved in the synthesis of labile methyl groups from more highly oxidized precursors such as C-α of glycine or C-β of serine.

Kislink (1961) showed that an enzyme containing a derivative of vitamin B_{12} is required for the synthesis of the methyl group of methionine. Recent evidence suggests that methylcobalamin may be the actual coenzyme for methionine synthetase (Guest, Friedman, Woods & Smith, 1962; Weissbach, Peterkofsky, Redfield & Dickerman, 1963; Foster, Dilworth & Woods, 1964). With regard to biotin, the only (indirect) evidence for its possible participation in the metabolism of C_1 units is that on its influence on folic acid synthesis. Luckey, Pleasants, Gordon & Reyniers (1955) and Noronha, Sreenivasan & Padval (1959) demonstrated that biotin administered to biotin- and folic acid-deficient rats caused an abnormal increase in folic acid excretion and an almost complete restoration of folic acid concentrations in liver.

Therefore the action of biotin on the metabolism of C_1 units might be due to its influence on the availability of folic acid or even more of folate cofactors in tissues.

The role of folate coenzymes in the metabolism of C_1 units is well known: the hydroxymethyl groups involved in serine glycine interconversion and in the biosynthesis of methyl groups of thymine, methionine and choline are transferred by tetrahydrofolic acid (O'Brien, 1962). Sakami & Ukstins (1961) and Larrabee, Rosenthal, Cathon & Buchanan (1963) have indicated that a folic acid derivative, N^5-methyltetrahydrofolic acid, can be considered as a precursor in methionine synthesis.

This present research has been undertaken to verify experimentally the hypothesis of the common participation of vitamin B_{12} and biotin in the metabolic pathway of C_1 units, and in particular in the metabolism of folic acid. For this purpose it was decided to study the effect of dietary biotin, compared with that of vitamin B_{12}, on the total content and on the distribution of the various folate derivatives in the liver of biotin-deficient rats. The effect of both vitamins on the conversion *in vitro* of folic acid into citrovorum factor was also examined.

MATERIALS AND METHODS

Weanling male rats of the Wistar strain were used. The animals were divided into three groups each of 12 rats, housed in cages with wire bottoms and fed *ad libitum* on the following diets respectively: biotin free diet; biotin-supplemented diet; biotin-free diet supplemented with vitamin B_{12}. The exact composition of these diets is given in Table 1.

After 60 days, six animals of each group were killed and the livers, removed as rapidly as possible, were used for the determination of the concentrations of folate derivatives and their distribution. The livers, wiped free from blood, were homogenized with 2 vol. of ice-cold water. To the homogenates 2 vol. of ice-cold acetone was added, and the precipitated material was immediately filtered on a Buchner funnel with suction, washed with acetone and ether, and dried under vacuum over P_2O_5. Suitable quantities of acetone-dried powders were extracted in 1% (w/v) potassium ascorbate, pH 6·0, by heating the suspensions (20 mg./ml.) for 30 min. in a water bath at 75°. After centrifugation at 0° samples of clear supernatant were put on a column of DEAE-cellulose–Hyflo Super-Cel (20 cm. × 1 cm.) prepared for use by successively washing with 25 ml. of 0·5 N-KOH followed by water until the rinse was neutral, and then with 25 ml. of 0·5 M-phosphate buffer, pH 6·0, followed by water until the rinse was free of phosphate. After the extract was absorbed the column was washed with 0·2% potassium ascorbate and then eluted with an ascorbate–phosphate eluent (0·2% potassium ascorbate, pH 6·0, and 0·5 M-potassium phosphate buffer, pH 6·0). The flow rate was 1 ml./min. Thirty 5 ml. fractions were collected in tubes containing 0·1 ml. of 10% (w/v) potassium ascorbate, and, after suitable dilution in 1% potassium ascorbate, were assayed for folic acid activities with *Pediococcus cerevisiae* ATCC 8081, *Lactobacillus casei* ATCC 7469 and *Streptococcus faecalis* R ATCC 8043. Media employed were those generally used and sterilized by Bakerman (1961). Furthermore, samples were added to basal media, each tube of which (final vol. 10 ml.) contained 10 mg. of potassium ascorbate. The tubes were steamed for 30 min., cooled and inoculated. Bacterial growth was measured turbidimetrically (at 660 mμ) after 20 hr. of incubation. Calcium leucovorin was used as a reference standard and the concentration was adjusted and corrected for the presence of the inactive isomer. The identification of various peaks in the elution pattern on DEAE-cellulose of acetone-dried liver powder was obtained by running individual chromatograms with reference samples. [N^{10}-Formyltetrahydrofolic acid was prepared by incubation of N^5-formyltetrahydrofolic acid with HCl at 37° and neutralization to pH 6·0; N^5-formyltetrahydrofolic acid was the DL-racemate, leucovorin (Lederle); tetrahydrofolic acid was purchased from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.] The identity of various folate derivatives was confirmed by their activities in stimulating the growth of the three test organisms and by their properties of stability (Silverman, Law & Kaufman, 1961).

The compound occurring in the region of tubes 8–9 (peak I) was identified as N^{10}-formyltetrahydrofolic acid. Further confirmatory evidence that peak I is that of N^{10}-formyltetrahydrofolic acid is that it possesses essentially equivalent growth activity for all three test organisms. In addition, when acetone-dried liver powder was extracted at 125° for 30 min. and then chromatographed, peak I disappeared almost completely, whereas peak III increased (isomerization of N^{10}-formyltetrahydrofolic acid to N^5-formyltetrahydrofolic acid).

The material that emerges at tubes 11 and 12 (peak II) corresponds in microbiological activity (active for *L. casei* and *S. faecalis* but not for *P. cerevisiae*) and stability to N^{10}-formyldihydrofolic acid and N^{10}-formylfolic acid.

The compound determining peak III (tube 13) was characterized as N^5-formyltetrahydrofolic acid by its stability at 125° as well as its activity in stimulating the growth of all three test organisms. The compound determining peak IV (tubes 14 and 15) was identified as N^5-methyltetrahydrofolic acid by its property of supporting the growth of *L. casei* but not of *S. faecalis* or *P. cerevisiae*.

The compound eluted in the region of tubes 16 and 17 (peak V) was characterized as tetrahydrofolic acid by its lability to heat in the absence of ascorbic acid and the different activity it showed in stimulating the growth of the three test organisms.

Finally, the folates occurring at tubes 19 and 20 (peak VI) and 22–28 (peak VII) could correspond, according to Wittenberg, Noronha & Silverman (1962), to N^{10}-formyl- and N^5-formyl derivatives of pteroylpolyglutamic acid.

For testing the conversion of folic acid into citrovorum factor *in vitro* six rats of each group were killed, and the livers were rapidly removed and homogenized with 4 vol.

Table 1. *Composition of experimental diets* *

Ingredients	Composition (%) Group 1	Group 2	Group 3
Casein (vitamin-free)	20	20	20
Sucrose	59	59	59
Salt mixture no. 4*	4	4	4
Groundnut oil	5	5	5
Autoclaved egg white	—	11	—
Raw dried egg white	11	—	11
Vitamin mixture†	1	1	1
Biotin	—	0·00001	—
Vitamin B_{12}	—	—	0·0001

* Hegsted, Mills, Elvehjem & Hart (1941).

† The vitamin mixture contained (mg./g.): thiamine hydrochloride, 0·2; riboflavine, 0·2; pyridoxine, 0·25; calcium pantothenate, 2·0; nicotinic acid, 5·0; inositol, 10·0; p-aminobenzoic acid, 25·0; folic acid, 0·02; choline hydrochloride, 100·0; vitamin K, 0·21; diluted in sucrose, 647. Two drops of vitamins A, D and E concentrate were fed orally to each rat once a week.

of 0·08M-phosphate buffer pH 6·3. Samples (5ml.) of homogenate were pipetted in 50ml. Erlenmeyer flasks containing 5ml. of the above-mentioned buffer, 10mg. of DL-homocysteine, 10mg. of L-serine and 100μg. of folic acid; the final volume was made up to 11ml. with water and the solutions were layered with toluene. Tests without adding folic acid were also prepared (control tests). The flasks were incubated in a reciprocating shaker at 37° for 2hr. under nitrogen. Then the samples were autoclaved, cooled, diluted and filtered. In the filtrates the citrovorum factor formed was assayed microbiologically with *P. cerevisiae*. Difco 'Qf' assay medium was used as medium and calcium leucovorin as standard. The cultures were incubated for 20hr. at 37° and bacterial growth was measured turbidimetrically.

The quantities of citrovorum factor formed in systems without folic acid were subtracted from those formed in systems with folic acid. All the results were analysed for statistical significance by Fisher's t test; a difference between two means was regarded as significant when P was smaller than 0·05.

RESULTS

From the results of Table 2 it appears that the total liver content of folic acid-active substances, measured microbiologically by *P. cerevisiae*, *S. faecalis* and *L. casei*, is significantly higher in the biotin-treated rats ($P < 0·001$, $P < 0·02$ and $P < 0·02$ respectively) and in the vitamin B_{12}-treated rats ($P < 0·01$, $P < 0·01$ and $P < 0·01$ respectively) than in biotin-deficient rats. Also, the distribution of various folate derivatives in liver is markedly modified in the three groups of tested animals. The results of Table 3 and Fig. 1 show significant increases in the amounts of various folate derivatives in the liver of rats receiving biotin compared with the deficient animals. Particularly increased are: N^{10}-formyldihydrofolic acid and N^{10}-formylfolic acid ($P < 0·001$), N^5-formyltetrahydrofolic acid ($P < 0·001$), N^5-methyltetrahydrofolic acid ($P < 0·01$), tetrahydrofolic acid ($P < 0·001$) and N^5-formyl derivatives of tetrahydropteroylpolyglutamic acid ($P < 0·001$).

Larger amounts of folic acid-active substances were also noted in the liver of rats receiving vitamin B_{12} than in those of deficient animals ($P < 0·001$). In the liver of vitamin B_{12}-treated rats N^{10}-formyltetrahydrofolic acid ($P < 0·02$) and N^{10}-formyl

Table 2. *Effects of dietary biotin and vitamin B_{12} on folic acid activities of rat liver*

Each value is given as the mean ± S.E.M. of six determinations on different animals. Experimental details are given in the text.

Group	Rat nutritional status	Folic acid activity (mμg./mg. of acetone-dried liver powder) By *P. cerevisiae*	By *L. casei*	By *S. faecalis* R
1	Biotin-deficient	4·48 ± 0·36	6·17 ± 0·59	5·63 ± 0·51
2	Biotin-treated (100μg./kg. of diet)	9·07 ± 0·43	8·72 ± 0·73	8·10 ± 0·57
3	Biotin-deficient vitamin B_{12}-treated (100μg./kg. of diet)	6·41 ± 0·51	9·55 ± 0·77	9·27 ± 0·59

Table 3. *Effects of dietary biotin and vitamin B_{12} on the distribution of various folate derivatives*

Each value is given as mean ± S.E.M. of the six determinations on different animals. Experimental details are given in the text.

Compound	Conc. of folic acid derivatives (mμg./mg. of acetone-dried liver powder) Biotin-deficient rat	Biotin-treated rat	Biotin-deficient vitamin B_{12}-treated rat
N^{10}-Formyltetrahydrofolic acid	4·09 ± 0·30	4·42 ± 0·43	5·52 ± 0·39
N^{10}-Formyldihydrofolic acid and N^{10}-formylfolic acid	0·60 ± 0·04	0·92 ± 0·05	1·07 ± 0·02
N^5-Formyltetrahydrofolic acid	0·19 ± 0·01	0·45 ± 0·03	0·56 ± 0·03
N^5-Methyltetrahydrofolic acid (prefolic A)	0·43 ± 0·04	0·67 ± 0·05	0·85 ± 0·09
Tetrahydrofolic acid	0·30 ± 0·01	0·90 ± 0·03	0·61 ± 0·02
N^{10}-Formyltetrahydropteroylpolyglutamic acid	0·65 ± 0·09	0·80 ± 0·18	1·20 ± 0·14
N^5-Formyltetrahydropteroylpolyglutamic acid	0·96 ± 0·12	2·30 ± 0·28	1·85 ± 0·21

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derivatives of tetrahydropteroylpolyglutamic acid ($P < 0{\cdot}001$) also showed a significant increase.

From the results of Table 4 it appears that the quantity of citrovorum factor formed is higher in systems with the liver homogenate of biotin-treated rats ($P < 0{\cdot}01$) and of vitamin B_{12} treated rats ($P < 0{\cdot}02$) than that with liver homogenate of biotin-deficient rats.

DISCUSSION

The present results confirm the participation of biotin in the metabolism of folic acid. Moreover, the same effect of biotin and vitamin B_{12} on folic acid metabolism is presumably a consequence of the common participation of the two vitamins in the metabolism of C_1 compounds.

The effect of vitamin B_{12} on folic acid metabolism and in particular on the formation of citrovorum factor has been much studied (Drysdale, Bethell, Lardy & Bauman, 1951; Dietrich, Monson & Elvehjem, 1951; Doctor *et al.* 1953; Doctor, Elam, Sparks, Lyman & Couch 1954; Dawbarn, Hine & Smith, 1958; Gardiner & Silverman, 1960; Marcucci *et al.* 1963). However, the results obtained do not as yet concur and this is probably due to different experimental conditions.

Besides, the exact nature of the effect is not yet known. Vitamin B_{12} might act directly or indirectly in one or more of the reactions of the enzymic process mentioned.

With respect to the action of biotin our results do not correspond to those of Halevy & Guggenheim (1958), who did not observe any differences either on the hepatic concentrations of citrovorum factor or in the conversion *in vivo* of folic acid into citrovorum factor. On the other hand, these authors noted no effect of biotin on the hepatic concentrations of folic acid.

Also, with regard to biotin the exact mechanism of action is not yet known: it might have a direct coenzymic effect or an indirect effect on a reaction of the enzyme system. It seems unlikely that biotin acts by increasing the tissue storage of cofactors such as ATP, NADPH, pyridoxal 6-phosphate and Mg^{++} involved in this process. The addition of suitable amounts of these substances to the reaction mixtures did not modify significantly the amount of citrovorum factor formed in the systems with liver homogenates of biotin-deficient animals (Marchetti, Landi & Pasquali, 1964).

In conclusion, the parallel effects of biotin and vitamin B_{12} on folic acid metabolism and therefore on numerous metabolic processes that involve the transfer and the utilization of C_1 units at the various oxidation levels could explain the nutritional and metabolic relationships between the two vitamins already observed by Marchetti & Testoni (1964).

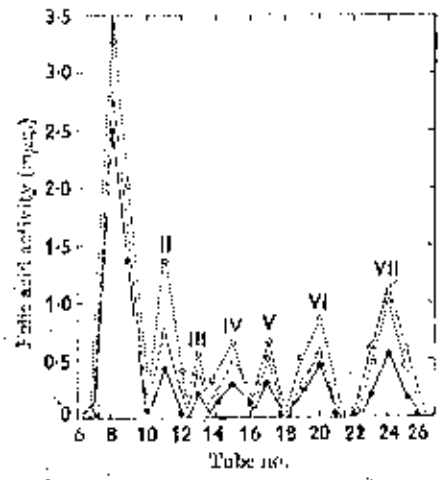

Fig. 1. Chromatograms of the liver folate derivatives of biotin-deficient rats (o——o), biotin-treated rats (△- - -△) and biotin-deficient vitamin B_{12}-treated rats (○- - -○). Peak I, N^{10}-formyltetrahydrofolic acid; peak II, N^{10}-formyldihydrofolic acid and N^{10}-formylfolic acid; peak III, N^5-formyltetrahydrofolic acid; peak IV, N^5-methyltetrahydrofolic acid; peak V, tetrahydrofolic acid; peak VI, N^{10}-formyltetrahydropteroylpolyglutamic acid; peak VII, N^5-formyltetrahydropteroylpolyglutamic acid.

Table 4. *Effects of dietary biotin and vitamin B_{12} on the conversion of folic acid into citrovorum factor by rat-liver homogenate*

Each value is given as the mean ± S.E.M. of six determinations on different animals. Experimental details are given in the text.

Group	Rat nutritional status	Citrovorum factor formed (μg./g. wet wt. of liver)
1	Biotin-deficient	2·28 ± 0·47
2	Biotin-treated (100 μg./kg. of diet)	4·86 ± 0·56
3	Biotin-deficient vitamin B_{12}-treated (100 μg./kg. of diet)	3·91 ± 0·35

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REFERENCES

[illegible]stein, H. R. V. (1955). *Symp. biochem. Soc.* 13, 92.

[illegible]kerman, H. A. (1961). *Analyt. Biochem.* 2, 568.

[illegible]wkers, M. C., Hine, D. C. & Smith, J. (1958). *Aust. J. exp. Biol. med. Sci.* 36, 511.

[illegible]trich, L. S., Monson, W. J. & Elvehjem, C. A. (1951). *Proc. Soc. exp. Biol., N.Y.*, 77, 93.

[illegible]trich, L. S., Monson, W. J. & Elvehjem, C. A. (1952). *J. biol. Chem.* 199, 765.

[illegible]ter, V. M., Elam, J. F., Sparks, P., Lyman, C. M. & [illegible], J. R. (1954). *Arch. Biochem. Biophys.* 48, 219.

[illegible]ter, V. M., Welch, B. E., Perret, R. W., Brown, C. L., [illegible], S. & Couch, J. R. (1963). *Proc. Soc. exp. Biol., N.Y.*, 84, 29.

[illegible]ydale, G. R., Bethell, J. J., Lardy, H. A. & Bauman, C. A. (1951). *Arch. Biochem. Biophys.* 33, 1.

[illegible], P., Marfatia, V. & Sreenivasan, A. (1955). *Indian J. med. Res.* 43, 43.

[illegible], M. A., Dilworth, M. J. & Woods, D. D. (1964). *Nature, Lond.*, 201, 39.

[illegible], R. C. & Silverman, M. (1960). *Arch. Biochem. Biophys.* 85, 313.

[illegible], J. R., Friedman, S., Woods, D. D. & Smith, E. L. (1962). *Nature, Lond.*, 195, 340.

[illegible], S. & Guggenheim, K. (1958). *J. Nutr.* 65, 77.

Hegsted, D. M., Mills, R. C., Elvehjem, C. A. & Hart, E. B. (1941). *J. biol. Chem.* 138, 459.

Kistiuk, R. L. (1961). *J. biol. Chem.* 236, 817.

Larrabee, A. R., Rosenthal, S., Cathon, R. E. & Buchanan, J. M. (1963). *J. biol. Chem.* 238, 1025.

Luckey, T. D., Pleasants, J. R., Gordon, H. A. & Reyniers, J. A. (1955). *J. Nutr.* 57, 169.

Marchetti, M., Laudi, L. & Pasquali, P. (1964). *Biochim. Biophys. Acta* (in the Press).

Marchetti, M., & Testoni, S. (1964). *J. Nutr.* 84, 249.

Marchetti, M., Viviani, R. & Rabbi, A. (1950). *Nature, Lond.*, 178, 805.

Morazzi, G., Marchetti, M. & Viviani, R. (1963). *Nature, Lond.*, 199, 695.

Noronha, J. M., Sreenivasan, A. & Padval, D. G. (1959). *Proc. Soc. exp. Biol., N.Y.*, 101, 803.

O'Brien, J. S. (1962). *Cancer Res.* 22, 267.

Opinsky, E. L. (1959). *Arch. Biochem.* 26, 327.

Ranke, B., Ranke, E. & Chow, B. F. (1962). *J. Nutr.* 76, 441.

Sakami, W., & Ukstins, I. (1961). *J. biol. Chem.* 236, PC50.

Silverman, M., Law, L. W. & Kaufman, B. (1961). *J. biol. Chem.* 236, 2530.

Weissbach, H., Peterhofsky, A., Redfield, G. B. & Dickerman, H. (1963). *J. biol. Chem.* 238, 3318.

Wittenberg, J. B., Noronha, J. M. & Silverman, M. (1962). *Biochem. J.* 85, 9.

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J. Nut. 70: 283-292

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The Influence of Vitamin B_{12} on the Content, Distribution and in Vivo Synthesis of Thiamine Pyrophosphate, Flavin Adenine Dinucleotide and Pyridine Nucleotides in Rat Liver[1]

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Apart from the well-known interrelationships among the B vitamins, there is reason to believe that folic acid and vitamin B_{12} may influence the functioning of other vitamins as cofactors. Thus, dietary folic acid has been known to determine rat liver stores of coenzyme A (CoA) and adenotriphosphate (ATP) (Popp and Totter, '52; Totter, '53); a decrease in liver DPN is also caused by aminopterin[2] (Strength et al., '54). The *in vivo* incorporation of nicotinamide into pyridinonucleotides in rat liver is affected in a deficiency of vitamin B_{12} (Nadkarni et al., '57). Low blood level of citrovorum factor in the hyperthyroid, vitamin B_{12}-deficient rat is corrected by administration of vitamin B_{12} (Pfander et al., '52). In liver homogenates from vitamin B_{12}-deficient hens, the synthesis of citrovorum factor from added folic acid is less than in those from animals injected with the vitamin (Doctor et al., '54). The potentiating effect of vitamin B_{12} in the mobilization of folic acid has also been reported from this laboratory (Sreenivasan, '51, Patterpaker et al., '55a). The general influence of vitamin B_{12} on carbohydrate and lipid metabolism has been linked to a primary relation to sulphydryl biosynthesis (Ling and Chow, '54; Register, '54; Kasbekar et al., '56, '59a). Distinguished from these apparently collateral findings is the reported elevation of CoA in livers of vitamin B_{12}-deficient rats and mice (Boxer et al., '53, '55; Wong and Schweigert, '56).

The present work relates to a study of the influence of vitamin B_{12} on the intracellular distribution of thiamine pyrophosphate (TPP), flavin adenine dinucleotide (FAD) and pyridine nucleotides (PN), and to their *in vivo* synthesis from the corresponding administered vitamins, in the rat liver. Data on the distribution of these cofactors in liver cells of the normal rat are available in the works of Goetbart ('52) and Dianzani and Dianzani Mor ('57) on TPP, of Carruthers and Suntzeff ('54) and Dianzani ('55) on pyridine nucleotides (PN) and of Schneider and Hogeboom (Schneider, '56) on FAD.

EXPERIMENTAL

Young, male Wistar rats weighing approximately 100 gm each were used. The animals, housed individually in raised mesh-bottom cages, were initially depleted of their vitamin B_{12} reserves by maintenance on a purified, iodo-casein ration. This consisted of the following percentage composition: hot, alcohol-extracted casein, 18; iodinated casein,[3] 0.15; arachis oil, 6; shark liver oil, 2; sucrose, 9.85; maize starch 60; and salt mixture (U.S.P. XIV), 4; with vitamins to provide in milligrams per kilogram of diet: thiamine·HCl, 6; riboflavin, 10; nicotinic acid, 30; calcium pantothenate, 20; pyridoxine·HCl 6; biotin, 1; folic acid, 5; *p*-aminobenzoic acid, 100; choline·Cl, 500; inositol, 500; 2-methyl-1, 4-naphthoquinone, 10; and α-tocopherol,

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[2] Strength, D. R., and N. I. Mondy 1953 Choline dehydrogenase activity and DPN content of rat livers following aminopterin injection. Federation Proc., 12: 276 (abstract).

[3] Protamone, obtained from Cerophyl Laboratories, Kansas City, Mo.

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50. The vitamin additions provided in this basal diet were considered adequate for the hyperthyroid condition. At the end of 4 weeks, animals were divided into two groups, one of which continued to receive the basal diet modified by the omission of iodinated casein and p-aminobenzoic acid and substitution by 2% of succinyl sulphathiazole. The latter addition was compensated for by adjusting the percentage of starch. The second, control group, received the modified diet with a supplement of vitamin B_{12} (150 μg/kg of diet). After a further 5 week period, the animals in both groups were divided into 4 sub-groups of 7 to 8 rats each, one of which was killed immediately to establish the liver distribution pattern of the cofactors in the vitamin B_{12}-deficient and replete states. The remaining three sub-groups of each group were used to study the synthesis of each of the three cofactors from the respective, administered vitamins. The rats were injected, intraperitoneally, on 5 successive days, with 5 mg of thiamine·HCl, 5 mg of riboflavin, or 1 mg of nicotinamide per rat per day. All animals were killed on the 5th day, 8 hours following the final injection of the test-vitamin.

The normal intracellular distribution pattern of the cofactors was secured on liver of 10 adult male rats weighing approximately 200 gm, maintained on the laboratory stock diet consisting of (gm per 100 gm of diet): whole wheat flour, 75; whole milk powder, 2; casein, 12; dried yeast, 2; arachis oil, 3; shark liver oil, 2; sodium chloride, 2; and calcium carbonate, 2.

Animals were exsanguinated and livers were perfused with ice-cold 0.25 M sucrose, promptly excised, blotted and made into 10% homogenates with 0.25 M sucrose in a Potter-Elvehjem homogenizer. The homogenates were separated by the differential centrifugation procedure of Schneider and Hogeboom ('50) into nuclear, mitochondrial and microsomal plus supernatant fractions using an International (PR-2) refrigerated centrifuge. Total and free thiamine in portions of whole homogenates and fractions were determined by a modification of the fluorometric method of Hennessy ('41) before and after hydrolysis of TPP complexes with taka-diastase for 18 hours at 37°C, the TPP content being calculated by difference. The total and non-FAD (flavin mono nucleotide, FMN plus free riboflavin) riboflavin were determined by the fluorometric procedure of Bessey et al. ('49) and the FAD content derived from these values with use of proper conversion factor, as indicated by these authors. The determination of total PN in homogenates and in fractions, as well as the differential determination of their oxidized and reduced forms in whole homogenates, was carried out fluorometrically by the procedure outlined by Dianzani ('55).

For determinations of blood erythrocyte count and hemoglobin content and of plasma vitamin B_{12} concentration, the animals were bled from tail veins and adequate samples collected in heparinated vials. The erythrocyte count was made by the standard method. Hemoglobin was determined by acid-hematin method in a Klett-Summerson photoelectric colorimeter (Kolmer et al., '51). Plasma vitamin B_{12} was determined by the method of Ross ('52) using *Euglena gracilis* as the test organism.

Portions of liver homogenates were incubated at 37°C for 12 hours under toluene with papain (25 mg/gm of fresh liver) to liberate the bound vitamin B_{12}, which was assayed using *E. gracilis* according to the method of Hoff-Jorgenson ('54).

The results were analyzed for statistical significance by calculating the t value of Fisher (Fisher and Yates, '53). Only the differences with a t value corresponding to a probability $P < 0.05$ were accepted as significant.

RESULTS

Content and distribution of cofactors and free vitamins in normal liver cytoplasm. The data obtained with liver homogenates of normal, stock diet-fed animals are reported in table 1. About 30% of the TPP of the homogenate was found contained in mitochondria, while the rest was present almost exclusively in the supernatant. The distribution pattern compares with data reported by others (Goethart, '52; Dianzani and Dianzani Mor, '57) under similar conditions. Free thiamine, also maximally localized in the supernatant

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TABLE 1

Content and distribution of cofactors and free vitamins in cytoplasm fractions of normal rat liver. Mean values from 10 independent determinations

Cytoplasm fraction	TPP		Free thiamine		FAD		FMN + free riboflavin		Total PN	
	μg/gm[1]	%	μg/gm	%	μg/gm	%	μg/gm	%	μg/gm	%
Homogenate	17.20 ± 1.31[2]		4.91 ± 0.34		24.80 ± 1.40		8.56 ± 0.61		645 ± 25	
Nuclear	0.69 ± 0.13	4.3 ± 0.9	1.01 ± 0.09	22.9 ± 2.1	4.26 ± 0.81	17.7 ± 2.5	1.80 ± 0.21	21.9 ± 2.3	164 ± 16	24.5 ± 2.1
Mitochondrial	5.01 ± 0.92	31.4 ± 4.9	0.51 ± 0.06	11.6 ± 1.4	13.94 ± 1.13	58.2 ± 4.7	1.80 ± 0.19	21.9 ± 2.3	121 ± 11	18.2 ± 1.2
Supernatant	10.26 ± 1.11	64.3 ± 5.6	2.89 ± 0.13	65.5 ± 4.3	5.75 ± 0.61	24.0 ± 3.1	4.53 ± 0.88	55.2 ± 4.3	442 ± 16	39.2 ± 2.9
Recovery, %		92.8 ± 2.6		89.8 ± 1.8		96.6 ± 3.4		98.3 ± 3.1		88.4 ± 3.4

[1] Gram weight of fresh tissue.
[2] Standard error of mean.

TABLE 2

Growth rate and blood and liver values in vitamin B_{12} deficiency[1]

Group	Body weight[2]		Erythrocytes	Hemoglobin	Vitamin B_{12}	
	Initial	Final			Plasma	Liver
	gm	gm	× 10⁶/mm³	gm/100 ml	μμg/ml	mμg/gm
Vitamin B_{12}-deficient	147 ± 4[3]	188 ± 4	4.0 ± 0.3	11.7 ± 0.5	87 ± 41	29.3 ± 9.2
Vitamin B_{12}-fed	146 ± 3	256 ± 5	6.9 ± 0.3	14.8 ± 0.7	834 ± 34	102.5 ± 11.3
Normal[4]	—	—	7.8 ± 0.4	15.1 ± 0.4	688 ± 29	66.4 ± 6.5

[1] Results are average values obtained from at least 7 independent determinations in each series.
[2] Initial weight refers to weight at time of grouping after 4 weeks of zein-casein feeding; final weight is 5 weeks after grouping and maintenance on succinyl sulphathiazole-containing diets.
[3] Mean value ± standard error of mean.
[4] Adult rats of approximately 200 gm maintained on laboratory stock diet.

TABLE 3

Effect of vitamin B_{12} on the content and distribution of TPP and free thiamine in liver cytoplasm and on the incorporation of thiamine administered into these components[1]

Cytoplasm fraction	Vitamin B_{12}-fed (8 rats)				Vitamin B_{12}-deficient (8 rats)				Thiamine-injected			
									Vitamin B_{12}-fed (7 rats)		Vitamin B_{12}-deficient (9 rats)	
	TPP		Free thiamine		TPP		Free thiamine		TPP	Free thiamine	TPP	Free thiamine
	μg/gm	%	μg/gm	%	μg/gm	%	μg/gm	%	Increase over uninjected control %	%	%	%
Homogenate	13.20 ± 1.01[2]		4.28 ± 0.13		7.23 ± 0.83		3.97 ± 0.30		78.7 ± 4.1	90.8 ± 3.8	48.2 ± 2.1	94.2 ± 3.9
Nuclear	0.62 ± 0.02	5.2 ± 0.4	0.76 ± 0.07	19.2 ± 1.6	0.56 ± 0.04	8.8 ± 0.6	0.63 ± 0.02	17.9 ± 1.0	124.2 ± 5.3	64.5 ± 3.0	12.5 ± 0.8	106.3 ± 8.8
Mitochondrial	3.49 ± 0.11	29.3 ± 2.1	0.34 ± 0.01	8.6 ± 0.6	0.86 ± 0.03	13.4 ± 1.2	0.33 ± 0.03	9.3 ± 0.8	100.6 ± 2.9	70.6 ± 3.1	62.8 ± 2.1	112.1 ± 7.3
Supernatant	7.81 ± 0.81	65.5 ± 6.2	2.86 ± 0.03	72.1 ± 1.8	4.90 ± 0.29	77.8 ± 5.3	2.57 ± 0.11	72.8 ± 4.3	74.4 ± 1.8	109.3 ± 4.3	51.4 ± 3.3	86.8 ± 2.3
Recovery, %		90.3 ± 3.3		92.6 ± 2.7		88.7 ± 3.1		88.9 ± 2.0	93.4 ± 2.9	95.8 ± 1.1	89.6 ± 2.0	88.2 ± 3.0

[1] Animals injected intraperitoneally with 5 mg of thiamine·HCl/rat/day for 5 consecutive days and killed 8 hours following final injection on the 5th day.

[2] Standard error of mean.

TABLE 4

Effect of vitamin B_{12} on the content and distribution of FAD and FMN + free riboflavin in liver cytoplasm and on the incorporation of riboflavin[1] administered into these components

Cytoplasm fraction	Vitamin B_{12}-fed (8 rats)				Vitamin B_{12}-deficient (8 rats)				Riboflavin injected			
									Vitamin B_{12}-fed (7 rats)		Vitamin B_{12}-deficient (9 rats)	
	FAD		FMN + free riboflavin		FAD		FMN + free riboflavin		FAD	FMN + free riboflavin	FAD	FMN + free riboflavin
	μg/gm	%	μg/gm	%	μg/gm	%	μg/gm	%	Increase over uninjected control %	%	%	%
Homogenate	24.50 ± 1.61[2]		10.13 ± 0.42		12.79 ± 1.83		12.48 ± 1.32		67.6 ± 2.6	75.1 ± 3.8	41.4 ± 2.9	68.9 ± 3.1
Nuclear	4.36 ± 0.33	19.5 ± 3.6	1.65 ± 0.01	16.9 ± 0.9	3.37 ± 0.03	29.4 ± 1.2	2.55 ± 0.04	23.3 ± 1.2	92.2 ± 3.7	38.8 ± 1.5	54.3 ± 2.2	27.8 ± 1.5
Mitochondrial	13.69 ± 0.58	61.3 ± 2.4	2.43 ± 0.02	24.8 ± 1.2	4.63 ± 0.05	40.4 ± 1.8	1.74 ± 0.04	15.9 ± 1.3	54.9 ± 1.4	53.1 ± 2.7	20.7 ± 2.1	70.7 ± 4.6
Supernatant	4.29 ± 0.17	19.2 ± 2.9	5.70 ± 0.32	58.3 ± 2.4	3.43 ± 0.05	30.1 ± 1.6	6.65 ± 0.21	60.8 ± 1.9	62.3 ± 2.9	75.6 ± 3.7	52.5 ± 3.9	84.2 ± 2.1
Recovery, %		91.2 ± 2.7		96.5 ± 1.3		89.5 ± 3.4		87.6 ± 3.1	91.1 ± 2.1	90.3 ± 3.9	88.8 ± 3.8	87.6 ± 3.9

[1] As for thiamine (table 3), with injections of 1 mg of riboflavin/rat/day.
[2] Standard error of mean.

TABLE 5

Effect of vitamin B_{12} on the content and distribution of total PN in liver cytoplasm and on its synthesis from administered nicotinamide[1]

Cytoplasm fraction	Vitamin B_{12}-fed (8 rats)		Vitamin B_{12}-deficient (8 rats)		Nicotinamide-injected	
					Vitamin B_{12}-fed (7 rats)	Vitamin B_{12}-deficient (9 rats)
	μg/gm	%	μg/gm	%	Increase over uninjected control %	%
Homogenate	641 ± 23[2]		392 ± 22		65.2 ± 3.9	43.8 ± 3.1
Nuclear	115 ± 11	20.1 ± 1.8	89 ± 5	26.2 ± 1.9	101.7 ± 4.2	13.5 ± 1.2
Mitochondrial	100 ± 8	17.4 ± 1.8	25 ± 4	7.6 ± 0.5	50.0 ± 3.8	60.0 ± 2.9
Supernatant	357 ± 11	62.5 ± 4.0	224 ± 13	66.2 ± 2.7	57.1 ± 3.1	56.2 ± 1.9
Recovery, %		83.2 ± 3.3		86.3 ± 1.8	89.0 ± 2.3	87.1 ± 2.1

[1] As for thiamine (table 3), with injections of 5 mg of nicotinamide/rat/day.
[2] Standard error of mean.

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fraction was, however, present in appreciable amounts in the nuclear fraction, its concentration in this fraction even exceeding that of TPP ($P < 0.05$).

The distribution pattern of FAD differed from that of TPP, in its greater association with the mitochondria than with the supernatant fraction. The proportion of FAD found in association with the mitochondria was similar to that reported (about 65%) by Schneider and Hogeboom (Schneider, '56). Although about 60% of the non-FAD riboflavin was contained in the supernatant, the proportion associated with mitochondria was small as compared with the FAD content of this fraction.

A major fraction of the total liver PN was localized in the supernatant, in confirmation of earlier observations (Carruthers and Scimzeff, '54; Dianzani, '55). A significant proportion was, however, contained in the nuclear fraction as well.

Alterations in vitamin B_{12} deficiency. The data on blood erythrocyte count and hemoglobin concentration and especially on plasma and liver content of vitamin B_{12} (table 2), apart from observations on growth noted below, connote the severity of the deficiency attained. During the 5-week period for which the animals were maintained on the succinyl sulphathiazole-containing diets, the vitamin B_{12}-deficient ration promoted an average gain in body weight of 8 gm/week compared with an average of 22 gm using the control ration with vitamin B_{12} (table 2). Data on blood erythrocyte count and hemoglobin concentration and especially on plasma and liver content of vitamin B_{12} (table 2) also connote the severity of the deficiency attained. Table 6 records the values obtained for oxidized and reduced forms of PN. The effects of vitamin B_{12} deficiency are summarized as follows: (a) there was a marked reduction in the liver content of the cofactors studied, being about 44% for TPP, 48% for FAD and 64% for total PN; (b) the mitochondria exhibited the largest depletion of the cytoplasm fractions. The reductions were 75, 66 and 75%, respectively, for TPP, FAD and PN. Significant reductions in the concentrations of these cofactors were also apparent in the other two fractions, especially in the supernatant; (c) the content and distribution of free thiamine remained essentially unaltered. With non-FAD riboflavin, there was small but significant elevation in both nuclear ($P < 0.005$) and supernatant ($P < 0.05$) fractions, the total being not significantly altered; and (d) the observed decrease in the proportion of oxidized to reduced forms of pyridine nucleotides (PN/PNH ratio) in livers of vitamin B_{12}-deficient animals (table 6) is in accordance with the findings of Nadkarni et al., '57). It is of interest to note that the changes in PNH content were much less pronounced in vitamin B_{12} deficiency.

Effect of vitamin B_{12} on incorporation of administered vitamins into liver cofactors. Tables 3 to 5 also include the results of experiments on the conversion by liver enzymes *in vivo* of the administered vitamins into their respective cofactors. Incorporation of thiamine, riboflavin and nicotinamide into their coenzyme forms is considerably less in the vitamin B_{12}-defi-

TABLE 6

Effect of vitamin B_{12} on oxidized and reduced PN in liver homogenates

Group	No. of rats	Oxidized pyridine nucleotides (PN)	Reduced pyridine nucleotides (PNH)	PN/PNH
		μg/gm	μg/gm	
Normal	10	639 ± 22[1]	206 ± 9	3.10 ± 0.09
Vitamin B_{12}-deficient	8	237 ± 18	155 ± 5	1.53 ± 0.11
Vitamin B_{12}-fed	8	476 ± 23	165 ± 6	2.88 ± 0.13
Vitamin B_{12}-deficient—nicotinamide injected	7	343 ± 26	221 ± 7	1.55 ± 0.14
Vitamin B_{12}-fed nicotinamide injected	8	809 ± 19	250 ± 11	3.24 ± 0.19

[1] Standard error of mean.

cient animals, although the extent of incorporation is appreciable even in this group. A relatively greater proportion of free thiamine and of non-FAD riboflavin was observed in the deficient group. The PN/PNH ratio was not significantly altered as a result of nicotinamide administration, the difference between the deficient and control groups being maintained (table 6).

The decreased ability in vitamin B_{12} deficiency of the liver enzymes for conversion of the administered vitamins into their respective cofactors is reflected in all of the cytoplasmic fractions and is largely seen in the nuclear fraction for TPP and PN and in the mitochondrial fraction for FAD. The deficient animals showed higher gains of free thiamine in the nuclear and mitochondrial fractions, whereas the differences with respect to non-FAD riboflavin were confined to the supernatant fraction.

DISCUSSION

The present observations demonstrate an impairment in the retention of TPP, FAD and PN in the vitamin B_{12}-deficient rat liver, as well as in their biosynthesis from the corresponding vitamins administered. In general, the observed effects point to greater depletion of cofactors from mitochondria than from other fractions. If these changes are not always reflected in liver levels of the free vitamins, it may be because of some losses through excretion; this may also imply a decreased use of the free vitamins for synthesis of coenzymes. Dianzani ('55) and Dianzani and Dianzani Mor ('57) have reported similar modifications in the distribution of TPP and PN in mitochondria from fatty livers caused by choline deficiency or CCl_4 poisoning. As discussed by these authors, such changes could result from decreased rates of synthesis of the cofactors or their increased degradation or from both causes. Thus it is possible that the synthesis of TPP through phosphorylation of thiamine and of DPN through the Kornberg reaction is diminished *in vivo* as a consequence of the reduced concentration of ATP in fatty livers; increased decomposition of TPP and DPN may be favored by increased acid phosphatase activity and through pyrophosphorolytic cleavage, respectively. Increased degradation of PN may also occur through partial displacement from the mitochondrial into the supernatant fraction, where DPNase is very active. Since both vitamin B_{12} and choline could exert similar lipotropic effects, it is probable that the same types of causes operate in either deficiency.

A rapid depletion of rat liver and its mitochondrial vitamin B_{12} could result from hyperthyroidism (Kasbekar et al., '59a) and acute carbon tetrachloride poisoning (Kasbekar et al., '59b). These conditions are known to cause morphological damage to mitochondrial structure with consequent displacement of intramitochondrial constituents into the surrounding medium (Dianzani, '54, '55; Dianzani and Dianzani Mor, '57; Maley and Lardy, '55; Kasbekar and Sreenivasan, '56). The protection afforded by prior administration of vitamin B_{12} under these conditions of stress (Fatterpaker et al., '55b; Kasbekar et al., '59a, '59b) could arise from its general lipotropic effect and from its known influence on sulphydryl conservation which, in turn, is essential for maintenance of mitochondrial integrity (Tapley, '56; Hunter et al., '56). It has also been suggested that vitamin B_{12} may be necessary for the synthesis of porphyrin-containing proteins of the cell (O'Dell et al., '55) which, apart from their function as respiratory carriers, are apparently also of importance in determining mitochondrial morphology (Gamble, '57). The observed alterations in mitochondrial cofactors in vitamin B_{12} deficiency may thus have an important though indirect, bearing on this function of the vitamin in the maintenance of mitochondrial organization in so far as the nucleotidation and phosphorylation reactions involved in the synthesis of cofactors are ATP-dependent and any structural damage to mitochondria renders the synthesis of ATP inoperative (Kielley and Kielley, '51; Dianzani, '54).

The effect of vitamin B_{12} also has to be assessed in terms of its known relationship to nucleotide biosynthesis. Although the nature of this relationship is obscure, evidence exists to suggest its involvement in the biosynthesis of both ribose[4] and de-

[4] Ling, C. T., and B. F. Chow 1954 Effect of vitamin B_{12} on ribose formation in erythrocytes. Federation Proc., 13: 253 (abstract).

oxyribose (Downing and Schweigert, '56; Wong and Schweigert, '57) moieties of nucleic acids.

It is interesting to note that the injection of a vitamin could enhance the liver content of the corresponding cofactor despite dietary adequacy of the vitamin concerned. Kaplan and coworkers ('56) had observed a 10-fold increase in liver PN following administration of nicotinamide into normal mice.

According to Hogeboom and Schneider ('52), the DPN synthesis is localized in the liver cell nucleus. In the vitamin B_{12}-deficient rats, the extent of impairment in PN synthesis from administered nicotinamide is more pronounced in the nuclear fraction (table 5). A similar impairment in TPP synthesis from administered thiamine is again better reflected in the nuclear fraction than in the mitochondria and least in the supernatant fraction (table 3); this nuclear impairment is accompanied by an appreciable rise in free thiamine in this fraction, suggesting that TPP, like DPN, may be synthesized in the liver nucleus from thiamine, a process susceptible to vitamin B_{12}-deficiency.

On the other hand, the impairment in FAD synthesis from riboflavin administered to the vitamin B_{12}-deficient rat (table 4) is better reflected in the mitochondrial fraction. The increase in the proportion of non-FAD riboflavin in the vitamin B_{12}-deficient animals, with or without riboflavin administration, is, however, confined mainly to the supernatant fraction. The synthesis of FAD from FMN and ATP is also localized in the supernatant fraction (Schneider, '56).

An increase in the proportion of reduced PN in fatty livers (Dianzani, '55) and in those from vitamin B_{12}-deficient rats (Nadkarni et al., '57) has been reported. Such a condition may provoke predomination of fatty acid synthesis as compared with breakdown (Lynen, '54). The decreased content (table 6) of oxidized PN in vitamin B_{12} deficiency (Nadkarni et al., '57) may point to its selective destruction by the DPNase (McIlwain and Rodnight, '49; Zatman et al., '53) contained in mitochondrial and supernatant fractions, as well as to decreased synthesis.

SUMMARY

Rats depleted of their vitamin B_{12} reserves were used to study the effects of dietary vitamin B_{12} on (1) the content and distribution in liver cytoplasm of thiamine pyrophosphate (TPP) and free thiamine, flavin adenine dinucleotide (FAD) and non-FAD riboflavin (FMN + free riboflavin) and total pyridine nucleotides (PN), and (2) the *in vivo* synthesis of these cofactors from the respective vitamins.

In the normal, stock diet-fed animals, about 30 % of total liver TPP was localized in the mitochondria while the rest was found almost exclusively in the supernatant fraction (including microsomes). Free thiamine was distributed between the supernatant (65%) and the nuclear fraction (23%). About 60% of total liver FAD content was associated with mitochondria while about 55% of the total non-FAD content was contained in the supernatant fraction, the balance in either case being almost equally distributed between the remaining two fractions. A major portion (60%) of the total liver PN was localized in the supernatant; the nuclear fraction also contained appreciable amounts (25%).

The liver content of the cofactors was markedly affected in vitamin B_{12} deficiency with average reductions of 45, 48 and 64%, respectively, in TPP, FAD and PN. The effects were largely confined to the mitochondrial fraction and were not accompanied by corresponding changes in the content of the free vitamins. A decrease in oxidized pyridine nucleotides in liver homogenates with proportional lowering of the ratio of oxidized to reduced pyridine nucleotides (PN/PNH) was observed.

The incorporation of injected thiamine, riboflavin and nicotinamide into the liver coenzymes was impaired in vitamin B_{12} deficiency. The impairment in TPP and PN synthesis was reflected to a greater extent in the nuclear fraction than in other fractions, while that in FAD synthesis was seen to an almost equal extent in all fractions.

In the vitamin B_{12}-deficient animal there was also appreciable incorporation of administered vitamins into their cofactors;

the concentration of the free forms of the vitamins was, however, relatively greater.

The results, suggesting an impairment in the biosynthesis of these cofactors in vitamin B_{12} deficiency, are discussed. Conditions arising out of possible damage to mitochondrial integrity, as well as the effects of the vitamin in relation to the mode and site of synthesis of the cofactors, are discussed.

LITERATURE CITED

Bessey, O. A., O. H. Lowry and R. H. Love 1949 The fluorometric measurement of the nucleotides of riboflavin and their concentration in tissues. J. Biol. Chem., 180: 755.

Boxer, G. E., W. E. Ott and C. E. Shonk 1953 Influence of vitamin B_{12} on the coenzyme A content of the liver of chicks. Arch. Biochem. Biophys., 47: 474.

Boxer, G. E., C. E. Shonk, E. W. Gilfillan, G. A. Emerson and E. L. Oginsky 1955 Changes in coenzyme A concentration during vitamin B_{12} deficiency. Ibid., 59: 24.

Carruthers, C., and V. Suntzeff 1954 The distribution of pyridine nucleotides in cellular fractions of some normal and malignant tissues. Cancer Res., 14: 29.

Dianzani, M. U. 1954 Uncoupling of oxidative phosphorylation in mitochondria from fatty livers. Biochim. Biophys. Acta, 14: 514.

——— 1955 Content and distribution of pyridine nucleotides in fatty livers. Ibid., 17: 391.

Dianzani, M. U., and M. A. Dianzani Mor 1957 Displacement of thiamine pyrophosphate from swollen mitochondria. Ibid., 24: 564.

Doctor, V. M., J. F. Elma, F. Sparks, C. M. Lyman and J. R. Couch 1954 Studies on the conversion of folic acid to citrovorum factor by avian liver homogenate. Arch. Biochem. Biophys., 49: 249.

Dowling, M., and D. S. Schweigert 1956 Role of vitamin B_{12} in nucleic acid metabolism. IV. Metabolism of C14-labeled thymidine by Lactobacillus leichmannii. J. Biol. Chem., 220: 521.

Fatterpaker, P., U. Marfatia and A. Sreenivasan 1955a Role of folic acid and vitamin B_{12} in transmethylations. I. Formation of creatine in vitro and in vivo. Ind. J. Med. Res., 43: 43.

——— 1955b Protective action of vitamin B_{12} in the hyperthyroid rat. Nature, 176: 165.

Fisher, R. A., and F. Yates 1953 Statistical Tables for Agricultural, Biological and Medical Research. Oliver and Boyd, Edinburgh.

Gamble, Jr., J. L. 1957 Aggregation of mitochondria, mitochondrial fragments, and microsomes by cytochrome C. Biochim. Biophys. Acta, 23: 306.

Gelehart, G. 1952 Thiamine pyrophosphate in fractions from rat-liver homogenates—the influence of thiamine deficient diet. Ibid., 8: 479.

Hennessy, D. J. 1941 Chemical methods for the determination of vitamin B_{12}. Ind. Eng. Chem., (Anal. ed.), 13: 216.

Jorgensen, E. 1954 Microbiological assay of vitamin B_{12}. In Methods of Biochemical Analysis, vol. 1. Interscience Publishers Inc., New York, p. 81.

Hogeboom, G. H., and W. C. Schneider 1952 Cytochemical Studies. VI. The synthesis of diphosphopyridine nucleotide by liver cell nuclei. J. Biol. Chem., 197: 611.

Hunter, Jr., F. E., J. Davis and L. Carlat 1956 The stability of oxidative and phosphorylative systems in mitochondria under anaerobic conditions. Biochim. Biophys. Acta, 20: 237.

Kaplan, N. O., A. Goldin, S. R. Humphreys, M. M. Ciotti and F. E. Stolzenbach 1956 Pyridine nucleotide synthesis in the mouse. J. Biol. Chem., 219: 287.

Kasbekar, D. K., and A. Sreenivasan 1956 Lability of intramitochondrial components in experimental liver injury. Nature, 178: 989.

Kasbekar, D. K., D. V. Rege and A. Sreenivasan 1956 Protective action of vitamin B_{12} in experimental liver injury. Ibid., 178: 989.

Kasbekar, D. K., W. V. Lavate, D. V. Rege and A. Sreenivasan 1959a A study of vitamin B_{12} protection in experimental thyrotoxicosis in the rat. Biochem. J., 72: 374.

——— 1959b A study of vitamin B_{12} protection in experimental liver injury in the rat by carbon tetrachloride. Ibid., 72: 384.

Kielley, W. W., and R. K. Kielley 1951 Myokinase and adenosinetriphosphatase in oxidative phosphorylation. J. Biol. Chem., 191: 485.

Kolmer, J. A., E. H. Spaulding and H. W. Robinson 1951 Approved Laboratory Technic, ed. 6, Appleton-Century-Crofts, New York.

Ling, C. T., and B. F. Chow 1954 The influence of vitamin B_{12} on carbohydrate and lipide metabolism. J. Biol. Chem., 206: 797.

Lynen, F. 1954 Participation of coenzyme A in the oxidation of fat. Nature, 174: 962.

McIlwain, H., and R. Rodnight 1949 Breakdown of oxidized forms of coenzymes I and II by an enzyme from the central nervous system. Biochem. J., 46: 337.

Maley, G. F., and H. A. Lardy 1955 Efficiency of phosphorylation in selected oxidations by mitochondria from normal and thyrotoxic rat livers. J. Biol. Chem., 215: 377.

Nadkarni, G. B., D. S. Wagle and A. Sreenivasan 1957 Vitamin B_{12} and the metabolism of pyridine nucleotides. Nature, 180: 659.

O'Dell, B. L., J. S. Gordon, J. H. Bruemmer and A. C. Hogan 1955 Effect of a vitamin B_{12} deficiency and of fasting on oxidative enzymes in the rat. J. Biol. Chem., 217: 1955.

Pfander, W. H., L. S. Dietrich, W. J. Monson, A. E. Harper and C. A. Elvehjem 1952 Citrovorum factor, vitamin B_{12}, and folic acid activity of whole blood of several species. Proc. Soc. Exp. Biol. Med., 79: 219.

Popp, E. M., and J. R. Totter 1952 Studies on the relationship between folic acid and coenzyme A. J. Biol. Chem., 199: 547.

Register, U. D. 1954 Effect of vitamin B_{12} on liver and blood non-protein sulphydryl compounds. Ibid., 206: 705.

Ross, G. I. M. 1952 Vitamin B_{12} assay in body fluids using *Euglena gracilis*. J. Clin. Pathol., 5: 250.

Schneider, W. C. 1956 Structural factors in metabolic regulations. Proc. 3rd Int. Congr. Biochem., Brussels, 1955. Academic Press, New York, p. 305.

Schneider, W. C., and G. H. Hogeboom 1950 Intracellular distribution of enzymes. V. Further studies on the distribution of cytochrome C in rat liver homogenates. J. Biol. Chem., 183: 123.

Sreenivasan, A. 1951 Metabolic inter-relationships between folic acid and vitamin B_{12}. Current Sci. (India), 20: 319.

Strength, D. R., N. I. Mondy and L. J. Daniel 1954 The effect of aminopterin on diphosphopyridine nucleotide. Arch. Biochem. Biophys., 48: 141.

Tapley, D. F. 1956 The effect of thyroxine and other substances on the swelling of isolated rat liver mitochondria. J. Biol. Chem., 222: 325.

Totter, J. R. 1953 The biological function of pteridine derivatives. J. Cell. and Comp. Physiol., Suppl. 1, 41: 241.

Wong, W. T., and B. S. Schweigert 1956 Role of vitamin B_{12} in nucleic acid metabolism. II. Liver coenzyme A levels in the rat. Arch. Biochem. Biophys., 60: 126.

——— 1957 Role of vitamin B_{12} in nucleic acid metabolism. V. Liver deoxyribosaldolase activity. Proc. Soc. Exp. Biol. Med., 94: 455.

Zatman, L. J., N. O. Kaplan and S. P. Colowick 1953 Inhibition of spleen diphosphopyridine nucleotidase by nicotinamide—an exchange reaction. J. Biol. Chem., 200: 197.

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Serum vitamin B_{12} in renal failure

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SYNOPSIS The serum vitamin B_{12} level was abnormally high in 14 out of 32 cases of renal failur This was probably due to impaired excretion of the vitamin, but the results of measurements of t e rate of excretion of radioactive vitamin B_{12} did not provide unequivocal evidence on this point; othe possible explanations are discussed. Renal failure must be added to the causes of high serum B_{12} level

During a study of the metabolism of vitamin B_{12} in diabetes mellitus (Matthews and Beckett, 1962), abnormally high serum B_{12} concentrations were found in some patients with renal failure due to diabetic glomerulosclerosis. Consequently it seemed important to investigate serum B_{12} concentrations in non-diabetic patients with renal failure, and the results are described here.

METHODS

Serum B_{12} concentrations were estimated in 32 patients with renal failure (Table I) and in 60 hospital control patients not suffering from renal disease or a condition known to affect the serum B_{12} concentration. Renal failure was considered to be present when, in the presence of primary or secondary renal disease, including urinary tract obstruction, the blood urea level was consistently raised above 45 mg./100 ml.

Serum B_{12} was estimated microbiologically with *Lactobacillus leichmanii* (Matthews, 1962); cyanide was used in the extraction of the vitamin from serum.

The rate of excretion of radioactive vitamin B_{12} was measured in some patients shortly after the blood sample had been taken for estimation of serum B_{12}. ^{57}Co-vitamin B_{12} 0·3 microcurie, in a total of 50 μg., was injected intramuscularly and the urine collected for an eight-hour period followed by a 16-hour period. Aliquots of the urine were counted in an annular scintillation counter, and the percentage excretion of the labelled vitamin calculated.

RESULTS

The mean serum B_{12} concentration in 60 hospital control patients was 461 $\mu\mu$g. per ml. (S.E. 25·3,

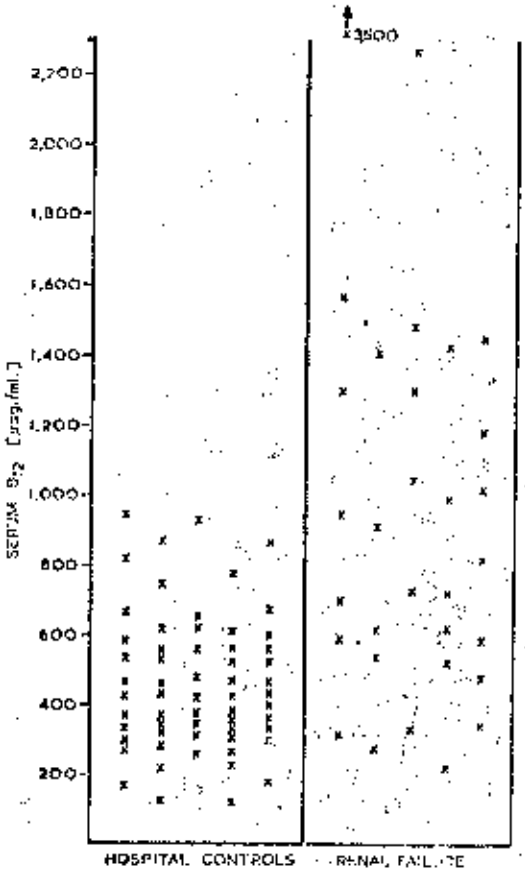

FIG. 1. *Serum B_{12} concentrations in hospital controls and in patients with renal failure.*

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TABLE I

SERUM VITAMIN B_{12} IN RENAL FAILURE

No.	Patient	Serum B_{12} (μμg./ml.)	Blood urea (mg./100 ml.)	Duration of Renal Failure (years)	Diagnosis
1	E.D.	1,500	50	About 1	Chronic pyelonephritis
2	A.C.	3[illegible]	110	> 5	Chronic pyelonephritis
3	V.H.	1,3[illegible]	135	> 5	Chronic pyelonephritis
4	L.B.	5[illegible]	75	> 10	Chronic pyelonephritis
5	A.C.	33[illegible]	55	< 1	Chronic renal failure secondary to arteriosclerosis
6	J.G.	2,2[illegible]	270	> 1	Chronic pyelonephritis
7	M.F.	5[illegible]	160	< 1	Type II nephritis
8	P.G.	9[illegible]	360	> 1	Chronic glomerulonephritis
9	P.M.	1,2[illegible]	120	> 2	Chronic pyelonephritis
10	S.K.	625	170	< 1	Malignant hypertension
11	A.P.	840	220	> 10	Polycystic kidneys
12	J.R.	1,5[illegible]	90	> 1	Nephrocalcinosis
13	J.S.	1,4[illegible]	130	> 1	Chronic pyelonephritis
14	F.T.	5[illegible]	200	> 8	Chronic pyelonephritis
15	E.L.	3,500	375	> 1	Chronic pyelonephritis
16	E.V.	5[illegible]	150	< 1	Carcinoma cervix; obstructive renal failure
17	A.C.	2[illegible]	420	< 1	Chronic glomerulonephritis
18	A.L.	6[illegible]	950	< 1	Carcinoma cervix; obstructive renal failure
19	S.H.	2[illegible]	340	> 1	Chronic nephritis ? type
20	W.B.	2[illegible]	65	< 1	Carcinoma prostate; obstructive renal failure
21	E.D.	1,7[illegible]	100	> 1	Malignant hypertension
22	E.L.	1,4[illegible]	52[illegible]	> 1	Chronic renal failure due to gout
23	A.H.	9[illegible]	185	> 1	Chronic pyelonephritis
24	J.S.	1,1[illegible]	370	> 1	Polycystic kidneys
25	A.N.	1,6[illegible]	240	About 1	Chronic renal failure; essential hypertension
26	R.M.	6[illegible]	185	> 2	Chronic renal failure; cause unknown
27	S.S.	215	165	> 5	Chronic glomerulonephritis
28	E.K.	4[illegible]	50	< 1	Type II nephritis causing malignant hypertension
29	H.M.	7[illegible]	70	< 1	Malignant hypertension
30	J.A.C.	5[illegible]	150	> 1	Chronic renal failure secondary to arteriosclerosis
31	E.L.	1,4[illegible]	150	[illegible]	Chronic renal failure; prostatic obstruction; urinary infection
32	J.O.	7[illegible]	310	< 1	Renal failure following appendicectomy ? cause

total range 115 to 950 μμg. per ml., 95% range 130 to 930 μμg. per ml.). In the 32 patients with renal failure, the mean serum B_{12} concentration was 949 μμg. per ml. (S.E. 115, total range 215 to 3,500 μμg. per ml.). The difference between the mean values (calculated by the t test) is highly significant ($P < 0{\cdot}001$).

The results in the individual patients with renal failure and the controls are compared in Fig. 1; those of the former group are given in Table I together with the diagnosis, the approximate duration of renal failure, and the blood urea level. Fourteen of these, with renal failure had a serum B_{12} concentration falling outside the upper limit of the 95% range for controls and 13 a value above the extreme limit of the control range.

The values of the patients with renal failure (Table I) show no clear relation to the height of the blood urea concentrations nor are they closely correlated with the duration of renal failure, though it is noticeable that no high values occur where the blood urea has been raised for less than a year.

The results of the excretion tests are illustrated in Fig. 2, and may be summarized as follows. In 13 control subjects the mean urinary excretion of labelled vitamin B_{12} in 24 hours was 8·3 ± 1·5%[1] of the dose of which 7·7 ± 1·6% was excreted in the first eight hours and 0·6 ± 0·2% in the next 16 hours. In seven patients with renal failure, 8·9 ± 2·8% was excreted in 24 hours, 5·7 ± 2·6% in the first eight hours and 3·2 ± 0·9% in the next 16 hours. Thus in renal failure the percentage of the labelled dose excreted in 24 hours is normal, but excretion is considerably delayed, and the patients excrete more of the dose in the latter part of the collection period than do control subjects. No correlation was observed between the results of the excretion test and the serum B_{12} concentration in individual cases.

[1] Standard error of the mean.

DISCUSSION

Several authors have shown that the urinary excretion of large doses of vitamin B_{12}, such as are used in the Schilling test, is delayed in renal failure (Rath, McCurdy, and Duffy, 1957; Dunn, Walsh, and Holthaus, 1958; Kuhlbäck and Gräsbeck, 1958; Kuhlbäck, Nyberg, and Gräsbeck, 1960) but the fact that serum B_{12} concentrations may be considerably raised has not, as far as we know, been reported. The simplest hypothesis to explain the raised values is that they are the result of failure of renal excretion of the vitamin. Although the results

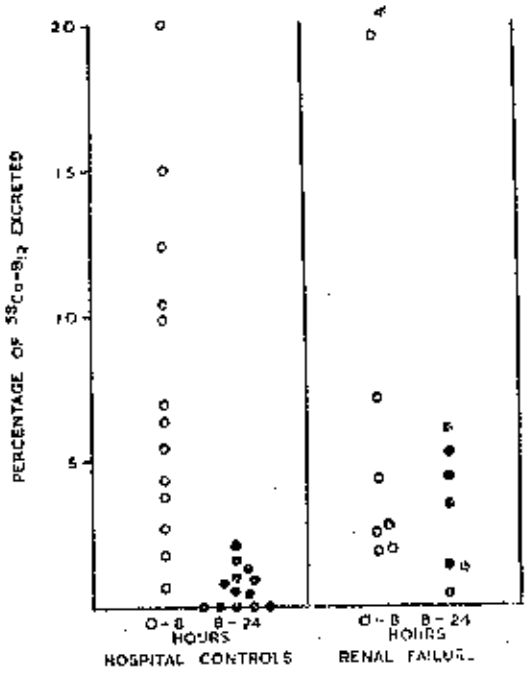

FIG. 2. *Urinary excretion of ^{58}Co-B_{12} in controls and in patients with renal failure.*

of the excretion tests which we have carried out in renal failure show that a normal percentage of the dose of labelled vitamin B_{12} is excreted in 24 hours, this does not necessarily mean that there is no serious impairment of excretion of the small amounts of vitamin B_{12} appearing in the urine under physiological conditions. The physiological relevance of tests in which very large doses of vitamin B_{12} are given, enough to raise the concentration of unbound vitamin B_{12} in the serum to a high level, is uncertain, since nothing is known of the normal mechanism of excretion of the vitamin. Since this work was carried out evidence has been presented suggesting that the excretion of large doses of vitamin B_{12} is mainly dependent on the renal filtration rate (Adams, 1961; Watkin, Barrows, Chow, and Shock (1961). The fact that no high serum B_{12} values have been observed in renal failure of short duration is in favour of the hypothesis of failure of excretion, but the occurrence of normal values in patients who have had high blood urea concentrations for over 10 years suggests that this is not the whole explanation.

Failure of excretion of vitamin B_{12}, though the most obvious explanation, is not the only possible cause of raised serum B_{12} values in renal failure. The kidney contains relatively large amounts of vitamin B_{12} (Halsted, Carroll, and Rubert, 1959), and release of the vitamin from necrosing renal tissue might raise the serum B_{12} by a similar mechanism to that operating in liver damage (Rachmilewitz, Aronovitch, and Grossowicz, 1956). Such increases would be expected to be greatest in acute renal damage such as extensive infarction or tubular necrosis; however, there are no cases of this acute type in the present series. Another possibility is that the serum B_{12} concentrations might be raised in association with an increase in serum B_{12} binding capacity, as they are in chronic myeloid leukaemia (Mollin and Ross, 1955; Miller, 1958). Such an increase in binding capacity might accompany the rise in α-globulins that is frequent in chronic renal disease (Owen, 1958). Failure of utilization of the vitamin by a bone marrow depressed by uraemia might play a part in raising the serum B_{12} concentration in the presence of inadequate excretion; however, in the present series, there was no relation between serum B_{12} and haemoglobin values. Finally, it is conceivable, though unlikely, that some unknown substance that is retained in the bloodstream in renal failure might stimulate the growth of the assay organism, so giving falsely high results. These possibilities remain to be investigated. Whatever the explanation of our results, it is evident that renal failure must be added to liver damage and certain myeloproliferative disorders as a possible cause of high serum B_{12} concentrations.

We wish to thank members of the staff of the Royal Free Hospital and Professor Clifford Wilson, London Hospital, for allowing us to study patients under their care; Mr. H. S. Williams and Miss Pamela Middleton for radioactive B_{12} excretion measurements. The work was supported by grants from the Endowment Fund of the Royal Free Hospital and the British Diabetic Association.

REFERENCES

Adams, J. F. (1961). *J. clin. Path.*, 14, 351.
Dunn, A. L., Walsh, J. R., and Holthaus, J. M. (1958). *A.M.A. Arch. intern. Med.*, 101, 927.
Halsted, J. A., Carroll, J., and Rubert, S. (1959). *New Engl. J. Med.*, 260, 575.
Kuhlback, B., and Gräsbeck, R. (1958). *Scand. J. clin. Lab. Invest.*, 10, 231.
——, Nyberg, R., and Gräsbeck, R. (1960). *Ibid.*, 12, 140.
Matthews, D. M. (1962). *Clin. Sci.*, 22, 101.
——, and Beckett, A. G. (1962). *Ibid.*, 23, 351.
Miller, A. (1958). *J. clin. Invest.*, 37, 556.
Mollin, D. L., and Ross, G. I. M. (1955). *Brit. J. Haemat.*, 1, 155.
Owen, J. A. (1958). In *Advances in Clinical Chemistry*, Vol. 1, edited by H. Sobotka and C. P. Stewart, p. 251. Academic Press, New York.
Rachmilewitz, M., Aronovitch, J., and Grossowicz, N. (1956). *J. Lab. clin. Med.*, 48, 339.
Rath, C. E., McCurdy, P. R., and Duffy, B. J. (1959). *New Engl. J. Med.*, 256, 111.
Watkin, D. M., Barrows, C. H. Jr., Chow, B. F., and Shock, N. W. (1961). *Proc. Soc. exp. Biol. (N.Y.)*, 107, 519.

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Cyanide metabolism and vitamin B_{12} in multiple sclerosis

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In the course of an investigation of vitamin B_{12} concentrations in body fluids in multiple sclerosis (Basil, Brown, and Matthews, 1965), it was noted that the proportion of total B_{12} extractable from serum in the absence of added cyanide was higher in cases of multiple sclerosis than in a group of patients with other neurological disorders. This finding might mean that the serum in multiple sclerosis contained an excess of cyanide, or might represent a disturbance in the normal proportions of cyanocobalamin and hydroxocobalamin in the blood (Matthews, 1961, 1962, 1964; Smith, 1961; Anderson, 1964; Basil *et al.*, 1965), and thus suggested the possibility of some disturbance in cyanide metabolism. Chronic exposure to cyanide can produce lesions of the central nervous system, and possibly demyelination, in animals (e.g., Hurst, 1942; Lumsden, 1950; Smith, Duckett, and Waters, 1963; Wilson, 1965), and it has been suggested that cyanide is the toxic factor in tobacco amblyopia, producing hydroxocobalamin deficiency by converting this substance to the cyano- form (Smith, 1961). Clinical and laboratory studies indicate that there is an abnormality in conversion of cyanide to thiocyanate in Leber's hereditary optic atrophy (Wilson, 1965). The present investigation was undertaken to see whether any abnormality of cyanide metabolism could be detected in multiple sclerosis, and whether there was any disturbance in the relationship between cyanide and vitamin B_{12} in this condition.

METHODS

The subjects of the present study comprised seven controls and six patients with multiple sclerosis, in-patients at The National Hospital, Queen Square.

The controls, four men and three women, were suffering from a variety of neurological disorders (idiopathic epilepsy, subarachnoid haemorrhage, chorea, meningioma, Arnold-Chiari malformation, acute infective polyneuritis and familial hypertrophic polyneuritis), and their ages ranged from 29 to 61 years (mean 46 years). The six patients with multiple sclerosis, four men and two women, were aged 18 to 51 years (mean 35 years).

Blood was drawn from each subject after an overnight fast. The specimens were drawn in pairs, one from the control group and one from a patient with multiple sclerosis, and the subsequent manipulations and analyses were carried through 'blind' on each pair. The controls were chosen as far as possible to resemble the patients with multiple sclerosis in weight, sex distribution, and degree of physical handicap. All patients were taking a normal ward diet, and none was bed-ridden. Seven patients with multiple sclerosis were originally included but one patient had to be excluded subsequently because she had been given vitamin B_{12} parenterally a few days before the blood specimen was taken. No other patient had received vitamin B_{12}.

All the patients were cigarette smokers, and smoking habits were very similar in both groups. To study the effect of smoking on cyanide metabolism, three additional patients, non-smokers, were investigated, but these were not included in the two main groups.

Plasma and urine cyanide and thiocyanate were estimated as described by Wilson (1965). Serum vitamin B_{12} was estimated with *Lactobacillus leichmannii* (Matthews, 1962); each sample was divided into two parts, one of which was assayed after extraction in the presence of cyanide (giving total serum B_{12}), and the other after extraction in the absence of cyanide.

The significance of differences between means was assessed by the *t* test.

RESULTS

The results in controls and patients with multiple sclerosis are compared in Table I. There was no significant difference between the two groups in plasma cyanide, plasma thiocyanate, urine thiocyanate, or serum vitamin B_{12}, though the urine cyanide was lower in the multiple sclerosis group ($P < 0.05 > 0.02$).

When the control patients, those with multiple

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TABLE I

PLASMA AND URINE CYANIDE AND THIOCYANATE AND SERUM VITAMIN B_{12} IN CONTROLS AND CASES OF MULTIPLE SCLEROSIS

	Controls	Multiple Sclerosis
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]

Mean ± S.E. Number of observations in parentheses.

tobacco, and the three non-smokers were grouped together, a very marked positive correlation between tobacco consumption, plasma thiocyanate concentration, and urinary thiocyanate excretion became apparent (Fig. 1) but there was no relationship between tobacco consumption and plasma or urine cyanide. No relationship was found between the proportion of serum B_{12} extractable in the absence of cyanide and tobacco consumption, or between this proportion and plasma or urine thiocyanate. There was no indication of a positive correlation between plasma cyanide and the proportion of serum B_{12} extractable without cyanide. The results did suggest, however, a negative correlation between plasma cyanide and total serum B_{12}. This correlation will be reported more fully after further investigation.

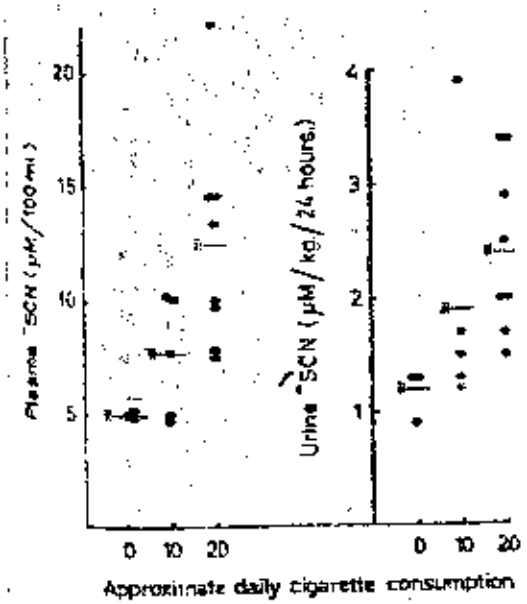

FIG. 1. *Plasma and urine thiocyanate in relation to smoking habits.*

DISCUSSION

The present results do not support the hypothesis that there is an abnormality of cyanide metabolism in multiple sclerosis. Smoking results in an increased cyanide load (Osborne, Adamek, and Hobbs, 1956), and one might expect any abnormality of cyanide metabolism to be accentuated by this, yet in these patients, all of whom were smokers, the values for plasma cyanide and thiocyanate, and for urine thiocyanate, were very similar in the control and multiple sclerosis groups. The values obtained in the two groups were also similar to those found in a larger group of normal smokers (Wilson, 1965). Though the urine cyanide was lower in multiple sclerosis than in controls, the significance of this is doubtful, since values for urinary cyanide can be markedly affected by the presence of bacteria (especially *E. coli*) and are therefore a relatively unreliable index of endogenous cyanide metabolism.

The serum B_{12} values obtained in the present work are essentially similar to those previously reported (Baril *et al.*, 1965). The total serum B_{12} in multiple sclerosis was completely normal. Though the proportion of B_{12} extractable without cyanide was apparently slightly higher in multiple sclerosis than in controls, the difference was not statistically significant in this small series. Even if this difference is real, its biological significance is uncertain. Recent work (Lindstrand and Ståhlberg, 1963; Lindstrand, 1964) suggests that B_{12} exists in blood in several forms and that hydroxocobalamin and cyanocobalamin are present only in relatively small amounts. Consequently the original hypotheses about the relationship between the proportion of B_{12} extractable from serum in the absence of added cyanide (Matthews, 1961; Smith, 1961) and the proportions of hydroxocobalamin and cyanocobalamin in blood may require modification. The possibility that these proportions are related primarily to the B_{12}-binding capacity of the plasma proteins has not yet been entirely excluded, and it has been claimed that B_{12}-binding capacity is reduced in multiple sclerosis (O'Connor, Davis, Langworthy, and Chow, 1960). Work on these problems is now in progress.

One relationship which emerges very clearly in this work is that between tobacco consumption and plasma and urine thiocyanate. This relationship is to be expected, since thiocyanate is probably the major detoxication product of cyanide (e.g., Himwich and Saunders, 1948; Hoxer and Richards, 1952). It is clearly essential to ensure that smoking habits

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are closely similar in test and control groups in any study of this type.

In conclusion, it may be pointed out that to produce neurological lesions in animals, it is necessary to give relatively large doses of cyanide over long periods, and that in some cases repeated sublethal doses have been used. This suggests that if abnormalities of cyanide metabolism were concerned in the aetiology of multiple sclerosis, they would be readily detectable. Consequently, though the present series of cases is small, the negative results suggest that further enquiry along these lines is unlikely to be fruitful.

SUMMARY

In view of observations suggesting that there might be an abnormality of cyanide/B_{12} metabolism in multiple sclerosis, plasma and urine cyanide and thiocyanate were measured in a group of patients with multiple sclerosis and in a control group with other neurological disorders. Total serum B_{12}, and serum B_{12} extractable without added cyanide, were also estimated using *Lactobacillus leichmannii*. The results did not indicate any abnormality of cyanide metabolism in multiple sclerosis. Total serum B_{12} concentrations were normal in this condition, and though there may be a slight increase in the proportion of serum B_{12} extractable without cyanide in multiple sclerosis, the significance of this is uncertain.

We are grateful to Miss J. S. Quick and to Mr. A. Pattison for technical assistance.

REFERENCES

Anderson, B. B. (1964). Investigations into the *Euglena* method for the assay of vitamin B_{12} in serum. *J. clin. Path.*, 17, 14-26.

Bazil, W., Brown, J. K., and Matthews, D. M. (1965). Observations on vitamin B_{12} in serum and C.S.F. in multiple sclerosis. *Brit.*, 18, 317-321.

Boxer, G. E., and Richards, J. C. (1952). Studies on the metabolism of the carbon of cyanide and thiocyanate. *Arch. Biochem.*, 39, 7-26.

Himwich, W. A., and Saunders, J. P. (1944). Enzymatic conversion of cyanide to thiocyanate. *Amer. J. Physiol.*, 143, 348-354.

Hurst, E. W. (1942). Experimental demyelination of the central nervous system. 3. Poisoning with potassium cyanide, sodium azide, hydroxylamine, narcotics, carbon monoxide, etc., with some consideration of bilateral necrosis occurring in the basal nuclei. *Aust. J. exp. Biol. med. Sci.*, 20, 297-312.

Lindstrand, K. (1964). Isolation of methylcobalamin from bacterial source material. *Nature (Lond.)*, 204, 188-189.

——, and Sjöberg, K. (1963). On vitamin B_{12} forms in human plasma. *Acta med. scand.*, 174, 665-669.

Lunsden, C. E. (1950). Cyanide leucoencephalopathy in rats and observations on the vascular and ferment hypotheses of demyelinating diseases. *J. Neurol. Neurosurg. Psychiat.*, 13, 1-15.

Matthews, D. M. (1961). Retrobulbar neuritis in Addisonian pernicious anaemia. *Lancet*, 1, 1289-1290.

—— (1962). Observations on the estimation of serum vitamin B_{12} using *Lactobacillus leichmannii*. *Clin. Sci.*, 22, 104-111.

—— (1964). *Vitamin B_{12} in Diabetes Mellitus*. M.D. Thesis, London.

O'Connor, J. S., Davis, R. L., Longworthy, O. R., and Chow, B. F. (1960). B_{12} metabolism and multiple sclerosis. *Proc. Soc. exp. Biol. (N.Y.)*, 103, 180-183.

Osborne, J. S., Adamek, S., and Hobbs, M. E. (1956). Some constituents of gas phase of cigarette smoke. *Anal. Chem.*, 28, 211-215.

Smith, A. D. M. (1961). Retrobulbar neuritis in Addisonian pernicious anaemia. *Lancet*, 1, 1001-1002.

——, Duckett, S., and Waters, A. H. (1963). Neuropathological changes in chronic cyanide intoxication. *Nature (Lond.)*, 200, 179-181.

Wilson, J. (1965). Leber's hereditary optic atrophy: a possible defect of cyanide metabolism. *Clin. Sci.*, in the press.

Lancet 7484: 544-545

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EFFECT OF INTRINSIC FACTOR ON ABSORPTION OF VITAMIN B_{12} FROM SMALL INTESTINE

Summary Hog intrinsic factor was found in fourteen patients to have apparently no effect on the absorption of physiological concentrations of vitamin B_{12} from segments of jejunum and ileum. The explanation of this unexpected finding may possibly be that intrinsic factor plays no part in the absorptive process of vitamin B_{12} in normal man.

INTRODUCTION

THE current view of cyanocobalamin absorption is that the vitamin must first be bound to intrinsic factor before it can be absorbed through the mucosa of human small intestine. Segments of human jejunum and ileum isolated with intestinal spring clamps at laparotomy have been shown to absorb appreciable amounts of radioactive vitamin B_{12}.[1,2] It seemed to us paradoxical that intrinsic factor should be necessary in the absorptive process when its molecular weight lies between 100,000 and 120,000,[3] whereas that of vitamin B_{12} is 1357. In view of this paradox we have investigated the effect of intrinsic factor on vitamin B_{12} absorption at laparotomy.

METHODS

During operation, segments of proximal jejunum and distal ileum were prepared by the method described by McColl and Nissim.[4] The segments were emptied by digital manipulation and washed out twice by injecting into the lumen normal saline solution at 37°C. By appropriate placing of intestinal spring clamps, segments of bowel measuring 75 sq. cm. were isolated without interfering with their blood-supply. Solutions of normal saline at 37°C containing 0·0002–0·0004 μg. of radioactive vitamin B_{12} per ml. were injected into four segments of equal surface area—two of jejunum and two of ileum. To one of the jejunal and one of the ileal segments hog intrinsic factor was added in ten patients and normal human gastric juice in one patient. The hog intrinsic factor was taken from three separate samples (prepared by Lederle Laboratories Ltd.) and used in concentrations of from 0·2 to 50 mg. per 100 ml., but mainly 20 mg. per 100 ml.

After an interval of fifteen minutes the fluid was aspirated from the segments, the volumes were noted, and the radioactivity was measured in a well scintillation counter. The percentage absorption was calculated from the amount of vitamin B_{12} which could not be retrieved. In an attempt to verify that this amount was really being absorbed, the liver uptake was estimated in two other patients. In one of these patients two segments of proximal jejunum were prepared, and into one was put ^{57}Co vitamin B_{12} and into the other ^{58}Co vitamin B_{12} plus hog intrinsic factor. In the other patient the same arrangement was made in the terminal ileum. The ratio of ^{57}Co vitamin B_{12} to ^{58}Co vitamin B_{12} in the liver was compared in the two patients with the ratio of the percentage absorption of ^{57}Co vitamin B_{12} to ^{58}Co vitamin B_{12} from the small-bowel segments. In the interval between injection and aspiration of the test solutions the surgeon was usually able to proceed with the operation, and during this time the segments under investigation were kept as near as possible to body-temperature. In five of the patients an assay was carried out on the aspirated fluid to verify that intrinsic factor was indeed absent from those segments to which it had not been added. The solutions of vitamin B_{12} were freshly made up and used within an hour. The serum-vitamin-B_{12} of most of the patients

1. McColl, I., Gacet, J.-C., Andrews, D., Raven, J., Streek, S. *Br. J. Surg.* 1966, 53, 996.
2. Gacet, J.-C., McColl, I. (1967) *Br. J. Surg.* 54, 128.
3. Grasbeck, R., Simons, K., Suokonen, J. *Acta chem. scand.* 1965, 19, 1517.
4. McColl, I., Nissim, J. A. *Nature, Lond.* 1965, 207, 949.

TABLE I—EFFECT OF INTRINSIC FACTOR (I.F.) ON ABSORPTION OF VITAMIN B_{12}

Patient no.	Conc. vitamin B_{12} put in segments (µg./ml.)	% absorption from jejunum		% absorption from ileum		Serum-B_{12} (µg./ml.)
		Without I.F.	With I.F.	Without I.F.	With I.F.	
1*	0·0002	33	21	9	11	1000
2	,,	13	6	8	5	780
3	,,	28	28	11	7	390
4	,,	20	17	7	15	442
5	0·0010	16	19	13	16	470
6	,,	20	24	7	7	950
7	,,	11	7	4	4	..
8†	0·0040	44	35	28	24	670
9	,,	31	26	33	27	..
10	,,	26	21	17	13	470

* Normal human gastric juice used instead of hog I.F. Control segments contained gastric juice which had been boiled for 10 minutes.
† Patient had treated addisonian pernicious anaemia.

was assayed using *Lactobacillus leichmannii*. The patients' permission was obtained after it had been fully explained that the proposed procedure extended the operation by ten minutes and was in no way therapeutic.

RESULTS

The results of the fourteen experiments are depicted in tables I–III. Intrinsic factor apparently made no obvious difference to the percentage absorption of vitamin B_{12}. The lowest of the three concentrations of vitamin B_{12}

TABLE II—EFFECT ON VITAMIN-B_{12} ABSORPTION OF VARYING CONCENTRATION OF I.F.

Patient no.	Conc. I.F. (mg./100 ml.)	% absorption from jejunum		% absorption from ileum	
		Without I.F.	With I.F.	Without I.F.	With I.F.
11	50	18	22	17	19
Mean values of patients 5–7	20	16	17	8	9
12	2·0	12	9	..	..
	0·2	12	8	..	..

Concentration of vitamin B_{12} put into segments was 0·001 µg. per ml.

TABLE III—VITAMIN-B_{12} ABSORPTION AND LIVER UPTAKE

Patient no.	Site of segments	I.F.	% of vitamin B_{12} absorbed	% of vitamin B_{12} detected in liver
13	Jejunum	Absent	7	1·5
		Present	6	1·3
14	Ileum	Absent	10	0·8
		Present	6	0·5

Concentration of vitamin B_{12} put into segments was 0·001 µg. per ml.

employed were less than the concentrations of vitamin B_{12} in the serum. The concentration of hog intrinsic factor was 20 mg. per 100 ml. in eleven patients. In two further patients, varying the concentration of intrinsic factor had no demonstrable effect (see table II). In another patient normal human gastric juice was used instead of hog's intrinsic factor with no appreciable difference (patient no. 1). The liver uptake of vitamin B_{12} in the two patients so studied is given in table III. The ratio of the two isotopes in the liver corresponded to the ratio of the percentage absorption of the two isotopes in the jejunum in one patient, and the same held true for the other patient in whom ileal absorption was studied. No intrinsic factor was detected in those segments to which hog intrinsic factor had not been added. The addition of 10 mg. calcium gluconate per 100 ml. in one patient did not affect absorption.

DISCUSSION

These preliminary results suggest that hog intrinsic factor does not enhance the absorption of vitamin B_{12} under the conditions of the experiment. The experiment, however, may contain a number of flaws—e.g.,

(1) Is it absorption that is being measured, and is it in any way comparable to physiological absorption?

(2) Is intrinsic factor absent from the control segments?

The liver uptake of vitamin B_{12} in the two patients so studied seemed to confirm that absorption was occurring. It is possible, however, that the vitamin B_{12} not retrieved on aspiration was absorbed later in a more physiological environment.

An attempt was made to simulate physiological conditions by using solutions containing concentrations of vitamin B_{12} which were approximately of the same order as those in the serum.

Although no intrinsic factor was detected in the segments after washing with normal saline solution it is possible that particles of intrinsic factor may have been present as suggested by Nabet and Wolff.[5] Two patients with pernicious anaemia were therefore included in the investigation, and vitamin B_{12} was absorbed whether hog intrinsic factor was added or not (patients no. 7 and 8, table I).

If absorption occurs under these circumstances, how can our findings be equated with the current view, that intrinsic factor does increase absorption of vitamin B_{12}? In pernicious anaemia the stomach and upper intestine may contain coliform organisms which compete successfully with the mucosa for vitamin B_{12},[6] and when intrinsic factor is present it combines with the vitamin B_{12} and protects it from coliform organisms and allows it to be absorbed. This implies that intrinsic factor has a protective role rather than a direct effect on absorption, and furthermore may have no function at all in normal man whose upper intestine is normally sterile.

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5. Nabet, P., Wolff, R. *C. r. Séanc. Soc. Biol. Strasbourg*, 1964, 158, 868.
6. Hoff-Jørgensen, E. *Archr Biochem. Biophys.* 1952, 38, 235.

Smoking in Pregnancy and Vitamin B_{12} Metabolism

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Summary

In pregnancy the level of serum vitamin B_{12} is lower in women who smoke than in non-smokers. This finding occurs independently of social class, parity, or level of haemoglobin. In addition, the mean serum B_{12} level tends to be less in women who are anaemic and is less in those women who have smaller babies. These findings may be an effect of the cyanide content of tobacco smoke, since cyanide may be detoxified by a mechanism which depletes the stores of vitamin B_{12} in the body.

Introduction

Two factors of importance in obstetrics which seem to be directly related to tobacco smoking are the increased incidence of small babies (Lowe, 1959; Herriot, Billewicz, and Hytten, 1963; O'Lane, 1963) and the decreased incidence of toxaemia and hypertension (Duffus and MacGillivray, 1968). Various ways have been suggested by which these effects may be produced, but little attention has been paid to the cyanide content of tobacco. However, Wilson and Matthews (1966) showed an association between tobacco smoking, cyanide detoxication, and serum vitamin B_{12} levels in both normal subjects and those with certain neurological disorders.

The present study was designed to investigate any relation between smoking in pregnancy and serum B_{12}.

Patients and Methods

All women attending an antenatal booking clinic at one Cardiff hospital had a serum B_{12} estimation. Patients were included in the study regardless of the stage of pregnancy at which they first booked, but subsequently results were subdivided according to the period of gestation. Obstetric data and details of smoking habit were obtained from the Cardiff Births Survey records. Inquiry into the cigarette smoking habits of each patient was made retrospectively during the puerperium.

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The method used in estimating the serum B_{12} was that utilizing *Euglena gracilis* (Hutner, Bach, and Ross, 1956). In all, 847 women were examined at the time of their first clinic visit. After exclusion of those pregnancies ending in multiple births or stillbirths, the results for the remaining 826 were recorded (Fig. 1).

Included were 22 patients of non-Caucasian extraction. As religious habits are particularly likely to influence serum B_{12} levels these patients were studied separately. Only 4 of the 22 had a serum B_{12} below 100 pg/ml (two Hindus, one Moslem, and one Sikh), and the mean level of the group was 146·7 pg/ml, compared with 172·2 pg/ml for the Caucasian population. These patients were then included in the general analysis as they form an increasingly large part of the population of this country.

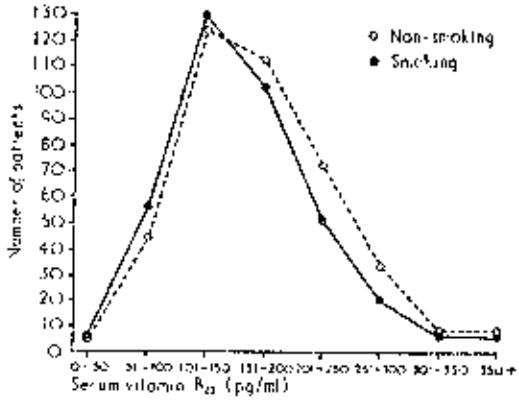

Fig. 1—Distribution of values of serum B_{12}

Results

Gestation Period.—Regardless of the period of gestation the mean level of serum B_{12} among smokers is significantly less than among non-smokers (Table I). The difference is greatest in early pregnancy. Table II shows the fall in mean serum B_{12} associated with increasing smoking. The 20 "ex-smokers" included in this table are those who stopped smoking in early pregnancy and are omitted from all other tables.

Parity.—No significant variation in serum B_{12} was found among women of different parity, although the B_{12} level tends to be lower among those who smoke regardless of their parity (Table III). The difference is least among women of high parity who, regardless of their smoking habit, may already have low B_{12} stores.

TABLE I—*Serum B_{12}, Gestation, and Smoking Habit*

	Period of Gestation			Any Gestation Period
	<20 weeks	20-30 weeks	>30 weeks	
Non-smokers:				
Number of cases	243	84	89	414
Mean serum B_{12} (pg/ml)	150·8	165·1	151·9	157·0
Standard deviation	70·9	73·8	54·9	70·8
% distribution	58·5	20·4	21·3	100
				*
Smokers:				
Number of cases	184	59	110	353
Mean serum B_{12} (pg/ml)	170·1	161·7	148·4	161·9
Standard deviation	66·4	67·9	69·9	67·7
% distribution	46·0	23·2	28·7	100
All cases:				
Number of cases	426	173	199	797
Mean serum B_{12} (pg/ml)	161·0	164·6	149·9	159·2
Standard deviation	69·7	70·7	62·8	69·5

Analysis of variance for B_{12} at period of gestation, F = 15·3. Significant at 1% level.

* Test between mean serum B_{12} at 20 weeks and at 30 weeks gestation:

	t	P	
Non-smokers	5·01	<0·001	
Smokers	2·74	<0·01	>0·001
All women	5·72	<0·001	

† Test between mean serum B_{12} of smokers and non-smokers tested at

	t	P	
20 weeks	2·09	<0·01	>0·001
20-30 weeks	<1·96	>0·05	
30 weeks	<1	>0·05	
Any stage	3·29	<0·01	>0·001

TABLE II—*Serum B_{12} and Amount Smoked*

	No.	Mean Serum B_{12} (pg/ml)	Standard Deviation
Non-smokers	414	177·9	70·8
Ex-smokers	29	172·3	59·0
Smokers:			
1-4 cigarettes	58	164·2	59·5
5-9 "	103	172·6	72·7
10-19 "	152	156·3	64·7
20+ "	70	150·3	67·2

Analysis of variance, F = 2·88.
Significant between 5% and 2% levels.

TABLE III—*Serum B_{12}, Parity, and Smoking Habit*

	Parity			
	0	1	2	3+
Non-smokers:				
Number	178	115	54	67
Mean serum B_{12} (pg/ml)	187·7	172·5	177·7	161·3
Standard deviation	74·6	68·6	63·5	70·8
Smokers:				
Number	149	108	54	72
Mean serum B_{12} (pg/ml)	170·4	150·3	172·3	153·6
Standard deviation	69·6	54·7	84·2	61·8
All cases:				
Number	327	223	108	139
Mean serum B_{12} (pg/ml)	179·8	161·7	175·0	157·4
Standard deviation	72·8	62·0	74·3	66·2

† Test between mean serum B_{12} of smokers and non-smokers:

	t	P	
Parity 0	2·17	<0·05	>0·01
" 1	2·73	<0·01	>0·001
" 2	0·33	>0·05	
" >3	0·66	>0·05	

Social Class.—There is a tendency towards a lower mean serum B_{12} in women of lower social class but this finding does not reach levels of significance (Table IV). However, the difference between the mean serum B_{12} of those women who smoke and those who do not is significant for all social classes except social class V.

Anaemia.—The mean serum B_{12} level falls progressively with the level of haemoglobin in pregnancy, but for any haemoglobin level the mean B_{12} level is less among women who smoke during pregnancy (Table V).

TABLE IV—*Serum B_{12}, Social Class, and Smoking Habit*

	Social Class			
	I and II	III	IV	V
Non-smokers:				
Number	123	93	114	81
Mean serum B_{12} (pg/ml)	183·3	180·3	170·7	155·8
Standard deviation	71·3	81·9	76·9	69·2
Smokers:				
Number	62	63	134	63
Mean serum B_{12} (pg/ml)	162·3	163·5	159·8	156·7
Standard deviation	68·6	61·9	79·7	65·0
All cases:				
Number	185	156	272	125
Mean serum B_{12} (pg/ml)	179·5	179·9	168·4	164·9
Standard deviation	71·0	75·3	79·8	66·9

Analysis of variance for social class, F = 1·33.
Not significant at 5% level.

† Test between mean serum B_{12} of smokers and non-smokers:

	t	P	
Social class I and II	2·03	<0·05	>0·01
" " III	2·74	<0·05	>0·01
" " IV	2·02	<0·05	>0·01
" " V	0·33	>0·05	

TABLE V—*Serum B_{12}, Anaemia, and Smoking Habit*

	Haemoglobin at First Clinic Visit		
	<60	60-69%	≥70%
Non-smokers:			
Number	7	34	373
% distribution	1·7	8·2	90·1
Mean serum B_{12} (pg/ml)	149	149	193·2
Standard deviation	38·2	70·9	69·9
Smokers:			
Number	2	38	343
% distribution	0·5	9·9	89·6
Mean serum B_{12} (pg/ml)	139	131·5	165·4
Standard deviation	42·4	59·0	67·1
All cases:			
Number	9	72	716
Mean serum B_{12} (pg/ml)	137·8	139·8	173·6
Standard deviation	36·6	67·4	69·0

Analysis of variance for anaemia, F = 9·18.
Significant at 1% level.

† Test for mean serum B_{12} of smokers and non-smokers.

	t	P	
Haemoglobin <60%	0·3	>0·05	
" 60-69%	1·69	>0·05	
" ≥70%	3·98	<0·001	

Serum B_{12} and Hypertension in Pregnancy.—In this study "hypertension" is defined as a diastolic blood pressure of 90 mm Hg or a systolic blood pressure of 150 mm Hg recorded on at least two occasions before pregnancy or before the twentieth week. Mild or moderate pre-eclampsia is recorded when after the twentieth week of pregnancy the diastolic blood pressure rises above 90 mm Hg on at least two occasions accompanied by clinical oedema and/or proteinuria of less than 1 g/litre. Severe pre-eclampsia is defined when the above findings are associated with proteinuria of more than 1 g/litre. This study again confirms the lower incidence of both hypertension and pre-eclampsia in those who smoke. The mean serum B_{12} is higher among hypertensive non-smoking women than among normotensive non-smoking women although lower in smokers who are hypertensive than in those who are normotensive (Table VI). The mean serum B_{12} levels in the two groups do not show any statistically significant difference.

Serum B_{12} and Infant Weight.—The increased incidence of small babies born to women smoking in pregnancy is again confirmed in this study. In those patients who had their B_{12} level estimated before the twentieth week of pregnancy there are pronounced differences in B_{12} levels between smokers and non-smokers, the greatest difference between the groups being found in women having babies weighing 2,500 g or less (Table VII). The mean B_{12} level for both groups of women rises with infant size although not to a significant level.

TABLE VI—*Serum B_{12}, Hypertension, and Smoking Habit*

	Hypertension		Pre-eclampsia	
	No	Yes	Mild and Moderate	Severe
Non-smokers:				
Number	328	32	49	6
% distribution	79·4	7·8	11·9	1·0
Mean serum B_{12} (pg/ml)	194·5	201	182·9	212·8
Standard deviation	70·1	81·1	65·9	51·0
Smokers:				
Number	344	16	22	—
% distribution	95·1	4·2	5·8	—
Mean serum B_{12} (pg/ml)	163·9	158·9	151·4	
Standard deviation	67·6	57·5	58·0	
All cases:				
Number	672	48	71	4
Mean serum B_{12} (pg/ml)	178·6	187·0	173·1	212·8
Standard deviation	69·0	64·8	51·0	
	(2 cases with renal disease excluded)			

t Test = 1·2045 P>0·05 Non-smokers—hypertension and no hypertension.
" = 0·2662 P>0·05 Smokers—hypertension and no hypertension.
" = 1·492 P>0·05 Non-smokers—pre-eclampsia and no pre-eclampsia.
" = 0·842 P>0·05 Smokers—pre-eclampsia and no pre-eclampsia.

TABLE VII—*Serum B_{12} before 20th Week, Infant Weight, and Smoking Habit*

	<2,500 g	2,500–2,950 g	>2,950 g
Non-smokers:			
Number	7	36	199
Mean serum B_{12} (pg/ml)	163·7	183·4	192·4
Standard deviation	37	55·7	73·7
Smokers:			
Number	7	42	132
Mean serum B_{12} (pg/ml)	142·9	167·0	171·7
Standard deviation	49·5	68·1	67·7
All cases:			
Number	14	78	331
Mean serum B_{12} (pg/ml)	163·8	174·6	184·1
Standard deviation	44·9	62·8	71·3

Analysis of variance for infant weight, F = 1·08.
Not significant at 5% level.

t Test for mean serum B_{12} of smokers and non-smokers:

	t	P
Infant weight <2,500 g	1·8	>0·05
" " 2,500–2,950 g	1·17	>0·05
" " >2,950 g	2·67	<0·05 >0·01

Serum B_{12} and Maternal Weight.—The weight of the patient at 20 weeks' gestation was recorded or computed on the basis of 0·5 kg a week weight gain taken from the nearest recorded weight. The mean serum B_{12} is always lower among smokers than non-smokers of similar weight, and there is also a fall of mean serum B_{12} with increasing maternal weight which does not reach levels of significance (Table VIII).

TABLE VIII—*Serum B_{12}, Maternal Weight, and Smoking Habit*

	Maternal Weight (kg)		
	<55	55–74	≥75
Non-smokers:			
Number	54	293	55
Mean serum B_{12} (pg/ml)	186·5	177·6	172·1
Standard deviation	75·5	73·0	59·7
Smokers:			
Number	63	264	51
Mean serum B_{12} (pg/ml)	174·8	159·5	156·7
Standard deviation	70·5	65·4	65·6
All cases:			
Number	117	557	106
Mean serum B_{12} (pg/ml)	181·2	169·0	164·7
Standard deviation	73·9	70·0	62·8

Analysis of variance for maternal weight, F = 1·84.
Not significant at 5% level.

t Test for mean serum B_{12} of smokers and non-smokers:

	t	P
Maternal weight <55 kg	1·0	>0·05
" " 55–74 kg	3·09	<0·01 >0·001
" " ≥75 kg	1·26	>0·05

Discussion

SERUM B_{12} LEVELS IN PREGNANCY

Various factors may affect the level of serum B_{12}. In pregnant women the levels are known to be lower than in non-pregnant women of similar age (Boger, Wright, Bech, and Bayne, 1956; Temperley, Meehan, and Gatenby, 1968). There is a progressive fall in serum B_{12} levels throughout pregnancy which is thought to be due to the concentration across the placenta of the vitamin by the fetus (Edelstein and Metz, 1969). A second possible cause is that the serum of pregnant women may contain an inhibitor to the growth of test organisms used in the assay of B_{12} (Lawrence and Klipstein, 1947). Further, the haemodilution of pregnancy may play a small part in the total fall in serum B_{12} level. There may also be changes in the serum B_{12}-binding capacity (Lowenstein, Brinton, and Hsieh, 1956).

Neither maternal age nor parity has been shown to be related to serum B_{12} levels in pregnancy (Ball and Giles, 1964; Temperley *et al.*, 1968). Ball and Giles found an association between anaemia (particularly megaloblastic anaemia) and reduction in serum B_{12} although Whiteside, Ungar, and Cowling (1968) found no association between serum B_{12} and haemoglobin levels.

Attempts have been made to relate variations in the level of serum B_{12} to abnormal pregnancy states. An increase in either premature delivery or retardation of infant growth, associated with low maternal B_{12} levels, has been described by several workers (Temperley *et al.*, 1968; Whiteside *et al.*, 1968). Temperley's other finding was of a relatively low serum B_{12} level among women with accidental haemorrhage unassociated with pregnancy toxaemia as compared with that for women having toxaemic accidental haemorrhage. Sure (1951), in a study of rats, was also able to show an increased incidence of stillbirths and neonatal deaths among animals fed on a vitamin-B_{12}-deficient diet.

EFFECTS OF SMOKING IN PREGNANCY

Smoking during pregnancy has been shown to affect the incidence of abortion, perinatal death, premature labour, infant weight, and hypertension in pregnancy.

The small baby born to a woman smoking during pregnancy may be born early or born small, the second effect being greater. Social class, poor maternal nutrition, and lack of appetite may affect fetal nutrition, but smoking may also have some direct inhibitory effect on fetal nutrition. Fetal nutrition may also be affected by the raised levels of carboxyhaemoglobin in those women who smoke (Heron, 1962). Montell (1964) described the part played by the enzyme carbonic anhydrase, which is diminished in amount in the cord blood of infants born to those women smoking in pregnancy.

Placental circulation may be affected either by the effect of nicotine or by actual placental separation. Nicotine has a complex effect on the circulatory system which according to Volle and Koelle (1970) is mainly vasoconstrictor although Coffman and Javett (1963) produced evidence that nicotine dilates calf muscle capillaries.

Constituents of tobacco other than nicotine have been largely ignored in discussions on the mode of action of smoking on fetal size and maternal blood pressure, although some animal studies have recently suggested that nicotine is either not or only partially responsible for the effects of smoking in pregnancy. Volle and Koelle (1970) described tobacco as consisting of between 0·5 and 8% nicotine, with pyridine and nitrogenous bases, plus only a very low concentration of hydrocyanic acid.

Yoshosai, Pelson, and Haworth (1969) exposed groups of pregnant rats to one of three types of cigarette, tobacco, lettuce leaf, and lettuce leaf with added nicotine. All the rats therefore inhaled similar amounts of carbon monoxide and fetuses of all rats were growth-retarded, but in the rats exposed to tobacco cigarettes fetal weight was reduced more than in other groups.

Kirschbaum, Dilts, and Brinkman (1970) performed experiments on pregnant ewes. The animals were injected with nicotine or the smoking of cigarettes was simulated and although changes were produced in the cardiovascular function of the ewe, these changes were not reflected in the fetus, and the authors concluded that the adverse effects of maternal smoking stemmed from some chronic effect of smoking, not measured in the study, as the fetus seemed to be protected from effects of both nicotine and carbon monoxide.

SMOKING AND SERUM B_{12}

Cyanide is detoxicated in the body by three possible routes and is also excreted unchanged by the lungs (Swinyard, 1970). The principal route for cyanide detoxication is by combination with thiosulphate and requires rhodanase, a liver enzyme. Thiocyanate thus becomes the principal detoxication product of cyanide. Cyanide may also be combined with cystine and other amino-acids, which might become important in women with poor nutrition where competition between fetal protein needs and detoxication of cyanide could occur. A further detoxication mechanism is possibly that cyanide becomes combined with hydroxycobalamin to form cyanocobalamin, which has been described as physiologically inactive (Smith, 1961; Lindstrand, Wilson, and Matthews, 1966).

Smith (1961) showed that sublethal doses of cyanide when administered to rats caused depletion of liver B_{12}. It has been shown that the plasma thiocyanate concentration is higher in smokers than in non-smokers (Stein, 1957; Monckosse and Wilson, 1966; Wilson and Matthews, 1966). The condition of tobacco amblyopia is caused by failure of the normal mechanisms of cyanide detoxication, which may be due to relative or absolute deficiency of B_{12}; the condition is also known to be associated with poverty and malnutrition (Darby and Wilson, 1967).

In the absence of such abnormalities B_{12} metabolism has been shown to be altered in smokers. Linnell, Smith, Smith, Wilson, and Matthews (1968) found the mean thiocyanate urinary excretion in smokers to be double that of non-smokers and was also able to show a negative correlation with serum B_{12} concentration. One mechanism suggested for these changes is that in subjects with relatively low serum B_{12} concentrations there is a reduced ability to detoxicate cyanide by pathways involving this vitamin so that detoxication by the thiocyanate pathway is increased. Alternatively, as shown by Wilson and Matthews (1966), the coexistence of high thiocyanate and B_{12} urinary excretion with the low serum B_{12} levels found among smokers may reflect B_{12} depletion resulting from the conversion of tissue cobalamin to cyanocobalamin. Such effects, although slight in normal subjects, might become significant in patients with marginal reserves of B_{12}.

Most of the work by Wilson (1969) and Wilson, Linnell, and Matthews (1971) has been in connexion with tobacco amblyopia, Leber's hereditary optic atrophy, and tropical neuropathy but Wilson also suggested a connexion between hypertension in pregnancy, cigarette smoking, and B_{12} metabolism.

SMOKING IN PREGNANCY, SERUM B_{12}, AND CYANIDE DETOXICATION

In suggesting a connexion between smoking in pregnancy and the level of serum B_{12} we have postulated a disorder related to cyanide detoxication.

Infant weight may be decreased in those cases where the mother already has poor body reserves of protein or serum B_{12}. In the former case the detoxication of cyanide, using essential amino-acids, may compete with fetal demand, and in the latter the already low B_{12} reserves of the mother may lead to increasing detoxication of cyanide by the thiocyanate pathway. It is also possible that the low serum B_{12} levels found among those women who smoke in pregnancy may only reflect B_{12} depletion resulting from conversion of tissue cobalamin to cyanocobalamin.

A further possibility is that the presence of higher levels of cyanide in the serum of women smoking in pregnancy adversely affects the test organism used in the method of estimating serum B_{12}. This last postulation seems unlikely as Matthews (1962) found no effect on the growth of the test organism when high concentrations of cyanide were added.

Concerning the mechanism of the relative hypotension found in pregnant women who smoke it is possible to postulate that the thiocyanate produced in detoxication of cyanide is responsible. Thiocyanate was at one time used as a hypotensive agent (Nickerson, 1970). Thus women with already low stores of B_{12} who also smoke are those in whom the detoxication of cyanide by the B_{12} pathway is minimal, with increased use of the thiocyanate pathway.

Further work is required to study thiocyanate and cyanide levels in pregnancy and radioimmune assay of serum B_{12}, and to relate the results to normal and abnormal conditions found in pregnancy. The present study does, however, seem to furnish further evidence that smoking during pregnancy is particularly contraindicated in the patient with existing poor nutrition such as exists in those women of higher parity, lower social class, or with coexistent anaemia.

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References

Ball, E. W., and Giles, C. (1964). *Journal of Clinical Pathology*, 17, 165.
Boger, W. P., Wright, L. D., Beck, G. D., and Bayne, G. M. (1956). *Proceedings of the Society for Experimental Biology and Medicine*, 92, 140.
Coffman, J. D., and Jayett, S. L. (1963). *Circulation*, 28, 932.
Darby, P. W., and Wilson, J. (1967). *British Journal of Ophthalmology*, 51, 336.
Duffus, G. M., and MacGillivray, I. (1968). *Lancet*, 1, 994.
Edelstein, T., and Meix, J. (1969). *Journal of Obstetrics and Gynaecology of the British Commonwealth*, 76, 945.
Heron, H. J. (1962). *New Zealand Medical Journal*, 61, 545.
Herriot, A., Hillewicz, W. Z., and Hytten, F. E. (1962). *Lancet*, 1, 771.
Hutner, S. H., Bach, M. K., and Ross, C. I. M. (1950). *Journal of Protozoology*, 3, 101.
Kirschbaum, T. H., Dilts, P. B., and Brinkman, C. R. (1970). *Obstetrics and Gynaecology*, 35, 527.
Lawrence, C., and Klopstein, F. A. (1967). *Annals of Internal Medicine*, 66, 25.
Lindstrand, K., Wilson, J., and Matthews, D. M. (1966). *British Medical Journal*, 2, 988.
Linnell, J. C., Smith, A. D. M., Smith, C. L., Wilson, J., and Matthews, D. M. (1968). *British Medical Journal*, 2, 215.
Lowe, C. R. (1959). *British Medical Journal*, 2, 613.
Lowenstein, L., Brinton, L., and Hsieh, Y. (1966). *Canadian Medical Association Journal*, 94, 636.
Matthews, D. M. (1962). *Clinical Science*, 22, 101.
Monckosse, G. L., and Wilson, J. (1966). *Lancet*, 1, 1062.
Montell, C. D. (1964). *New Zealand Medical Journal*, 63, 601.
Nickerson, M. (1970). In *Pharmacological Basis of Therapeutics*, ed. L. S. Goodman and A. Gilman, 4th edn., p. 738. New York, Macmillan.
O'Lane, J. M. (1964). *Obstetrics and Gynaecology*, 22, 181.
Smith, A. D. M. (1961). *Lancet*, 1, 1001.
Stein, K. F. (1957). *Årbok for Universitetet i Bergen*, 2, 1.
Stare, F. (1951). *Journal of the American Dietetic Association*, 27, 564.
Swinyard, E. (1970). In *Pharmacological Basis of Therapeutics*, ed. L. S. Goodman and A. Gilman, 4th edn., p. 930. New York, Macmillan.
Temperley, I. J., Mechan, M. J. M., and Gardiner, P. B. B. (1968). *Journal of Obstetrics and Gynaecology of the British Commonwealth*, 75, 511.
Volle, R. L., and Koelle, G. B. (1970). In *Pharmacological Basis of Therapeutics*, ed. L. S. Goodman and A. Gilman, 4th edn., p. 585. New York, Macmillan.
Wilson, J. (1968). *Lancet*, 1, 1357.
Wilson, J., Linnell, J. C., and Matthews, D. M. (1971). *Lancet*, 1, 259.
Wilson, J., and Matthews, D. M. (1966). *Clinical Science*, 31, 1.
Whiteside, M. G., Unger, R., and Cowlum, D. C. (1966). *Medical Journal of Australia*, 1, 338.
Youssefzadeh, M. K., Peloso, J., and Haworth, J. C. (1969). *American Journal of Obstetrics and Gynaecology*, 104, 1207.

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CONGENITAL PERNICIOUS ANEMIA: EFFECTS ON GROWTH, BRAIN, AND ABSORPTION OF B_{12}

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ABSTRACT. Three Irish children, two of them siblings, developed megaloblastic anemia around one year; deficiency of intrinsic factor (I.F.), presumably congenital, was shown by Schilling tests and assay of gastric juice. Congenital pernicious anemia (P.A.), in contrast to adult P.A., shows normal gastric mucosa and acidity and no antibodies to gastric mucosa or I.F. Adult type P.A., with gastric atrophy and achlorhydria, if occurring in childhood, can be called "juvenile P.A." "congenital P.A." being reserved for the type described here. P.A. associated with polyendocrinopathy and the type described by Imerslund due to specific B_{12} malabsorption should be named accordingly. Congenital P.A.'s inheritance is thought to be autosomal recessive.

Growth retardation and acceleration appeared to be related to B_{12} deficiency and treatment. I.Q.'s of around 70 in each child may represent the effects of B_{12} deficiency on cerebral growth. An initial malabsorption of B_{12} was shown to improve markedly with B_{12} treatment. In diagnosis of megaloblastic anemia, only that due to B_{12} deficiency will respond to dosage of 2 to 5 μg B_{12} (intramuscular) daily. Early diagnosis and treatment may prevent brain damage. *Pediatrics*, 42:149, 1968. ANEMIA, PERNICIOUS, CONGENITAL, GROWTH, BRAIN, VITAMIN B_{12}.

Three types of pernicious anemia (P.A.) due to vitamin B_{12} deficiency arising from endogenous causes are known to occur in childhood.[1] The type dealt with in this paper could best be called congenital pernicious anemia (C.P.A.),[2] the primary fault being failure to secrete gastric intrinsic factor (I.F.) despite normal gastric mucosa and acidity. An alternative name for C.P.A. would therefore be congenital intrinsic factor deficiency. "Adult" pernicious anemia (P.A.) may occur in later childhood and could then be called juvenile pernicious anemia. Another variety of P.A. with gastric atrophy and achlorhydria may occur in association with hypofunction of the thyroid, parathyroid, or adrenal glands,[1,3-5] but antibodies to I.F. are usually found, and return of I.F. secretion has been described following treatment with corticosteroids and thyroid hormone.[1] A different type of chronic relapsing megaloblastic anemia due to B_{12} deficiency was described by Imerslund and Bjornstad,[6,7] the defect is thought to be a selective malabsorption of B_{12}, but secretion of I.F. is normal and proteinuria and renal defects are usually found. Twenty-five confirmed cases of C.P.A. have been described,[2,8-24] our 3 in Ireland make 28.

The diagnosis of C.P.A. should rest on a megaloblastic anemia, usually presenting around the age of 1 year, which recurs and is potentially fatal or causative of neurological damage if untreated; low serum levels of B_{12}, which respond to small doses of B_{12}, are found. Gastric mucosa and acidity are characteristically normal, I.F. is absent or minimal, and antibodies to I.F. and gastric mucosa are absent. In contrast, adult P.A. implies gastric atrophy and achlorhydria, antibodies to gastric mucosa are found in the majority of instances, and antibodies to I.F. in over a half.[25] Exogenous causes of B_{12} deficiency, such as malnutrition, severe malabsorption, blind loops, or infection with the fish tapeworm, should be excluded.

In describing three further cases of this rare disease, emphasis is laid on the favorable effect of B_{12} on growth, on the adverse effects of B_{12} deficiency on cerebral development, and on the mechanism for its own absorption.

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SPECIAL INVESTIGATIONS

Intrinsic factor was assayed by the method of Gottlieb, *et al.*[26] following augmented histamine stimulation (0.04 mg/kg); fasting and subsequent samples were collected on ice, brought to pH 11.0 with NaOH and then to pH 7.0 with HCl, packed in "dry ice" and flown to New York on the same day. In the Schilling tests,[27] 0.5 μCi Cobalt 58 B_{12} was given fasting; this was followed 2 hours later by 1 mg intramuscularly of ordinary B_{12} and urine was collected for 24 or 48 hours (a second 1 mg of B_{12} was given before a second 24-hour collection). After July 1964 0.25 mg carbachol was given 15 minutes before the isotope; hog I.F. was given with isotope in the second and third tests in each child (Table 1). In test meals, gastric juice was collected fasting and at half hour intervals after 0.04 mg/kg histamine. Serum B_{12} was assayed with Lactobacillus Leichmanii, and serum folate was assayed with Lactobacillus Casei. Bone age was assessed using the radiological atlas of Greulich and Pyle.[28] Height and weight were plotted on the growth charts of Tanner and Whitehouse,[29] from which figures 1, 2, and 3 have been constructed.

CASE REPORTS

Case 1

A female weighing 2.95 kg. at birth in June 1957 was the fifth of seven children; she had no vitamin supplements and from 6 months had intermittent vomiting with increasing apathy, as well as a seborrheic type of dermatitis. Able to sit at 6 months she later was unable to do so. Hospitalized elsewhere at 1 year with diarrhea and vomiting, she weighed 7 kg. (Fig. 1) and, when seen by us a month later, the hemoglobin was 4.7 gm/100 ml, erythrocytes were 1.9 million/mm³; leucocytes were 4,000/mm³, neutrophils were 14%, lymphocytes were 84%, monocytes were 2%, and platelets were "reduced" in number. Two hundred milliliters of packed erythrocytes were transfused. The bone age was normal. The bone marrow was moderately megaloblastic 2 weeks later, and a marked clinical and hematological response followed intramuscular multiple vitamin preparations containing B_{12} together with oral folic and ascorbic acids. She was walking by 15 months. Further megaloblastic anemic episodes occurred at 1¾ and 3¼ years; these were attributed to folate deficiency possibly due to celiac disease even though fat excretion in two 3-day periods was between 1 and 2 gm per day. Good hematologic response occurred with oral folic acid, 15 mg daily, for 2 months in each episode; in the second period 1 mg of B_{12} was given intramuscularly and a gluten free diet was started. At 3¾ years Schilling tests (Table 1) were thought to indicate C.P.A. and 250 μg cyano-cobalamin was given monthly for 29 months. B_{12} was then stopped and 18 months later, although the hemoglobin was 12.0 gm/100 ml, the serum B_{12} had fallen to 120 pg. In April 1966 the bone age was again normal; the I.Q. was 68.

Case 2

A male sibling of Case 1 weighed 3.6 kg. at birth in September 1958. Following adequate diet with vitamin supplements, he stood in his crib at age 10 months. Anorexia, vomiting, and intermittent diarrhea caused his admission at 12 months; he weighed 6.8 kg and his growth was also retarded (Fig. 2), although the bone age was normal. His hemoglobin was 10 gm/100 ml with slight macrocytosis. He was also treated for possible celiac disease despite fecal fat of 2.9 gm daily; B_{12} 1 mg, was given intramuscularly, and oral folic acid and a gluten-free diet were started. Eighteen months later an additional 1 mg of B_{12} was given. At age 4 years, no B_{12} having been given for 18 months, the hemoglobin was 6.5 gm/100 ml, erythrocytes were 1.85 million/mm³; leucocytes were 6,500 mm³, neutrophils were 50%; the bone marrow was markedly megaloblastic and the serum B_{12} was 40 pg. A satisfactory response to oral folic acid occurred before the serum B_{12} level was known. Schilling tests were interpreted as indicating C.P.A. or mal-absorption. At 6¾ years megaloblastic anemia recurred with lemon-yellow skin and mild atrophic glossitis, the hemoglobin was 5.7 gm/100 ml and the serum B_{12} was 40 pg. No abnormal neurological signs other than some apathy were present. The anemia was partially refractory to B_{12}, 50 μg intramuscularly. A week later 100 μg B_{12} produced no rise in hemoglobin or reticulocytes; 250 μg B_{12} then produced a slow rise in hemoglobin to 10.5 gm in 4 weeks. The serum folate 1 month later was 3.2 ng, suggesting that the refractory phase may have been due to concomitant folate deficiency. In April 1966 the bone age was again normal; the I.Q. was 70.

Case 3

A male infant weighing 3.9 kg at birth in October 1961 was the second of three children and he began to walk before one year. After some weeks of vomiting he was hospitalized at 14½ months when his hemoglobin was 3.2 gm/100 ml, erythrocytes were 940,000/mm³, leucocytes were 10,000/

mm³, neutrophils were 55%, lymphocytes were 42%, and monocytes were 3%. A whole blood transfusion was given. Growth was retarded (Fig. 3), but the bone age was advanced about 9 months. Fecal fat excretion in two separate 3-day periods was 4.6 and 3.8 gm per day, respectively. Six weeks later the bone marrow was megaloblastic and the serum B_{12} was 50 pg; a prompt hematological response followed 100 µg of B_{12} intramuscularly. Two further anemic episodes responded fully to 25 µg and 500 µg of B_{12}, respectively. Schilling tests in 1964 (Table 1) were suggestive of C.P.A. In April 1966 the bone age was 5 years and actual age was 4½ years; the I.Q. was 71.

SOME DATA IN COMMON

There was no consanguinity and the two families were not related. Both Cases 2 and 3 showed normal gastric acid secretion on three occasions; Case 1 showed no free acid at ages 1½, 3½, and 8 years. Absence of I.F. in gastric juice in all three children was shown by the method of Gottlieb, *et al.*[25] in August 1965, when serum antibodies to I.F. and gastric juice were also absent. Gastric and jejunal biopsies were normal in each child, using 8 mm Crosby Kugler capsules. Serum folate was assayed several times in each child from September 1962 onwards, the levels being over 10 ng on every occasion except in Case 2 in February 1965. Since February 1965, normal blood pictures (hemoglobin levels averaging 12.0 gm/100 ml) have been maintained in each child with 500 µg cyanocobalamin every 3 months; this was changed to the hydroxy form in April 1966. On one occasion recently when Cases 1 and 2 did not return for 5 months, the serum B_{12} levels fell to 210 and 150 pg, respectively, without fall in hemoglobin. No albuminuria was found on repeated urinalysis and urinary chromatograms were normal.

TABLE 1

TWENTY-FOUR URINARY EXCRETION FOLLOWING 0.5 µCi COBALT 58 B_{12} ORALLY, ALONE, AND WITH INTRINSIC FACTOR

Patient	% Alone (date)	% with I.F. (date)	% with I.F. (date)
Case 1	0.2 (Mar 1964)	7.5* (Mar 1964)	12.0† (Feb 1965)
Case 2	0.1 (Nov 1963)	1.1* (Nov 1962)	11.0† (Feb 1965)
Case 3	1.0 (May 1961)	2.5‡ (Feb 1965)	36.0‡ (Sep 1966)

* 3.5 gm hog I.F. (Armour).
† 100 mg (2 N F) hog I.F. (Lederle).
‡ 500 mg (6 N F) hog I.F. (Lederle).

DISCUSSION

The diagnosis of congenital pernicious anemia appears to be acceptable for these three children. The occurrence of megaloblastic anemia around the end of the first year was followed by demonstration of absent I.F. by Schilling tests at ages 3 years 9 months, 4 years 2 months, and 2 years 7 months, respectively. Absence of I.F. was later confirmed by assay of gastric juice. Other diagnostic criteria which were satisfied were normal gastric mucosa, absent antibodies to gastric mucosa and I.F., and eventual normal absorption of B_{12} when combined with I.F. A satisfactory hematological response to a small dose of B_{12} was shown in Case 3. The persisting achlorhydria in Case 1 is atypical, since, although it was present initially in some other cases, it later returned following B_{12} treatment.[10,11,14] Pohl[1] described initial achlorhydria but no subsequent gastric studies. The refractory phase in Case 2 if not explicable by concomitant folate deficiency may implicate some other unrecognized factor; an analogous situation seemed to occur in Dimson's[24] patient. Diagnosis should have been made earlier, but it was delayed by our assumption of folate deficiency as the cause of the anemia and by the repeated responses to massive folate treatment. The maintenance of normal blood pictures for long periods in Cases 1 and 2 was also disconcerting; however, even longer periods without B_{12}, from 2 to 5 years, have been reported.[11,13,14,17,24] Even without presently available assays of B_{12} and folate, separation of the main causes of megaloblastic anemia should be made on

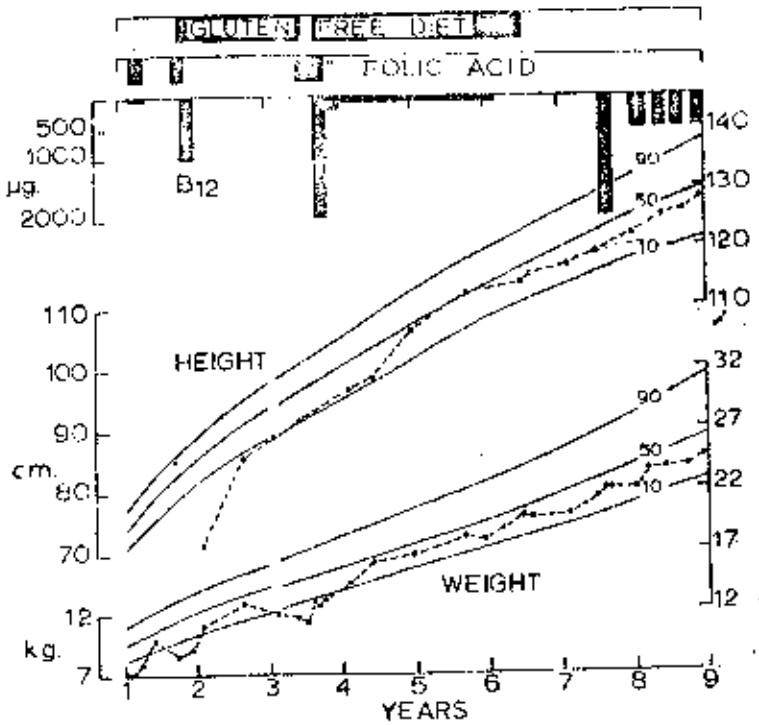

FIG. 1. Height and weight graph for Case 1.

clinical grounds. For instance, only the anemia of B_{12} deficiency should respond to intramuscular doses of the order of 2 to 5 µg of B_{12} daily or to a single dose of 10 µg. Similarly, only the anemia of folate deficiency should respond to minute doses of folic acid parenterally, e.g. 100 µg daily.[29,30]

GROWTH

Vitamin B_{12} is considered necessary for the normal growth of all species so far studied,[31] whereas there is no satisfactory evidence that the vitamin has any influence on appetite or growth in healthy subjects.[32] Improved growth following B_{12} treatment has been reported in five cases of C.P.S.,[10,13,17,20,21] although in none was the improvement as marked as in Cases 1 and 3. Case 1 showed a marked increase in height from well below the 0 percentile to the 40th percentile, and the accelerated growth phases appeared to coincide with B_{12} administration (Fig. 1). Case 2 had not passed the 10th percentile following a moderate early improvement (Fig. 2). Case 3 showed a clear-cut increase from the 20th to the 75th percentile for height (Fig. 3). Their present heights are consistent with family stature. The parents of Cases 1 and 2 are both 168 cm and the other siblings range from below zero to the 70th percentile. The father of Case 3 is 183 cm, the mother is 158 cm, and the two siblings are on the 60th and 70th percentiles. The normal bone age in the first two cases and the advanced bone age in the third would seem to exclude deficiency of growth hormone or thyroxine, and also malabsorption as causes of the impaired growth; it also suggests that B_{12} deficiency does not have any adverse effect on epiphyseal maturation. The bone age has only been recorded in three cases of C.P.A.;[17,24] in two of these it was retarded and probably associated with malabsorption and in the third it was normal. The metabolic effect of B_{12} appears to be mainly concerned with the incorporation

of amino acids into protein,[24] and probably primarily in nucleic acid synthesis. There is a close relationship to folate metabolism, since this will not proceed normally in B_{12} deficiency.[24] Vitamin B_{12} may therefore influence growth primarily through its effect on folate and nucleic acid metabolism, and it is interesting that a deficiency at this level does not seem to affect epiphyseal maturation. C.P.A. provides what is probably the only example of the isolated effect of B_{12} deficiency on growth in the human.

CENTRAL NERVOUS SYSTEM COMPLICATIONS

The spinal cord and cerebral lesions of B_{12} deficiency are well-known and may occur in C.P.A.[10,12,17,28] Continued treatment of B_{12} deficiency with folic acid is known to increase the neurological damage, and an instance of this in C.P.A. has been described.[29] Our first two patients may have been spared serious effects of this nature due to the occasional 1 mg doses of B_{12} given in the early stages as part of the treatment for their suspected celiac disease and for the Schilling tests. However, the fact that their I.Q.'s are at the borderline of subnormality (about 70) suggests that at some stage minor cerebral damage may have occurred: this may have been in the latter part of their first years when they showed regression or slowing in motor development, or during their long periods without B_{12}. As these children come from isolated rural families in which education ceased at the primary stage and as they are in the appropriate classes for age at school, some of the poor performance at intelligence testing may have an environmental background. The I.Q. has been recorded in only two other cases of C.P.A.,[15,17] when it was 88 and 60; so minor degrees of cerebral damage may well exist in some patients in whom intelligence was not formally tested. C.P.A. must therefore be added to the list of inborn metabolic errors capable of causing mental retardation. The

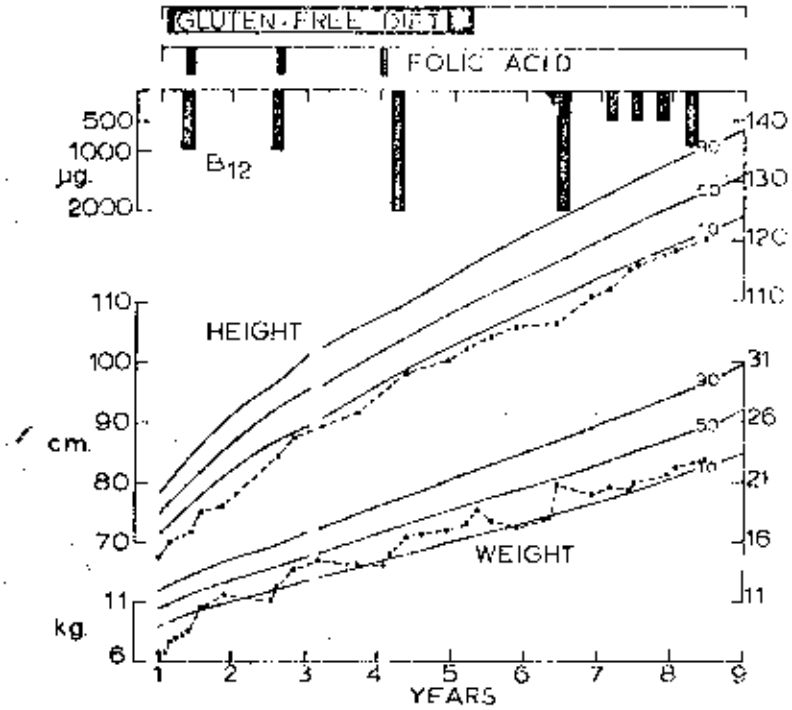

FIG. 2. Height and weight graph for Case 2.

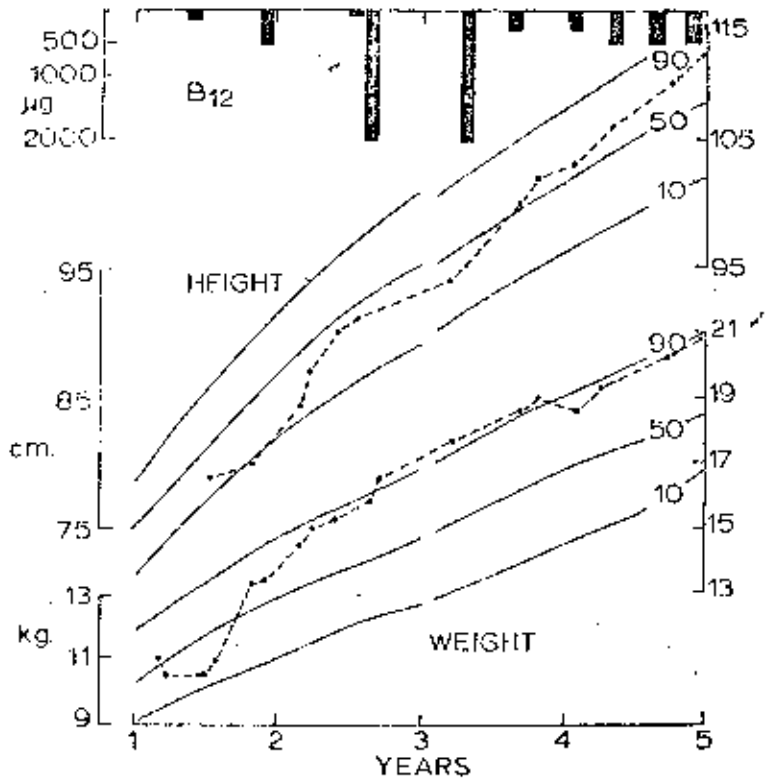

FIG. 3. Height and weight graph for Case 3.

eldest sibling in the first family, a girl of 12 years, has moderate mental handicap and marked growth retardation; she has a normal blood picture, serum B_{12} and folate but has not been studied further.

SPECIFIC MALABSORPTION OF B_{12}

No significant general malabsorption was demonstrated in our patients; fecal fat excretion and jejunal biopsies were within normal limits. Case 2 had a borderline d-Xylose excretion of 16.3% in 12 hours in 1966 and a flat lactose tolerance curve; these tests were normal in the other children. The specific malabsorption of B_{12}, first studied by Castle, *et al.*[9] and later by others[10-13] was clearly shown (Table I); this is thought to be due to the adverse effect of B_{12} deficiency on the mechanism for its own absorption. In Cases 2 and 3, addition of I.F. to the second dose of radioactive B_{12} led only to a moderate increase in uptake, whereas the third dose of B_{12} combined with I.F. showed a decided increase, particularly in Case 3, representing the improvement in absorption of B_{12} following B_{12} treatment.

INHERITANCE

The familial incidence of C.P.A. is well established and relevant studies have been recently reviewed;[2,24] the most favored explanation of the mode of inheritance is that of an autosomal recessive defect, although incomplete dominance has also been suggested.[27] Our studies contribute little to the question of inheritance. None of the parents or siblings of our patients were anemic and all had normal serum levels of B_{12} and folate. Because of slightly low levels of B_{12} and folate in the father of Case 3—these were normal on retesting—a Schilling test was made with a normal urinary excretion of 33% in 24 hours. The father of Cases 1

and 2 had slight and equivocal antibodies to gastric mucosa, the other parents being negative. Neither Schilling tests nor I.F. assays have been done on other relatives. The question of whether adult P.A. and C.P.A. are related is undecided, although the most recent suggestion[8] is that they are not. This theory is based on the grounds that cases of C.P.A. followed for up to 29 years still had normal gastric mucosa and acidity, and that antibodies to gastric mucosa and I.F. (common in the relatives of patients with classical P.A.) are rare in relatives of patients with C.P.A. Our Case 1 seems to occupy an intermediate position with persistent histamine-fast achlorhydria despite normal gastric mucosal histology.

IMPLICATIONS

Since cerebral damage may result from delay in recognition and treatment, early diagnosis of pernicious anemia is important and can be precisely made with present day methods. In the absence of full diagnostic facilities, response of a severe anemia to minute doses of B_{12} is a clear indication of deficiency of this vitamin and of the need for treatment, which will not preclude more precise diagnosis later. The megaloblastic anemia of folate deficiency should also respond only to minute doses of folic acid and occurs much more frequently than B_{12} deficiency. When having to treat megaloblastic anemia in the absence of full diagnostic facilities, B_{12} and folic should both be given, in view of the adverse effect of folate treatment on the central nervous system manifestations of B_{12} deficiency, if B_{12} is not also given.

SUMMARY

Three children, two of them siblings, developed pernicious anemia around 1 year, which was shown to be due to a presumably congenital deficiency of intrinsic factor, by means of Schilling tests and assay of gastric juice.

The adverse and beneficial effects on growth of B_{12} deficiency and treatment are shown graphically. I.Q.'s of around 70 may be attributed to adverse effects of B_{12} deficiency on cerebral growth; more severe effects have been reported. Intrinsic factor deficiency must be added to the list of inborn errors that may cause mental retardation. An initial specific malabsorption of B_{12} was shown to improve markedly with B_{12} treatment. The mode of inheritance of congenital pernicious anemia is probably autosomal recessive, and it is probably not related to adult pernicious anemia.

REFERENCES

1. Herbert, V., Streiff, R. R., and Sullivan, L.: Notes on vitamin B_{12} absorption: autoimmunity and childhood pernicious anemia; relation of intrinsic factor to blood group substance. Medicine, 43:679, 1964.
2. Miller, D. R., Blum, C. E., Streiff, R. R., LeBoglio, A. F., and Diamond, L. K.: Juvenile "congenital" pernicious anemia. New Eng. J. Med., 275:978, 1966.
3. Reisner, D. J., and Ellsworth, R. M.: Coexistent idiopathic hypoparathyroidism, and pernicious anemia in a young girl. Ann. Intern. Med., 43:1146, 1955.
4. Wilkins, L.: The Diagnosis and Treatment of Endocrine Disorders in Childhood and Adolescence. Springfield, Illinois: Charles C Thomas, p. 430, 1957.
5. Morse, W. I., Cochrane, W. A., and Landrigan, P. L.: Familial hypoparathyroidism with pernicious anemia, steatorrhoea and adrenocortical insufficiency. New Eng. J. Med., 264:1021, 1961.
6. Hung, W., Migeon, C. J., and Parott, R. H.: A possible autoimmune basis for Addison's disease in three siblings, one with idiopathic hypoparathyroidism, pernicious anemia and moniliasis. New Eng. J. Med., 269:658, 1963.
7. Imerslund, O.: Idiopathic chronic megaloblastic anaemia in children. Acta Paediat. (Uppsala), (Suppl. 119), 49, 1960.
8. Imerslund, O., and Bjornstad, P.: Familial vitamin B_{12} malabsorption. Acta haemat. (Basel), 30:1, 1963.
9. Pohl, C.: Uber perniziose anamie in Kindersalten. Mschr. Kinderheilk., 84:192, 1940.
10. Benjamin, B.: Infantile forms of pernicious (Addisonian) anemia. Amer. J. Dis. Child., 75:143, 1948.
11. Reisner, E. H., Wolff, J. A., McKay, R., and Doyle, E. F.: Juvenile pernicious anemia. Pediatrics, 8:88, 1951.
12. Mollin, D. L., Baker, S. J., and Doniach, I.: Addisonian pernicious anaemia without gas-

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tric atrophy in a young man. Brit. J. Haemat., 1:278, 1955.

13. Stevenson, T. D., Little, J. A., and Langley, L.: Pernicious anemia in childhood. New Eng. J. Med., 255:1219, 1956.

14. Harris-Jones, J. N., Swan, H. T., and Tudhope, G. R.: Pernicious anaemia without gastric atrophy and in the presence of free hydrochloric acid. Blood, 12:461, 1957.

15. Leiken, S. L.: Pernicious anemia in childhood. Pediatrics, 25:91, 1960.

16. Clement, D. H., Nichol, C. A., and Welch, A. D.: A case of juvenile pernicious anaemia; study of the effects of folic acid and vitamin B_{12}. Blood, 17:618, 1961.

17. Lambert, H. P., Frankeel, T. A. J., and Smellie, J.: Pernicious anaemia in childhood. Quart. J. Med., 30:71, 1961.

18. Oehme, J., Hundeshagen, H., and Willenbuckel, U.: Über die perniziöse Anämie des Kindes. Z. Kinderheilk., 87:289, 1962.

19. Waters, A. H., and Murphy, M. E. B.: Familial juvenile pernicious anaemia. A study of the hereditary basis of pernicious anaemia. Brit. J. Haemat., 9:1, 1963.

20. Pearson, H. A., Vinson, R., and Smith, R. T.: Pernicious anemia with neurologic involvement in childhood. J. Pediat., 65:334, 1964.

21. Wilson, J. F., cited in Herbert, V., Streiff, R. R., and Sullivan, L.: Notes on vitamin B_{12} absorption, autoimmunity and childhood pernicious anemia; relation of intrinsic factor to blood group substance. Medicine, 43:679, 1964.

22. McIntyre, O. R., Sullivan, L. W., Jeffries, G. H., and Silver, R. H.: Pernicious anemia in childhood. New Eng. J. Med., 272:981, 1965.

23. Moody, E. A.: Juvenile pernicious anemia. Northwest Med., 64:191, 1965.

24. Dimson, S. B.: Juvenile pernicious anemia. Arch. Dis. Child., 41:216, 1966.

25. Ardeman, S., and Chanarin, I.: A method for the assay of human gastric intrinsic factor and for the detection and titration of antibodies against intrinsic factor. Lancet, 2:1350, 1963.

26. Gottlieb, C., Kam Seng Lau, Wasserman, L. R., and Herbert, V.: Rapid charcoal assay for intrinsic factor, gastric juice unsaturated B_{12} binding capacity, antibody to intrinsic factor, and serum unsaturated B_{12} binding capacity. Blood, 25:875, 1965.

27. Schilling, R. F.: A new test for intrinsic factor activity. J. Lab. Clin. Med., 42:946, 1953.

28. Greulich, W. W., and Pyle, S. I.: Radiographic Atlas of Skeletal Development of the Hand and Wrist. Stanford, California: Stanford University Press, 1959.

29. Tanner, J. M., and Whitehouse, R. H.: Standards for height and weight of British children from birth to maturity. Lancet, 2:1086, 1959.

30. Herbert, V.: Current concepts in therapy: megaloblastic anemia. New Eng. J. Med., 268:201 and 368, 1963.

31. Smith, E. L.: Vitamin B_{12}. London: Methuen and Co., 1965.

32. Committee on Nutrition: Appraisal of the use of vitamins B_1 and B_{12} as supplements promoted for the stimulation of growth and appetite in children. Pediatrics, 21:860, 1958.

33. Peterson, E. W., and Register, V. D.: Effect of major dietary constituents. Proc. Soc. Exp. Biol. Med., 98:851, 1958.

34. Will, J. J., Mueller, J. F., Glazer, H. S., Friedman, R. I., and Viller, R. M.: The alteration of ascorbic acid oxidation in pernicious anemia. J. Lab. Clin. Med., 42:967, 1953.

35. Castle, W. B., Heath, C. W., and Strauss, M. B.: Observations on etiologic relationship of achylia gastrica to pernicious anemia. Amer. J. Med. Sci., 182:741, 1931.

36. Mollin, D. L., Booth, C. L., and Baker, S. J.: The absorption of vitamin B_{12} in control subjects, in Addisonian pernicious anaemia and in the malabsorption syndrome. Brit. J. Haemat., 3:412, 1957.

37. Callender, S. T., and Denborough, M. A.: A family study of pernicious anaemia. Brit. J. Haemat., 3:88, 1957.

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Effect of Folic Acid and Vitamin B_{12} Supplementation on Tests of Folate and Vitamin B_{12} Nutrition in Pregnancy

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MEGALOBLASTIC anemia is not an uncommon complication of pregnancy,[1,2] and is usually associated with folate deficiency.[3-11] While the recognition of folate deficiency of sufficient severity to cause megaloblastic anemia is not usually difficult, there is need for a test of folate nutrition during pregnancy that will accurately reflect deficiency prior to the development of anemia. Attempts have been made to demonstrate folate deficiency in pregnancy before the development of anemia.[12-14] The search for a suitable index of folate nutrition in pregnancy, however, has been complicated by the recognition that tests of folate nutrition may be affected by physiology changes in pregnancy, independent of changes in folate nutrition.[15,16]

The present study was undertaken to evaluate the significance of tests of folate and vitamin B_{12} nutrition during pregnancy by studying the effect of folic acid and vitamin B_{12} supplements on the results of the tests. If changes in the test results of folate and vitamin B_{12} nutrition in pregnancy do in fact reflect deficiency then adequate supplements of these vitamins should reverse the changes. The hemoglobin and hematocrit in groups receiving folic acid and vitamin B_{12} supplements were also determined to gain further information on the effect of supplementation during pregnancy on the incidence of anemia.

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MATERIAL AND METHODS

Subjects Studied

A total of 175 pregnant white subjects attending the antenatal clinic at South Rand Hospital, Johannesburg (altitude 5,740 feet), were studied. These subjects were subsisting on a good Western-type diet, with over 75 per cent of them taking fruit, green vegetables, meat and milk daily.

Supplementation

In the antenatal clinic, patients in early pregnancy were allocated by random numbers to one of three groups. Group 1 consisted of fifty-seven patients who were given 200 mg. of iron orally daily, either as ferrous sulfate or ferrous fumarate. Group 2 was composed of sixty patients who were given iron as in group 1 together with 5 mg. of folic acid orally daily. Group 3 was made up of fifty-eight patients who were given 50 μg. of vitamin B_{12} orally daily in addition to iron and folic acid as in group 2.

Supplementation was started after the twenty-fourth week of pregnancy, and discontinued at delivery. All tablets were dispensed in identical gelatin capsules. The vitamin B_{12} and folic acid contents of the tablets was checked by microbiologic assay. At each visit, patients were given enough tablets for twenty-five days, and instructed to return for follow-up on the twenty-eighth day. They were told not to take any tablets for two days prior to their return for follow-up examinations.

Procedure

Patients were tested at intervals of four weeks during pregnancy and at six weeks postpartum. The duration of pregnancy was determined from the date of the last menstrual period and the size of the uterus, and was checked with the date of delivery.

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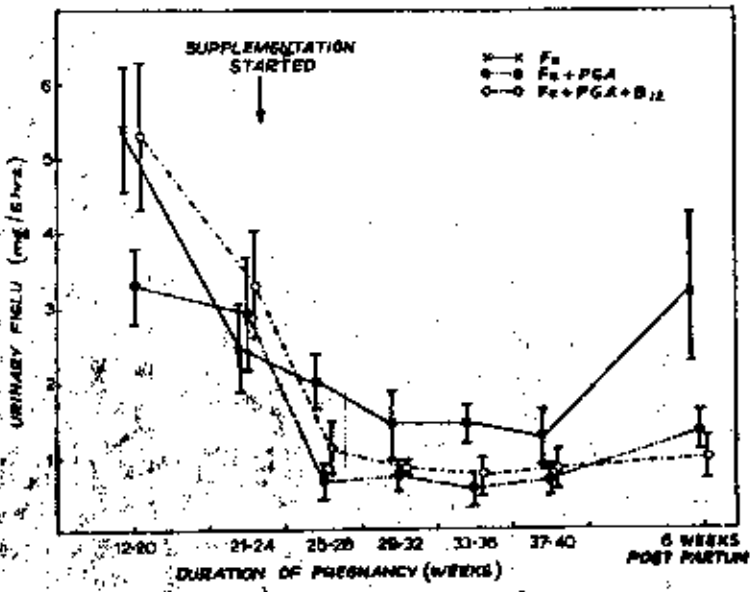

FIG. 1. Urinary FIGLU excretion after histidine loading in pregnant subjects receiving various types of supplementation.

At each visit the hemoglobin, hematocrit, serum vitamin B_{12} level, serum Lactobacillus casei folate activity, and the urinary excretion of formiminoglutamic acid (FIGLU) and urocanic acid after histidine loading were determined. On arrival at the clinic, it was ascertained that no folic acid or vitamin B_{12} tablets had been taken on the previous two days. The loading dose of 10 gm. l-histidine monohydrochloride was then given orally, and all urine voided over the ensuing 6 hours was collected. A blood sample for the determination of serum L. casei folate activity, vitamin B_{12} concentration, hemoglobin and hematocrit was then drawn.

METHODS

The 6 hour urine samples were acidified with hydrochloric acid. The urinary volume was recorded, and an aliquot stored at −20°c. until assayed. FIGLU and urocanic acid content were assayed by the spectrophotometric method of Chanarin and Bennett.[17] By this method, healthy subjects excreted less than 6 mg. FIGLU per 6 hours, and no detectable urocanic acid.

The blood samples for assay of folate and vitamin B_{12} concentration were allowed to clot and the serum separated and stored at −20°c. until assayed. The serum folate activity was assayed with L. casei according to the method of Herbert[18] and Waters and Mollin.[19] In healthy subjects, serum folate activity ranged from 3 to 15 μmg. per ml. Serum vitamin B_{12} concentration was assayed with L. leichmanii.[19] In healthy subjects, serum vitamin B_{12} concentration ranged from 214 to 1150 μμg. per ml. The blood samples for hemoglobin and hematocrit estimation were mixed with dried sequestrene. Hemoglobin was estimated as oxyhemoglobin in a photoelectric colorimeter standardized with a commercially obtained hemoglobin standard. The hematocrit was measured in an International microhematocrit centrifuge. Normal values for healthy adult women resident at this altitude are 12.3 to 17.6 gm. per cent for hemoglobin and 37 to 49 per cent for hematocrit.

RESULTS

Urinary FIGLU (Fig. 1)

Urocanic acid and FIGLU content were measured in a total of 1,169 urine specimens. Urocanic acid did not comprise a significant proportion of the total excretion of histidine metabolites, and the results are thus expressed as FIGLU excretion only. The mean FIGLU excretion was greatest in early pregnancy, and after twenty weeks fell steadily until delivery. This pattern was apparent irrespective of the type of supplementation. In subjects receiving iron supplements only, there was an increase in the mean FIGLU excretion at six weeks postpartum. This increase was negligible in the groups receiving folic acid supplements. At all stages of pregnancy, the mean

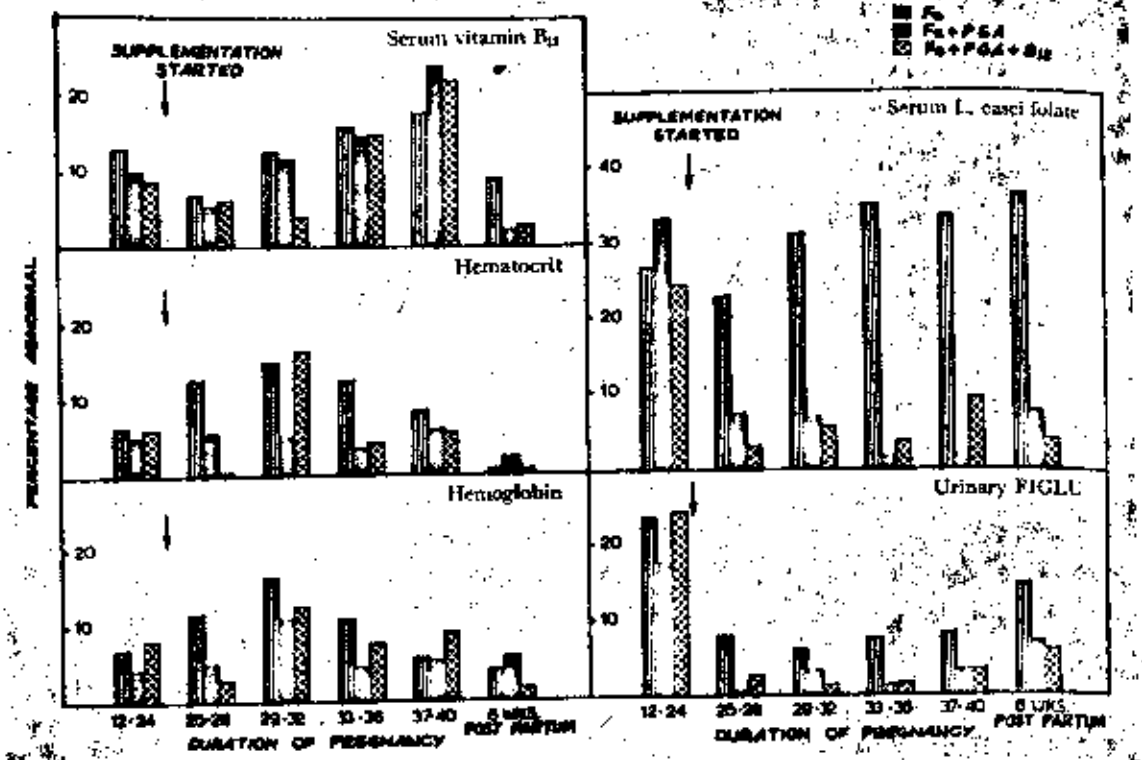

FIG. 2.—Percentage of abnormal results at different stages of pregnancy in subjects receiving various types of supplementation. Criteria were as follows: serum vitamin B_{12}, 200 μμg. per ml.; hemoglobin, 12.0 gm. per cent; hematocrit, 38 per cent; serum L. casei folate activity, 3.0 μμg. per ml.; and urinary FIGLU, 6 mg. per 6 hours.

FIGLU excretion was less in the groups receiving folic acid, but the addition of vitamin B_{12} had no further effect. In the group receiving iron only, the number of patients with excessive FIGLU excretion as compared to nonpregnant control subjects was highest in early pregnancy, fell sharply after the twenty-fourth week and then remained fairly constant up to the time of delivery (Fig. 2). At six weeks postpartum there was an increase again. In the patients receiving folic acid with or without vitamin B_{12}, the number of subjects with excessive excretion was reduced at all stages of pregnancy and postpartum. There was no obvious difference in patients receiving vitamin B_{12}, folic acid and iron and those receiving folic acid and iron only.

Serum L. casei folate activity (Fig. 3)

The L. casei activity was measured in 1,246 serum specimens. In patients receiving iron supplements only the serum L. casei levels were highest in early pregnancy, and then fell slightly as pregnancy advanced, rising again postpartum. The rate of fall was slow, and the difference between the mean value in early pregnancy and at term was less than 1 μμg. per ml. In subjects receiving folic acid supplements, there was a prompt and marked rise in the mean serum L. casei folate levels, which continued up to delivery, when supplementation ceased. At six weeks postpartum there was a fall in the mean L. casei levels, but the levels were still approximately twice that of the group that received iron only. In subjects receiving iron only, the number of subnormal values rose as pregnancy progressed (Fig. 2). There was a marked reduction in the number of subjects with subnormal levels in the groups receiving folic acid supplements. The addition of vitamin B_{12} to the folic acid supplement did not afford any further advantage.

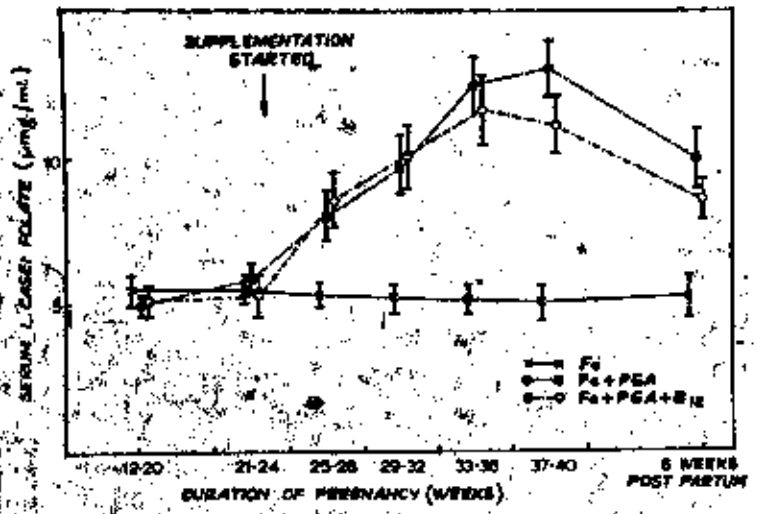

FIG. 3. Serum L. casei folate levels in pregnant subjects receiving various types of supplementation.

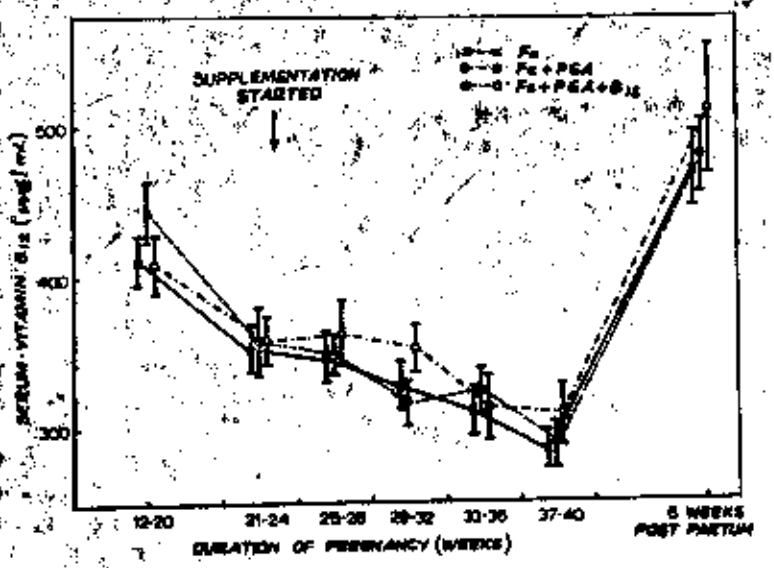

FIG. 4. Serum vitamin B_{12} concentration in pregnant subjects receiving various types of supplementation.

Serum Vitamin B_{12} Concentration (Fig. 4)

Serum vitamin B_{12} concentration was measured in 1,233 serum samples. In subjects not receiving the vitamin B_{12} supplement, the mean serum vitamin B_{12} concentration was highest in early pregnancy and then decreased progressively toward delivery rising again at six weeks postpartum. The number of subnormal values increased as pregnancy progressed, but decreased markedly at six weeks postpartum (Fig. 2). In subjects receiving vitamin B_{12}, the pattern was essentially similar, except for a transient rise in the mean serum levels together with a decrease in the number of subnormal values in the eight week period immediately following the institution of supplements.

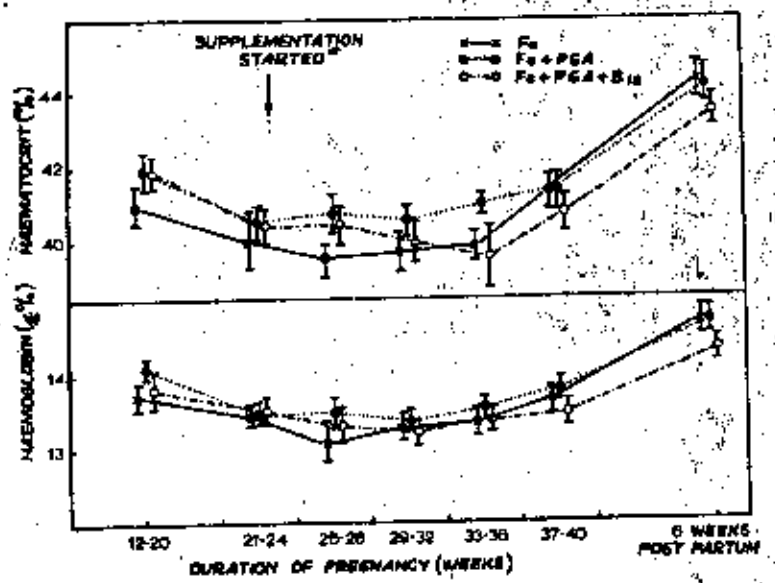

FIG. 5. Hematocrit and hemoglobin values in pregnant subjects receiving various types of supplementation.

Hemoglobin and Hematocrit Values (Fig. 5)

The hemoglobin concentration was measured in 1,221 specimens and the hematocrit in 1,208. During the period between the twelfth and twenty-fourth week, there was a decrease in the mean hemoglobin and hematocrit values. After twenty-four weeks, when supplementation was started, the mean values remained fairly constant up to the time of delivery, and then increased at six weeks postpartum. This pattern was apparent, irrespective of the type of supplement. There was no consistent difference in the numbers of subjects with subnormal hemoglobin and hematocrit values at different stages of pregnancy in the groups receiving various supplements (Fig. 2). However, during the period between the thirty-third and fortieth week the number of subjects with subnormal hematocrit values was less in the groups receiving folic acid.

Statistical Analysis

The mean values of all the observations in the third trimester were compared for the three groups receiving various types of supplements. The differences in the hemoglobin, hematocrit and serum vitamin B_{12} concentrations were not statistically significant. The mean FIGLU excretion was significantly less ($p = <0.01$) and the mean L. casei folate activity significantly greater ($p = <0.001$) in the group receiving iron and folic acid compared to the group receiving iron only. The addition of the vitamin B_{12} supplement had no further significant effect on mean FIGLU and L. casei values.

COMMENTS

The results of the present study confirm the previously described pattern of high FIGLU excretion in early pregnancy, with a subsequent decrease toward delivery.[10,20] Berry et al.[14] suggested that the progressive decline of FIGLU excretion during pregnancy was due to the utilization of increasing amounts of histidine for protein synthesis. Changes in histidine metabolism in pregnancy provide the most likely explanation of the observed pattern of FIGLU excretion. Increased histidinuria occurs in pregnancy[21] and reaches a peak at twenty-eight to thirty-six weeks.[22] This increased histidinuria is related to an increased glomerular filtration rate and diminished tubular reabsorption of histidine.[23] Thus the urinary loss of histidine following an oral histidine load is greater in pregnant subjects than in nonpregnant control subjects.[15] Absorption

of histidine from the gut decreases during pregnancy,[16,34] and this, together with increasing urinary excretion of absorbed histidine, means that following the administration of a constant histidine load to pregnant subjects, the amount of histidine presented for degradation to FIGLU decreases as pregnancy advances. Thus FIGLU excretion might be expected to decrease after the twenty-eighth week of pregnancy, and increase again following delivery when histidine absorption and excretion return to normal. The fact that supplementation with folic acid caused a decrease in the mean FIGLU excretion and in the number of patients with excessive excretion, compared to patients who received iron only, indicates that excessive FIGLU excretion in late pregnancy is related to folate nutrition. Thus, while the pattern of FIGLU excretion in pregnancy, with maximum excretion in early pregnancy and a decrease toward delivery, is related to physiologic changes in histidine metabolism, differences in FIGLU excretion in subjects at the same stage of pregnancy do reflect differences in folate nutrition. The actual amount of FIGLU excreted by a pregnant patient is not necessarily directly related to her folate nutrition, but increased excretion in late pregnancy is most likely due to folate deficiency.

The pattern of decreasing serum L. casei folate activity with increasing duration of pregnancy found in the present study of patients who did not receive folic acid is similar to that reported by other workers,[14,25–27] although the actual fall was less. The serum L. casei activity is less affected by metabolic changes during pregnancy than FIGLU excretion. Further evidence in favor of this is the observation that no obvious change occurs following delivery. This does not necessarily mean that the L. casei level is insensitive to changes in folate nutrition during pregnancy. In fact, in subjects receiving folic acid supplements, the mean L. casei levels rose rapidly, and there was a marked reduction in the number of subjects with subnormal values. In the present study, all patients received iron supplements, and it is possible that in groups not receiving iron, the fall in the L. casei levels during pregnancy may be more marked.

A number of previous studies[28–32] have demonstrated a fall in serum vitamin B_{12} levels during pregnancy, with a rise following delivery. In the present study, this pattern was again evident, and the fall in mean levels during pregnancy was not prevented by supplementation with vitamin B_{12}. Similar findings were reported by Young et al.[32] who were unable to prevent the fall in serum vitamin B_{12} levels during pregnancy with supplements of 100 μg. of the vitamin daily, started as early as the seventeenth week. This indicates that either the fall in serum vitamin B_{12} level during pregnancy is independent of the dietary intake of the vitamin, or that the amount of the supplement administered was inadequate to cover the fetal demands for vitamin B_{12} during pregnancy. The minimal daily requirements for vitamin B_{12} in the nonpregnant subject is approximately 1 μg. per day.[33] The amount of vitamin B_{12} absorbed from a 50 μg. oral dose by pregnant subjects is not known, but a supplement of this magnitude seems adequate to cover maternal and fetal requirements during pregnancy.

The weight of evidence then suggests that the fall in serum vitamin B_{12} levels in pregnancy represents a change in vitamin B_{12} metabolism independent of the dietary intake of vitamin B_{12}, and does not necessarily reflect depletion of maternal vitamin B_{12} stores. The rapid rise in vitamin B_{12} levels following delivery is further evidence in favor of this concept, and probably reflects removal of whatever factor(s) caused the depression of the level during pregnancy.

The effect of folic acid and vitamin B_{12} supplements on the incidence of megaloblastic anemia and on the hemoglobin levels at term has been reported. While supplementation with folic acid during pregnancy is effective in preventing megaloblastic anemia[35,36] the effect on the hemoglobin value at term is less convincing. In the studies by Giles and Burton[36] and Dawson et al.[37] the mean hemoglobin value at term in subjects given folic acid and iron supplements was of the order of 0.3 gm. per cent higher than that of subjects given iron supplements only. In the present study of subjects subsisting on a good Western type diet, even this small difference could not

be demonstrated although here was a reduction in the number of subjects with subnormal hematocrit values in the groups receiving folic acid. There is little doubt that the mean hemoglobin value in large groups of subjects during pregnancy is dependent mainly on iron nutrition, although it is possible that the beneficial effects of iron supplements may be exerted to some degree by improving folic acid nutrition.

While sensitive indices of folate and vitamin B_{12} nutrition may ultimately have wide clinical application in pregnant patients, at present anemia is the main proved deleterious effect of deficiency of these substances in pregnancy. Thus any argument for or against the routine use of folic acid and vitamin B_{12} supplements during pregnancy depends on the effect on the incidence of anemia. Diminution in the numbers of patients showing biochemical or microbiologic evidence of folate deficiency is not necessarily an indication for routine supplementation. Witts[38] has warned of treating laboratory tests rather than patients. While severe megaloblastic anemia in pregnancy can be detected and treated in patients regularly attending antenatal clinics, in populations in which attendance is erratic, routine supplementation with folic acid may be warranted.

SUMMARY

The effect of folic acid and vitamin B_{12} supplements in pregnant subjects subsisting on a good Western type diet has been studied. The serum L. casei folate activity, serum vitamin B_{12} concentration, urinary FIGLU excretion after histidine loading, and the hemoglobin and hematocrit have been determined at regular intervals in subjects given supplements of either iron, iron and folic acid, or iron, folic acid and vitamin B_{12}.

Urinary FIGLU excretion was highest in early pregnancy, and then decreased steadily up to the time of delivery. In patients receiving folic acid, FIGLU excretion was significantly reduced. While FIGLU excretion after histidine loading in pregnant subjects is related to physiologic changes in histidine metabolism during pregnancy, increased excretion in late pregnancy probably reflects folate deficiency. The serum L. casei folate level showed little change during pregnancy, but the administration of folic acid resulted in a prompt and significant increase in the serum values. The serum vitamin B_{12} concentration decreased markedly during pregnancy and increased again after delivery; this pattern was not changed by vitamin B_{12} supplementation.

The administration of folic acid and vitamin B_{12} to groups of pregnant subjects receiving iron did not result in a significant increase in the mean hemoglobin or hematocrit values.

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REFERENCES

1. Gilbs, C. and Shuttleworth, E. M. Megaloblastic anaemia of pregnancy and the puerperium. *Lancet*, 2: 1341, 1958.
2. MacKenzie, A. and Abbott, J. Megaloblastic erythropoiesis in pregnancy. *Brit. M. J.*, 2: 1114, 1960.
3. Chanarin, I., Mollin, D. L. and Anderson, B. B. The clearance from the plasma of folic acid injected intravenously in normal subjects and patients with megaloblastic anaemia. *Brit. J. Haemat.*, 4: 435, 1958.
4. Metz, J., Stevens, K., Krawitz, S. and Brandt, V. The plasma clearance of injected doses of folic acid as an index of folic acid deficiency. *J. Clin. Path.*, 14: 622, 1961.
5. Lubby, A. L., Cooperman, J. M. and Teller, D. N. Histidine metabolic loading test to distinguish folic acid deficiency from Vit. B_{12} in megaloblastic anaemias. *Proc. Soc. Exper. Biol. & Med.*, 101: 360, 1959.
6. Spray, G. H. and Witts, L. J. Excretion of formiminoglutamic acid as an index of folic acid deficiency. *Lancet*, 2: 702, 1959.
7. Kohn, J., Mollin, D. L. and Rosenbach, L. M. Conventional voltage electrophoresis for formiminoglutamic acid determination in folic acid deficiency. *J. Clin. Path.*, 14: 345, 1961.
8. Metz, J., Stevens, K. and Brandt, V. Urinary formiminoglutamic acid in the megaloblastic anaemias associated with pregnancy and malnutrition. *Brit. M. J.*, 2: 1440, 1962.
9. Herbert, V., Baker, H., Frank, O., Pasher, I., Sobotka, H. and Wasserman, L. R. The

measurement of folic acid activity in serum: a diagnostic aid in the differentiation of the megaloblastic anaemias. *Blood*, 15: 228, 1960.

10. Waters, A. H. and Mollin, D. L. Studies on the folic acid activity of human serum. *J. Clin. Path.*, 14: 335, 1961.
11. Stevens, K., Metz, J., Brandt, V. and Van Broekhuizen, L. Serum vitamin B_{12}, folic acid and urinary formiminoglutamic acid in megaloblastic anaemias in South African Bantu adults. *East African M. J.*, 39: 222, 1962.
12. Chanarin, I., MacGibbon, B. M., O'Sullivan, W. J. and Mollin, D. L. Folic acid deficiency in pregnancy. The pathogenesis of megaloblastic anaemia of pregnancy. *Lancet*, 2: 634, 1959.
13. Hibbard, B. D. FIGLU excretion. A screening test for folic acid deficiency in pregnancy. *J. Obst. & Gynec. Brit. Emp.*, 69: 739, 1962.
14. Solomons, E., Lee, S. L., Wasserman, M. and Malkin, J. Association of anaemia in pregnancy and folic acid deficiency. *J. Obst. & Gynaec. Brit. Emp.*, 69: 724, 1962.
15. Chanarin, I., Rothman, D. and Watson-Williams, E. J. Normal formiminoglutamic acid excretion in megaloblastic anaemia in pregnancy. Studies on histidine metabolism in pregnancy. *Lancet*, 1: 1068, 1963.
16. Berry, V., Chanarin, I., Booth, M. A. and Rothman, D. Urinary formiminoglutamic acid excretion in pregnancy. *Brit. M. J.*, 2: 1103 1963.
17. Chanarin, I. and Bennett, M. C. A spectrophotometric method for estimating formiminoglutamic acid and urocanic acid. *Brit. M. J.*, 1: 27, 1962.
18. Herbert, V. The assay and nature of folic acid activity in human serum. *J. Clin. Invest.*, 40: 81, 1961.
19. Brandt, V. and Metz, J. Serum vitamin B_{12} levels in South African White and Bantu subjects. *South African J. M. Sc.*, 26: 1, 1961.
20. Metz, J., Stevens, K., Edelstein, T., Brandt, V. and Baumblag, N. Changes in serum vitamin B_{12}, plasma folic acid clearance, and urinary formiminoglutamic acid and urocanic acid during pregnancy, with observations on the effect of vitamin B_{12} and folic acid supplementation. *South African J. M. Sc.*, 28: 74, 1963.
21. Hönpa, M. Examination of the urine of pregnant women. *J. Biochem.*, 2: 351, 1923.
22. Page, E. W. Excretion ratio of histidine in pregnant and non-pregnant women. *Western J. Surg.*, 51: 482, 1943.
23. Page, E. W., Glendening, M. B., Dignam, W. and Harper, H. A. Causes of histidinuria in normal pregnancy. *Am. J. Obst. & Gynec.*, 68: 110, 1954.
24. Page, E. W. Mechanism of histidinuria of pregnancy. *Am. J. Obst. & Gynec.*, 51: 553, 1946.
25. Hansen, A. H. and V. Klewesahl-Palm, H. Blood folic acid levels and clearance rate of injected folic acid in normal pregnancy and puerperium. *Scandinav. J. Clin. & Lab. Invest.*, 15 (supp. 69): 78, 1963.
26. Martin, J. D. and Davis, R. E. Serum folic acid activity and vaginal bleeding in early pregnancy. *J. Obst. Gynaec. Brit. Emp.*, 71: 400, 1964.
27. Ball, E. W. and Giles, C. Folic acid and vitamin B_{12} levels in pregnancy and their relation to megaloblastic anaemia. *J. Clin. Path.*, 17: 165, 1964.
28. Karlin, R. Sur la Vitamine B et la methionine du serum sanguin chez la femme, pendant la gestation et la lactation. *Compt. rend Soc. Biol., Paris*, 149: 971, 1955.
29. Boger, W. P., Wright, L. D., Beck, G. D. and Bayne, G. M. Vitamin B_{12} correlation of serum concentrations and pregnancy. *Proc. Soc. Exper. Biol. & Med.*, 92: 140, 1956.
30. Okuda, K., Hellinger, A. E. and Chow, B. F. Vitamin B_{12} serum level and pregnancy. *Am. J. Clin. Nutrition*, 4: 441, 1956.
31. Baker, H., Erdberg, R., Pasher, I. and Sobotka, H. Study of folic acid and vitamin B_{12} in blood and urine during normal pregnancy. *Proc. Soc. Exper. Biol. & Med.*, 94: 513, 1957.
32. Lowenstein, L., Lalonde, M., Deschenes, E. B. and Shapiro, L. Vitamin B_{12} in pregnancy and the puerperium. *Am. J. Clin. Nutrition*, 8: 265, 1960.
33. Young, J. E., Barrows, C., Okuda, K. and Chow, B. F. Vitamin B_{12} serum level in pregnancy. *Obstet. & Gynec.*, 14: 149, 1959.
34. Sullivan, L. W. and Herbert, V. Delineation of minimal daily requirement and relative potency of vitamin B_{12} analogues using minimal dosage therapeutic trials. *Am. J. Clin. Nutrition*, 10: 354, 1962.
35. Lowenstein, L., Pick, C. and Philpott, N. W. Megaloblastic anaemia of pregnancy and the puerperium. *Am. J. Obst. & Gynec.*, 70: 1309, 1955.
36. Giles, C. and Burton, H. Observations on the prevention and diagnosis of anaemia in pregnancy. *Brit. M. J.*, 2: 636, 1960.
37. Dawson, D. W., More, J. R. S. and Aird, D. C. Prevention of megaloblastic anaemia in pregnancy by folic acid. *Lancet*, 2: 1015, 1962.
38. Witts, L. J. The blood in pregnancy. *J. Obstet. & Gynaec. Brit. Emp.*, 69: 714, 1962.

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Dynamics of Serum Binding of Intravenously Administered ^{57}Co Vitamin B_{12}[1] (35624)

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Following the addition of ^{57}Co B_{12} to normal serum, three binders have been isolated by DEAE–cellulose column chromatography. The order of elution of these binders in phosphate buffer of increasing ionic strength and decreasing pH is transcobalamin II (TCII), a binder eluted with the main protein peak, and transcobalamin I (TCI), according to the terminology used by Hall and Finkler (1). Differences in clearance rates of intravenously administered ^{57}Co B_{12} have been reported from several laboratories for normal subjects and patients with pernicious anemia (P.A.) in relapse and untreated chronic myelocytic leukemia (C.M.L.) (1–9). Hall and Finkler (1) reported that following intravenous administration of ^{57}Co B_{12} to two normal subjects the percentage of residual ^{57}Co B_{12} in the serum bound to TCI increased with time as measured by DEAE–cellulose column chromatography. Maximal labeling of both binders was seen in the first sample which was drawn 1.5 min after administration of the radioactive vitamin. Hom *et al.* (8) showed that 24 hr after intravenous administration of ^{57}Co B_{12} to one normal subject, 55 to 72% of the residual radioactivity calculated on the basis of recovered TCII and TCI, was bound to TCI depending on which of three techniques was employed, *viz.*, DEAE Sephadex chromatography, G-200 Sephadex gel filtration, and ammonium sulfate precipitation. Plasma clearance analyses of ^{57}Co B_{12} bound to TCII and TCI have been reported from several laboratories (1, 3, 10). In all studies, the clearance rate of TCII-bound ^{57}Co B_{12} was faster than that of TCI-bound ^{57}Co B_{12}. Hom and Olesen (10) reported that ^{57}Co B_{12} bound to TCII reappeared bound to TCI, following intravenous injection of ^{57}Co B_{12}-labeled TCII. In this study, clearance rates of intravenously administered ^{57}Co B_{12} were measured in 17 normal subjects, nine P.A., and four C.M.L. patients. Distribution of ^{57}Co B_{12} into TCII and TCI at various time intervals after the intravenous administration of ^{57}Co B_{12} was determined by DEAE–cellulose column chromatography. Purpose of the study was the investigation of the plasma clearance rates of *in vivo*-labeled TCII and TCI in normal subjects, P.A. patients, and C.M.L. patients. Observation that absolute amounts of ^{57}Co B_{12} bound to TCI increased with time would provide a proof that ^{57}Co B_{12} bound to TCII reappears as bound to TCI.

Materials and Methods. Clearance. Clearance studies were performed as described by Meyer *et al.* (9). Two μCi of ^{57}Co B_{12}, sp act, 10–18 μCi/μg (supplied by Squibb Co.) in a volume of 2.0 ml were rapidly injected. At intervals of 1, 2, 5, 15, 30, 45, and 60 min and 2, 4, 8, 12, 24, and 48 hr thereafter, 20 ml of blood were withdrawn. Ten ml were added to sodium and potassium oxalate and rotated thoroughly to prevent clotting. Four ml of plasma from each specimen were counted in a well-type scintillation spectrometer. Serum was separated from the remaining 10 ml of clotted blood and stored at −20° before chromatographic separation. Plasma volume was calculated by estimating total blood volume based on height, weight, and sex of the subjects, using plasmacrit observed on first day of clearance study.

DEAE–cellulose column Chromatography To 1.0 ml of serum obtained from a blood sample drawn at zero time (prior to injection of labeled vitamin), 200 pg of ^{57}Co B_{12} were added and allowed to incubate for 15

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min. All serum samples were treated with hemoglobin-coated charcoal prior to application on the column to remove free vitamin B_{12} (11). One-ml serum aliquots from samples obtained during clearance studies were chromatographed on 5.0-ml DEAE–cellulose columns as described by Retief *et al.* (11). The method was modified in that eleven 2.0-ml samples were collected with the 0.06 *M* phosphate buffer, pH 6.35, and six 2.0-ml samples with 1.0 *M* NaCl. DEAE–cellulose (purchased from Schleicher and Schuell, Keene, N.H.) of ion-exchange capacity between 0.90 and 0.95 meq/g, was used. The information obtained from the serum sample drawn at zero time was used as an indication of how much of the injected ^{57}Co B_{12} was bound to each of the two binding proteins.

Results. Clearance of ^{57}Co B_{12} in normal subjects and distribution to TCII and TCI. Table I (a) shows the percentage of residual radioactivity at zero time, 1 min, and 1,24, and 48 hr; percentage distribution of ^{57}Co B_{12} bound to TCII and TCI at the same time intervals, and ^{57}Co B_{12} bound to TCII and TCI as percentage of injected activity. Values in the last two columns are the products of percentage residual values and percentage distribution of TCII and TCI. Values in parenthesis in the last two columns express circulating ^{57}Co B_{12} bound to TCII and TCI as percentage of values at zero time.

An initial decrease in circulating and radioactivity to 55% of injected dose was observed. Circulating radioactivity decreased as clearance time advanced but at a slower rate per unit time than in the first minute.

A good agreement was observed between values of TCII and TCI at zero time and 1 min. It appeared that the binding pattern of injected ^{57}Co B_{12} was identical to that of the 200 pg of ^{57}Co B_{12} added per ml of serum *in vitro*. The percentage of ^{57}Co B_{12} bound to TCI increased, and consequently, the percentage bound to TCII decreased as clearance time advanced. Due to decrease in residual radioactivity, the absolute amount of ^{57}Co B_{12} bound to both TCI and TCII decreased at all time intervals studied.

Clearance of ^{57}Co B_{12} in pernicious anemia patients and distribution as TCII and TCI. Table I (b) shows clearance data in 9 P.A. patients to whom tracer doses of ^{57}Co B_{12} were administered.

Circulating radioactivity decreased to 60% during the first minute. This continued for the remainder of the 48-hr period of study. Percentage of TCI-bound ^{57}Co B_{12} increased, but because of decrease in total circulating radioactivity the absolute amount of ^{57}Co B_{12} bound to TCI and TCII decreased at all time intervals studied. Plasma disappearance of ^{57}Co B_{12} bound to TCII is clearly faster than ^{57}Co B_{12} bound to TCI.

Clearance of ^{57}Co B_{12} in chronic myelocytic leukemia patients and distribution as TCII and TCI. Table I (c) shows clearance data in four C.M.L. patients to whom tracer doses of ^{57}Co B_{12} were administered.

TCI content at zero time is higher than in normal and P.A. patients. Decrease in residual activity after the first minute was less than in the other two groups studied. Percentage of ^{57}Co B_{12} bound to TCI increased at all time intervals except at 48 hr (it is possible that this discrepancy is due to the high standard deviation). The last two columns in Table I (c) indicate an absolute decrease in the percentage of ^{57}Co B_{12} bound to TCII and TCI. As in normal and P.A. patients, disappearance of ^{57}Co B_{12} bound to TCI was slower than disappearance of ^{57}Co B_{12} bound to TCII.

Discussion. A rapid decrease in residual radioactivity in the first minute of the clearance was observed when ^{57}Co B_{12} was injected to normal subjects.

Clearance rate of *in vivo*-labeled TCII after injection of free ^{57}Co B_{12} resembled closely that of *in vitro*-labeled TCII when administered to normal subjects (3). Clearance rate of TCI labeled *in vivo* was faster in normal persons than that of *in vitro*-labeled TCI prepared from normal serum and injected into normal subjects (3). In the latter case, less than 20% of the administered dose was cleared in the first minute, while in the former instance 50% had been cleared. It is possible that injected free ^{57}Co B_{12} was removed from the circulation before it was bound to TCI and TCII. Instantaneous absorption of cyanocobalamin on cell mem-

TABLE I. TCI and TCII Levels in Serum Following Intravenous Injection of ^{57}Co B_{12} Into 17 Normal Subjects, 9 P.A. and 4 C.M.L. Patients.

Time	Res. activity[a] (%)	TCII[b] (%)	TCI[b] (%)	TCII % inj. act[c]	TCI % inj. act[c]
		(a) Normal			
0[d]		88.7 ± 3.4	11.3 ± 3.4		
1 min	54.6 ± 10.9	87.9 ± 3.8	12.3 ± 3.8	48.2 ± 15.8 (53.0)[e]	6.4 ± 2.8 (56.3)
1 hr	13.6 ± 3.0	80.8 ± 7.6	19.2 ± 7.6	11.0 ± 2.6 (12.1)	2.6 ± 1.2 (32.9)
24 hr	4.5 ± 1.2	66.5 ± 12.1	33.5 ± 12.1	3.0 ± 0.9 (3.3)	1.5 ± 0.8 (13.2)
48 hr	3.5 ± 1.0	60.1 ± 12.0	39.9 ± 12.0	2.0 ± 0.8 (2.4)	1.4 ± 0.8 (12.4)
		(b) P.A.			
0		80.9 ± 5.1	19.1 ± 5.1		
1 min	60.9 ± 4.7	88.7 ± 2.4	11.3 ± 2.4	53.9 ± 4.0 (63.6)	6.9 ± 1.7 (36.0)
1 hr	17.4 ± 4.2	71.8 ± 6.3	28.3 ± 6.3	12.3 ± 2.3 (14.8)	5.0 ± 2.3 (26.1)
24 hr	8.1 ± 2.5	60.9 ± 6.6	39.1 ± 6.6	5.0 ± 1.9 (6.0)	3.1 ± 0.8 (16.0)
48 hr	6.4 ± 2.3	55.4 ± 7.2	44.6 ± 7.2	3.5 ± 1.1 (4.2)	2.9 ± 1.3 (15.2)
		(c) C.M.L.			
0		59.9 ± 15.3	40.1 ± 15.3		
1 min	63.7 ± 37.3	66.4 ± 13.3	33.6 ± 13.3	39.5 ± 19.7 (65.9)	24.8 ± 30.2 (60.3)
1 hr	45.9 ± 13.3	56.1 ± 14.0	43.9 ± 14.0	25.8 ± 9.8 (43.1)	20.5 ± 7.2 (51.1)
24 hr	29.8 ± 19.9	41.9 ± 21.0	58.1 ± 21.0	12.0 ± 6.4 (20.0)	17.8 ± 9.6 (44.4)
48 hr	23.1 ± 10.9	45.3 ± 23.7	54.7 ± 23.7	10.6 ± 6.6 (16.7)	12.5 ± 6.9 (31.8)

[a] Res. activity shows percentage injected activity in plasma at indicated time interval.

[b] TCII% and TCI% represent percentage distribution of residual activity of these two binders, determined by DEAE-cellulose chromatography.

[c] Values in columns marked TCII% inj. act and TCI% inj. act are derived by multiplying values of Res. activity by values of TCII% and TCI%.

[d] Zero-time values are calculated by chromatographing patient's serum to which ^{57}Co B_{12} has been added (200 pg/ml).

[e] Numbers in parentheses were derived by dividing "% inj. act" by corresponding zero-time activity and multiplying by 100.

branes of Ehrlich ascites cells has been reported by Paranchych and Cooper (12).

Clearance rates of *in vivo*-labeled TCII and TCI after injection of ^{57}Co B_{12} into P.A. patients were slower than those of labeled TCII and TCI after injection of ^{57}Co B_{12} to normal subjects. The difference does not appear to be significant at the 5% level as estimated by the student *t*-test. High standard deviations were observed in the data from C.M.L. patients, probably due to the smaller number of cases studied or to differ-

ent stages of disease. TCI content was found markedly elevated in accordance with previous reports (1, 13). Plasma clearances of ^{57}Co B_{12} and those of *in vivo*-labeled TCII and TCI were strikingly delayed. Rate of plasma clearance of *in vivo*-labeled TCI was similar to that observed when *in vitro*-labeled TCI was administered to normal subjects.

High TCI content probably hindered clearance of ^{57}Co B_{12}-labeled TCII. The findings in this study differ from those of Horn and Olesen (10). It is possible that these differences are due to the fractionation procedures. It has been reported from several laboratories (14, 15, 16) that the 120,000 molecular size fraction from Sephadex columns does not always correspond to TCI. The presence in serum of a binder with beta-globulin electrophoretic mobility and molecular size similar to TCI has been demonstrated (15, 16). Since a continuous decrease in the amount of ^{57}Co B_{12} bound to TCI as determined by DEAE-cellulose column chromatography was found in this study and an increase of the amount of ^{57}Co B_{12} bound to the 120,000 fraction (gel filtration) was observed by Horn and Olesen, ^{57}Co B_{12} possibly reappeared in circulation bound to this binder (10).

Summary. Following intravenous injection of ^{57}Co vitamin B_{12}, clearance rates of endogenously labeled TCII and TCI were delayed in patients with C.M.L., compared to normal subjects and cases of P.A. If ^{57}Co B_{12} from TCII reappeared bound to TCI, it occurred at a rate slower than that of plasma disappearance of ^{57}Co B_{12} bound to TCI.

1. Hall, C. A., and Finkler, A. E., J. Lab. Clin. Med. 65, 459 (1965).
2. Brody, E. A., and Wasserman, L. R., Blood 18, 806 (1961).
3. Gizis, E. J., Arkun, S. N., Miller, I. F., Choi, G., Dietrich, M. F., and Meyer, L. M., J. Lab. Clin. Med. 74, 574 (1969).
4. Hall, C. A., Blood 18, 717 (1961).
5. Hall, C. A., and Finkler, A. E., J. Lab. Clin. Med. 60, 765 (1962).
6. Brody, E. A., Estren, S., and Wasserman, L. R., Blood 15, 646 (1960).
7. Hall, C. A., and Kulonen, M., Oka, M. J., Acta Med. Scand. 172, 147 (1962).
8. Horn, B. L., Olesen, H., and Lous, P., J. Lab. Clin. Med. 68, 958 (1966).
9. Meyer, L. M., Osofsky, M., and Miller, I. F., Scand. J. Haematol. 4, 301 (1967).
10. Horn, B. L., and Olesen, H. A., Scand. J. Clin. Lab. Invest. 23, 201 (1969).
11. Retief, F. P., Gottlieb, C. W., Kochwa, S., Pratt, P. W., and Herbert, V., Blood 29, 501 (1967).
12. Paranchych, W., and Cooper, B. A., Biochim. Biophys. Acta 60, 393 (1962).
13. Hall, C. A., and Finkler, A. E., Blood 27, 618 (1966).
14. Cooper, B. A., Blood 35, 829 (1970).
15. Lawrence, C., Blood 34, 774 (1969).
16. Gizis, E. J., Dietrich, M. F., Choi, G., and Meyer, L. M., J. Lab. Clin. Med. 75, 673 (1970).

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relapse was 'less severe', even though serum vitamin B_{12} levels in each case was 40 pg/ml. A sixth patient with megaloblastic anaemia due to vitamin B_{12} dietary deficiency showed a normal clearance rate. Anaemia was severe and serum B_{12} value was 80 pg/ml. The authors suggested that plasma clearance is related to degree of serum unsaturation, which is high both in pernicious anaemia and chronic myelocytic leukaemia.

Brody et al. (1960) reported that four of five patients with malabsorption syndrome in relapse showed values similar to those in pernicious anaemia in relapse. They noted that in three patients with pernicious anaemia under treatment, plasma clearance of intravenously administered radioactive B_{12} was more rapid but did not reach normal levels.

Hall (1961) showed that in patients with untreated pernicious anaemia or anaemia due to *Diphyllobothrium latum* infestation, plasma clearance of intravenously administered radioactive vitamin B_{12} was slower than in normal subjects. Following treatment patients with pernicious anaemia showed normal clearance of the test dose, as did patients who were carriers of the tapeworm but escaped B_{12} deficiency (Hall et al. 1962). They suggested that slow disappearance rate in pernicious anaemia was related to tissue and/or plasma vitamin B_{12} deficiency and not to some permanent defect associated with this disease. They further suggested that the fundamental abnormality was either (1) an increased binding of vitamin B_{12}, (2) an abnormally firm binding, (3) a defective tissue extraction, or (4) a shift of vitamin B_{12} back to plasma from tissue. None of these suggestions has been proved or disproved.

Brody et al. (1960) suggested there may be a substance in plasma whose function is to permit transfer of circulating vitamin B_{12} from blood to tissues. They proposed that this hypothetical substance ('B_{12}-transferase') is present in adequate amounts in the normal person and is diminished in the blood of patients with pernicious anaemia in relapse and to a lesser degree in remission. The authors suggested there may be some relationship between this 'factor' and intrinsic factor of gastric juice which may account for slow clearance of B_{12} in patients with total gastrectomy and malabsorption syndrome, conditions in which intrinsic factor is either not produced or not absorbed.

The purpose of this study is to determine the effect of normal serum on clearance rate of intravenously administered Co^{57} vitamin B_{12} in patients with pernicious anaemia.

MATERIALS AND METHODS

Co^{57} cyanocobalamin had a specific activity ranging from 10 to 18 $\mu C/\mu g$. Fourteen patients with well documented pernicious anaemia and one case of post-gastrectomy megaloblastic anaemia were studied. They varied in degree of relapse from fresh un-

treated subjects to known patients who had failed to receive therapy for longer than three months. Haematologic parameters at the time of clearance included estimation of Hgb., R.B.C., W.B.C., haematocrit, platelet count, reticulocyte count, differential count, serum iron, L.D.H., intrinsic factor antibodies, vitamin B_{12} and folic acid. Sera obtained from patients and donors for future studies to be described were stored at $-20°$ C.

On day of clearance a Cournand needle was placed in an accessible vein of one arm for one hour. Through another vein a tracer dose of $Co^{57}B_{12}$ (0.065 to 0.25 μg), volume 0.5 to 1.0 ml, was rapidly injected. The syringe was flushed twice with an equal volume of recipient's blood after injection. At intervals of 1, 2, 5, 15, 30, 45, and 60 minutes thereafter 10 ml of blood were withdrawn, added to sodium and potassium oxalate, and rotated thoroughly to prevent clotting. After removal of the needle additional samples of blood were obtained at 2, 4, 8, 12, 24, and 48 hours and similarly treated. All specimens were centrifuged. Two ml of plasma from each specimen were placed in Visking casing bags and subjected to dialysis against running tap water for 48 hours. Aliquots similarly prepared but not dialyzed served as controls. Each bag (dialyzed and undialyzed) was placed in a test tube, two ml of concentrated sulfuric acid added to disintegrate casing, thoroughly mixed, and counted in a three inch well-type sodium iodide crystal scintillation counter. Counting error was less than 1 per cent. Since blood volume in pernicious anaemia is not significantly different from normal, plasma volume was calculated by estimating total blood volume based on height, weight and sex of patient, using the plasmacrit observed on the day of clearance study (Meyer & Bertcher 1952). Haematologic values did not vary during periods of study. The percentage of administered dose in plasma at any given time was determined by the formula:

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

(1) In three cases 100 ml of patient's own serum were incubated with 0.06 to 0.1 μg of Co^{57} vitamin B_{12} for one hour. A 10 ml aliquot was withdrawn part of which was subjected to dialysis against running tap water for 48 hours, and another portion shaken with charcoal (Norit A), to determine presence of 'free $Co^{57}B_{12}$' (Meyer et al. 1963). The remainder of the serum (90 ml) was administered rapidly intravenously and samples of blood taken at intervals indicated in the above paragraph.

(2) In another study 0.06 to 0.1 μg of $Co^{57}B_{12}$ was added to 100 ml of (a) normal clearing pernicious anaemia serum (3 cases) and (b) slow clearing pernicious anaemia (3 cases), incubated for one hour, examined for 'free $Co^{57}B_{12}$' (as above), and administered rapidly to six patients with pernicious anaemia in relapse. Samples of blood were obtained following injection as described in the second paragraph of 'Methods' and radioactivity estimated.

(3) In two cases 100 ml of serum from pernicious anaemia patients were incubated with 0.06 to 0.1 μg of $Co^{57}B_{12}$ for one hour. A sample was removed to determine if the labelled vitamin was completely bound. Varying amounts of normal serum, ranging from 25 to 100 ml were added and allowed to mix for an additional hour. An aliquot of this mixture was removed to determine the presence of 'free $Co^{57}B_{12}$' (using dialysis and charcoal) and the remainder administered to each of two patients with pernicious anaemia in relapse. Samples of blood were taken at designated intervals to quantitate plasma activity.

(4) $Co^{57}B_{12}$ clearance studies were performed on a patient with megaloblastic

TABLE I

Haematologic and biochemical data on 14 patients with untreated pernicious anaemia showing plasma clearance rates 1, 24 and 48 hours after intravenous administration of 0.1 to 0.25 μg of Co^{57} vitamin B_{12}

Name	Age years	Sex	Last treatment	Haematocrit vol. per cent	Serum B_{12} pg/ml	Serum F.A. ng/ml	L.D.H.U.	I.F. antibody	Per cent uncleared		
									1 hr	24 hr	48 hr
Normal range									7.2–14.0	3.5–7.0	2.2–4.8
COL	70	F	3 mos.	34	108	14.0	500	−	12.2	4.3	2.7
HEB	50	M	9 mos.	44	120	5.4	280	+	14.0	7.0	4.0
HIR	80	F	none	15	70	5.3	280	−	12.5	5.8	3.7
HOR	86	M	6 mos.	41	80	21.1	225	−	10.1	3.5	2.6
MCD	73	F	none	9	36	2.1	580	−	7.2	3.7	2.2
MAT	75	F	5 mos.	40	88	3.5	300	−	11.9	5.1	4.8
WAS	75	F	none	24	25	21.0	1625	−	10.0	5.0	4.0
Mean									11.1	4.9	3.4
Slow range									16.0–29.0	7.8–17.3	5.4–12.7
KAP	67	M	10 mos.	44	80	6.6	380	−	19.0	12.0	9.0
LEE	56	F	none	25	25	33.5	1930	+	22.0	12.6	8.9
MCN	25	F	none	34	105	7.6	1300	+	22.0	17.3	12.7
PIE	44	M	8 mos.	49	76	9.1	460	+	18.5	7.8	5.4
RIV	49	M	none	18	50	33.0	2000	+	16.0	12.0	10.0
RUD	70	F	none	19	37	3.8	2100	−	27.0	13.0	12.0
SIN	72	M	8 mos.	44	55	9.3	400	+	24.0	9.0	8.0
Mean									21.2	11.9	9.4

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anaemia 19 years following gastrectomy. The effect of normal serum was also examined (as described in paragraph 2).

Normal values for plasma clearance activity following intravenous tracer doses of Co^{57} vitamin B_{12} are based on previous studies in this laboratory (Meyer et al. 1965).

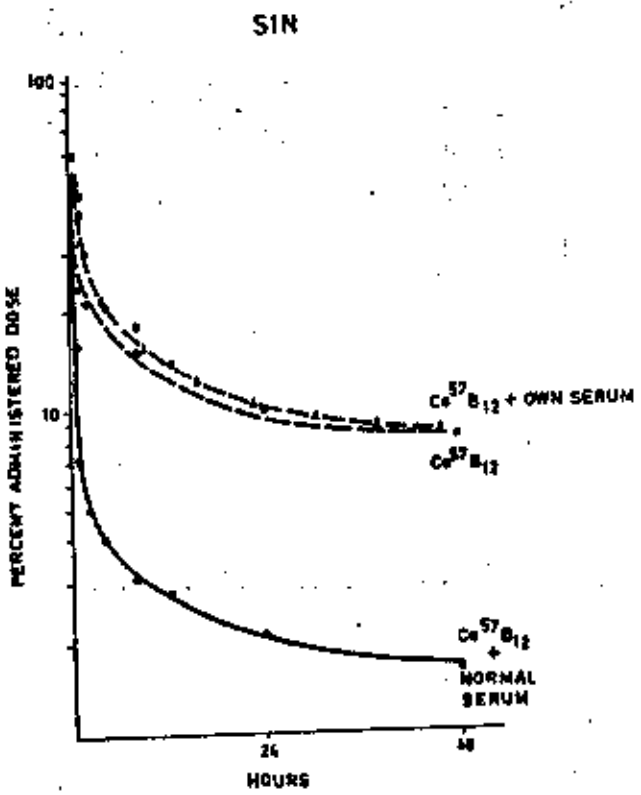

Figure 1. 'Slow' clearance of intravenous $Co^{57}B_{12}$ in patient with pernicious anaemia in relapse. When labelled vitamin is pre-bound to patient's own serum prior to injection the clearance pattern is unaltered. When normal serum is substituted the clearance rate becomes normal.

RESULTS

Table 1 shows individual values for 1, 24, and 48 hour clearances for all patients with pernicious anaemia in relapse. Also listed are pertinent laboratory data and length of time from last injection of vitamin B_{12} (usually 1 mg) to the performance of clearance study. In some instances this study was performed more than once in the same patient and clearance patterns were unchanged. There was no apparent relationship between haematologic values, serum vitamin B_{12}, folic acid, L.D.H. or I.F. antibody content. Duration of time since untreated patients received therapy had no apparent bearing on

clearance rate of radioactive B_{12}. There appear to be two groups of patients. Mean values for normal and slow rates are clear-cut and ranges within each category at 1, 24 and 48 hours shown no over-lap.

The same three patients with 'slow' clearance rates were used throughout the following investigations. Dialysis and charcoal studies indicated that there

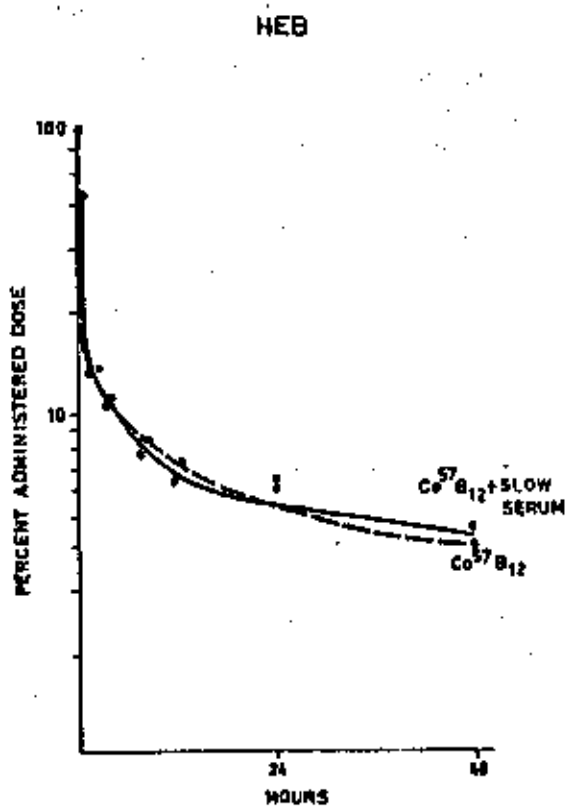

Figure 2. Normal clearance rate in patient with pernicious anaemia in relapse following intravenous injection of tracer dose of $Co^{57}B_{12}$. When labelled vitamin is pre-bound to serum from 'slow clearing' pernicious anaemia patient and infused into patient HEB, the pattern is normal. Similar results are noted when $Co^{57}B_{12}$ pre-bound to 'slow serum' is infused into a normal subject.

was complete binding of all labelled vitamin B_{12} to serum prior to administration.

(1) Infusion of $Co^{57}B_{12}$ bound to 100 ml of patient's own serum showed a clearance rate similar to that observed when 'free' labelled B_{12} was injected (Figure 1). This study was performed in two other persons with pernicious anaemia in relapse who showed 'slow' clearance rates and similar results were obtained.

(2a) Figure 1 also shows 48-hour clearance rate in this patient following intravenous administration of the tracer dose bound to 100 ml of normal serum. A rapid clearance, achieving normal levels is seen. Similar results were noted in two other subjects with pernicious anaemia in relapse.

(2b) When $Co^{57}B_{12}$ was bound to 100 ml of serum from a pernicious anaemia patient in relapse with a slow clearance and administered to a

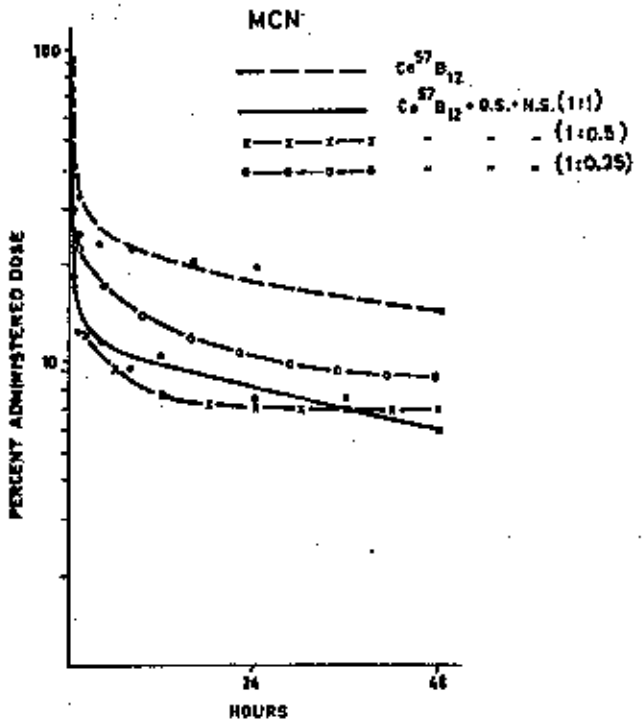

Figure 3. Slow clearance rate in patient (MCN) with pernicious anaemia in relapse following intravenous injection of tracer dose of $Co^{57}B_{12}$. When labelled dose is pre-bound to patient's own serum (0.5) to which normal serum (N.S.) is added correction of slow rate occurs when ratio of 0.5 to N.S. is 1:1 and 1:0.5, but only slightly improved with ratio of 1:0.25.

pernicious anaemia subject with normal clearance the clearance rate of 'bound' $Co^{57}B_{12}$ was normal. This is shown in Figure 2. Similar results were obtained in two other subjects with pernicious anaemia in relapse.

(3) In two cases where $Co^{57}B_{12}$ was bound *in vitro* to 100 ml of serum from a pernicious anaemia patient with slow clearance, to which 100 or 50 ml of normal serum were subsequently added, allowed to mix for an additional hour and re-infused into the same subject, normal clearance rates were observed. In one of these patients 25 ml of normal serum was ineffective (Figure 3).

(4) A post-gastrectomy patient (nineteen years after the operation) with serum vitamin B_{12} level of 70 pg/ml and megaloblastic bone marrow showed 'slow' clearance similar to that observed in half of pernicious anaemia patients in relapse. Administration of a tracer dose of $Co^{57}B_{12}$ fully bound to 100 ml normal serum was followed by normal clearance (Figure 4).

In no instance was free radioactivity found in the plasma, after administration of labelled vitamin B_{12} (pre-bound or free).

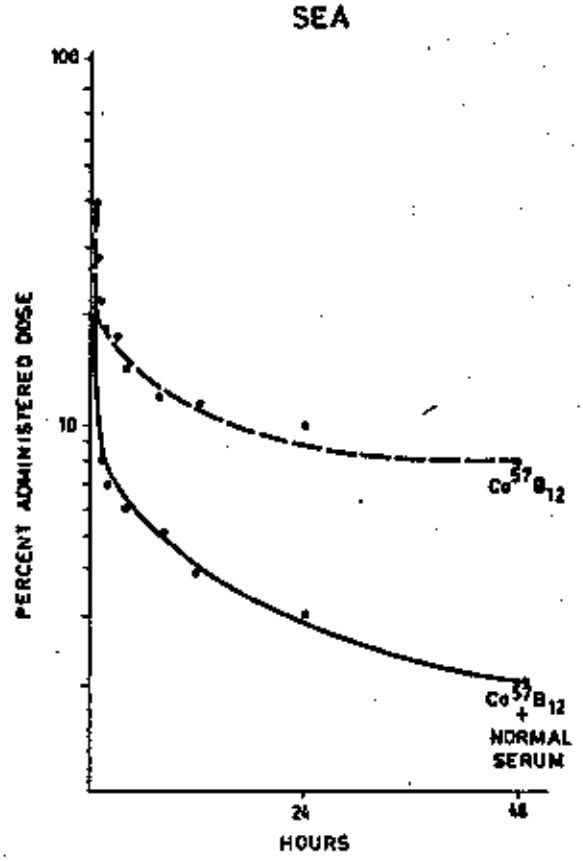

Figure 4. Slow clearance rate in patient 19 years post-gastrectomy who received intravenous tracer dose of $Co^{57}B_{12}$. Correction of clearance to normal levels occurred when labelled vitamin was pre-bound to normal serum prior to injection.

DISCUSSION

The clearance rate of intravenously administered radioactive vitamin B_{12} from plasma of patients with pernicious anaemia in relapse falls into two categories, namely slow and normal. The differences may depend on variations in quantity of unsaturated vitamin B_{12} binding α and β globulins.

The above studies suggest that there is an agent present in normal serum which enhances clearance of radioactive vitamin B_{12} from plasma of pernicious anaemia patients with slow clearance rates. This factor is supplied when the labelled vitamin is added to normal serum prior to injection, or if previously bound to slow clearing serum, is supplied when introduced into the circulating blood of a patient with normal clearance. It is also possible to supply this 'factor' *in vitro*, as the addition of normal serum to B_{12} bound to slow clearing serum is cleared at a normal rate in 'slow' recipients. This observation in pernicious anaemia is different from the experience of Brody et al. (1961) who found that radioactive vitamin B_{12} bound to serum of a patient with chronic myeloid leukaemia (slow clearance) injected into a normal subject was followed by slow clearance in the recipient. We have recently noted similar results using serum from a person with myeloid metaplasia (slow $Co^{57}B_{12}$ clearance) bound with $Co^{57}B_{12}$ which was introduced into the two 'normal subjects'. This too was followed by slow clearance rates in each recipient (Meyer et al. unpublished). Conversely labelled vitamin B_{12} bound to normal serum was cleared slowly from plasma of a patient with myeloid metaplasia. The mechanisms are undoubtedly different as increased binding capacity of serum in chronic myeloid leukaemia (untreated) and some cases of myeloid metaplasia but not pernicious anaemia has been demonstrated in this and other laboratories (Meyer et al. 1961).

That there may be a factor in normal plasma necessary for utilization of vitamin B_{12} was suggested by Horrigan & Heinle (1952) who described a case of unexplained macrocytic anaemia of unknown origin who could not be maintained in haematologic remission with vitamin B_{12} or folic acid. Plasma alone induced a rise in haemoglobin and haematocrit. Administration of plasma prior to injection of a tracer dose of $Co^{60}B_{12}$ was followed by reduction in urinary excretion of labelled vitamin. The authors suggested that (a) anaemia resulted from deficiency of proper binding of vitamin B_{12} or (b) normal plasma contains a factor which corrects this defect. This factor may be (a) the binding substance itself, (b) a substance necessary for the combination of vitamin B_{12} and endogenous binding substance, or (c) amounts of the vitamin in bound form is sufficient to promote haematopoiesis. From the current studies reported there appears to be a quantitative relationship between the amount of 'clearance factor' and 'clearance rate', as a ratio of normal to pernicious anaemia serum of 1:4 *in vitro* does not correct slow clearance. Proportions of 1:2 and 1:1 are followed by normal or near-normal clearance rates.

On the other hand, Herbert & Spaet (1958), using a technique of $Co^{60}B_{12}$ uptake by rat liver slices, showed that serum from normal subjects, pernicious anaemia patients, and persons with vitamin B_{12} deficiency not due to lack of

intrinsic factor, enchanced uptake of labelled vitamin to the same degree. The authors did not state whether the latter subjects were in relapse or remission, nor do we have information as to serum B_{12} levels or clearance rates of these persons. They suggested the presence of a 'circulating intrinsic factor' in the blood of patients studied.

Investigations of Hall & Finkler (1965) and Laurence (1966) have shown alterations in binding of vitamin B_{12} by α_1 and β globulins (Transcobalamin I and II) with elevations of the former in pernicious anaemia and chronic myelocytic leukaemia. However, the present study shows that normal serum will correct 'slow clearance' in pernicious anaemia but has no effect in chronic myelocytic leukaemia (or myeloid metaplasia).

Current efforts in this laboratory are directed towards further characterization of vitamin B_{12} binding proteins and identification of 'clearance factor'.

Co^{57} Vitamin B_{12} was supplied by Dr. Elmer Alpert, Merck, Sharpe and Dohme, West Point, Pennsylvania.

REFERENCES

Brody, E. A., Estren, S. & Wasserman, L. R. (1960) 'The kinetics of intravenously injected radioactive vitamin B_{12}: Studies on normal subjects and patients with chronic myelotic leukemia and pernicious anemia.' *Blood* **15**, 646–61.

Brody, E. A., Estren, S. & Wasserman, L. R. (1961) 'The disappearance of intravenously injected radioactive vitamin B_{12} in pernicious anemia in relapse and in remission.' *Blood* **18**, 806.

Hall, C. A. (1961) 'The plasma disappearance of radioactive vitamin B_{12} in myeloproliferative diseases and other blood disorders.' *Blood* **18**, 717–26.

Hall, C. A., Kulonen, M. & Oka, M. J. (1962) 'The plasma disappearance of intravenous cobalt-57 vitamin B_{12} in vitamin B_{12} deficiencies.' *Acta med. scand.* **172**, 147–50.

Hall, C. A. & Finkler, A. E. (1965) 'The dynamics of Transcobalamin II. A vitamin B_{12} binding substance in plasma.' *J. Lab. clin. Med.* **65**, 459–68.

Herbert, V. & Spaet, T. H. (1958) 'Distribution of 'Intrinsic factor' activity.' *Amer. J. Physiol.* **195**, 194–96.

Horrigan, D. L. & Heinle, R. W. (1953) 'Refractory macrocytic anemia with defect in vitamin B_{12} binding and with response to normal plasma.' *J. Lab. clin. Med.* **40**, 811.

Laurence, C. (1966) 'The binding of vitamin B_{12} by serum proteins in normal and B_{12} deficient subjects.' *Brit. J. Haemat.* **12**, 569–77.

Meyer, L. M. & Bertcher, R. (1952) 'Blood volume studies in pernicious anemia and non-tropical sprue.' *Proc. Soc. exp. Biol.* (N.Y.) **81**, 511–13.

Meyer, L. M., Bertcher, R. W., Cronkite, E. P., Suarez, R. M., Miller, I. F., Mulzac, C. W. & Olivarreta, S. T. (1961) 'Co^{60} vitamin B_{12} binding capacity of serum in persons with hematologic disorders, various medical diseases and neoplasms.' *Acta med. scand.* **169**, 557–75.

Meyer, L. M., Mulzac, C. W., Miller, I. F. & Brennan, B. L. (1963) 'Determination of

Co^{60} vitamin B_{12} binding capacity of serum by charcoal absorption.' *Acta haemat.* (Basel) **29**, *229–32*.

Meyer, L. M., Schiffer, L. M., White, D. A. & Cronkite, E. P. (1965) 'The plasma clearance of radioactive cyanocobalamin: Effect of prior administration of vitamin B_{12} analogous.' *Brit. J. Haemat.* **11**, 370–79.

Meyer, L. M., Osofsky, M. & Miller, I. F. Unpublished data.

Miller, A., Corbus, H. F. & Sullivan, J. F. (1957) 'The plasma disappearance, excretion and tissue distribution of cobalt 60 labelled vitamin B_{12} in normal subjects and patients with chronic myelogenous leukemia.' *J. clin. Invest.* **36**, 18–24.

Mollin, D. L., Fitney, W. R., Baker, S. J. & Bradley, J. E. (1956) 'The plasma clearance and urinary excretion of paranterally administered $Co^{58}B_{12}$.' *Blood* **11**, 31–43.

Mollin, D. L. & Booth, C. C. (1959) 'The plasma clearance of intravenous doses of radioactive vitamin B_{12}.' *Hematologica Latina* **2**, 257–64.

Riiz, N. D. & Meyer, L. M. (1960) 'Clearance of intravenously injected radioactive cobalt-labelled vitamin B_{12} in chronic myeloid leukemia and other conditions.' *Cancer* **13**, 1000–07.

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Co^{60} Vitamin B_{12} Binding Capacity of Serum in Persons with Hematologic Disorders, Various Medical Diseases and Neoplasms

By

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Utilizing microbiologic assay or radioactive labelled vitamin many investigators have demonstrated that normal human serum has the capacity to bind additional vitamin B_{12} in vitro (4, 13, 20, 23, 24, 27). By these same methods it has been shown that in untreated chronic myelocytic leukemia, and in some cases of acute myeloblastic leukemia, polycythemia vera, lymphocytic leukemia, multiple myeloma and idiopathic leukopenia, there is enhancement of this phenomenon (4, 15, 17, 20, 24). Using plasma clearance of intravenously administered radiovitamin as a measure of in vivo binding capacity similar results were observed in patients with chronic myelocytic leukemia (6, 13, 18, 26).

The present report is an extension of previous studies from this laboratory on serum vitamin B_{12} binding capacity in persons with hematologic disorders, various medical diseases and neoplasms (15, 16).

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Methods

Sera were obtained from 113 normal subjects, and 427 patients with various medical disorders classified as: hematologic 218, nonhematologic 138, and neoplastic 71. In the groups designated as blood disorders serial examinations were carried out on those patients showing abnormalities, to determine the effect of therapy and relation of Co^{60} B_{12} binding capacity to hematologic findings and clinical course. A total of more than 1,000 measurements was made.

In general, the Co^{60} B_{12} had a specific activity of approximately 1.0 mc/mg. Sera not examined immediately were stored at —20° C. until used. To 1.0 ml aliquots of serum in Visking casing bags were added 1.0 to 100 mμg of radiovitamin B_{12}. The mixture was incubated at room temperature for 2

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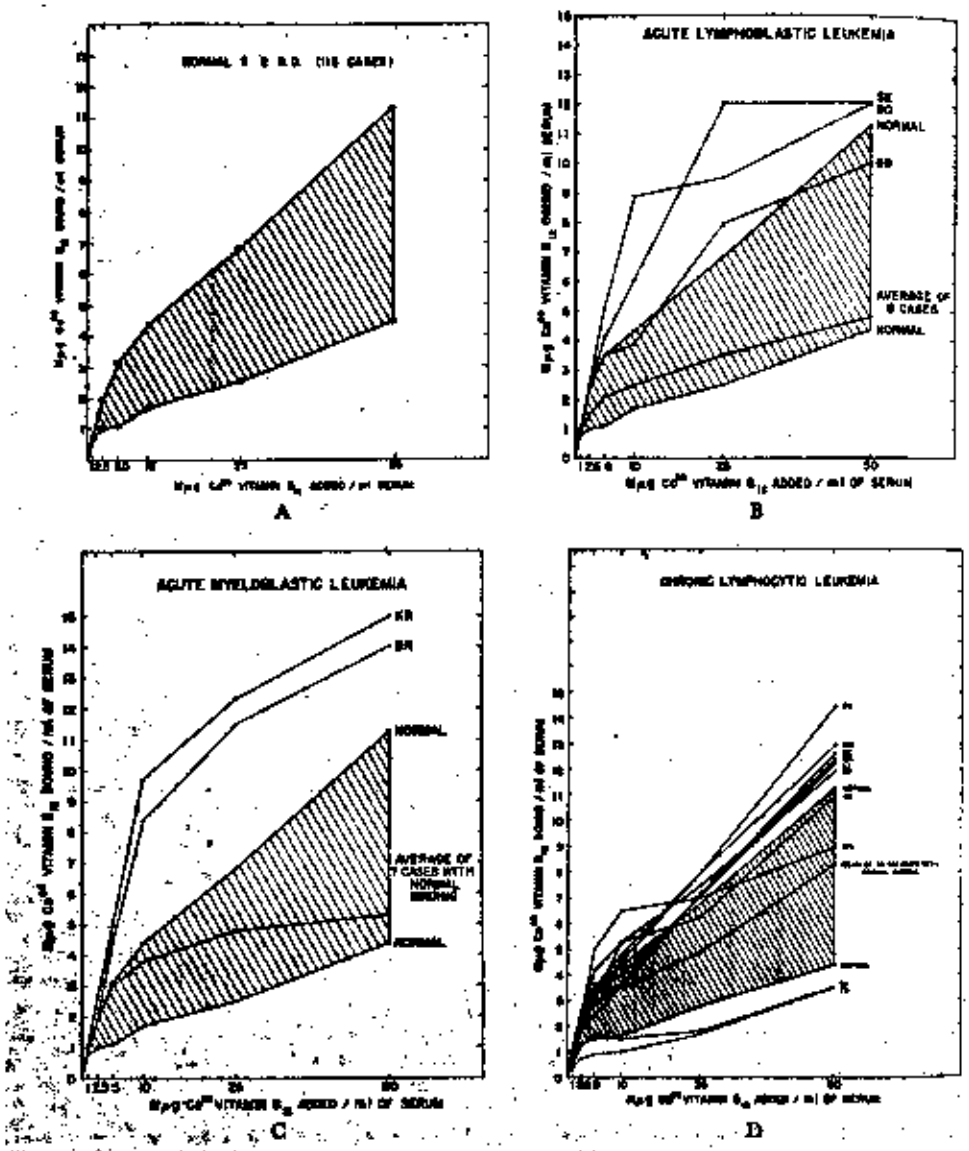

Fig. 1. Serum Co^{60} vitamin B_{12} binding capacity in (A) normal subjects, (B) acute lymphoblastic leukemia, (C) acute myeloblastic leukemia, and (D) chronic lymphocytic leukemia.

hours, and dialyzed against cold, running tap water for 48 hours. At the end of this period each sample was placed in a 4 ml vial which was filled with concentrated sulfuric acid, dissolving the bag and allowing for complete mixing. Standards were prepared in a similar fashion. Serum from a normal person was examined approximately every other day to serve as a control. Counting was performed in a well-type scintillation counter. "Bound" vitamin B_{12} was calculated as activity retained in the bag after dialysis.

Results

Normals. In 113 normal blood donors a range of unsaturated binding capacity within two standard deviations is shown in fig. 1. Nine cases were above and an equal number below these ranges. Inspection of the graph shows a rapidly ascending limb and a second less acute rise. Previous studies in the laboratory have shown continuation of this second phase when as much as 500 mμg of the radiovitamin were added (21). This curve has been interpreted as suggesting a specific binding mechanism which becomes saturated, following which non-specific binding takes place.

Acute leukemia. Twenty-three cases are included in this group, of which 11 were classified as lymphoblastic, 10 myeloblastic, and 2 monoblastic. The data show there were 3 instances in the lymphoblastic and 2 in the myeloblastic groups in which increased B_{12} binding was observed (fig. 1). Of 3 patients in the lymphoblastic group binding was markedly elevated in a single observation in 1 moribund subject (S. E.), moderately elevated as a preterminal event in a second (B. O.), and at upper limits of normal (5 separate determinations) in the third (G. O.). In the second patient (B. O.) binding capacity was within normal range at the onset of the disease and during most of the course, and rose only shortly before death. Sera from the third patient (G. O.) showed persistently elevated binding levels throughout the illness even when clinical and hematologic remissions occurred. Leukocyte counts in these 3 patients were 12,000/mm³, 2,000/mm³, and 4,500/mm³ with 41 %, 80 % and 28 % lymphoblasts, respectively. In 8 other patients with acute lymphoblastic leukemia with normal serum B_{12} binding, leukocytes ranged from 5,300/mm³ to 130,000/mm³, with 14 % to 98 % lymphoblasts. Their average binding capacity is shown in fig. 1.

Of the 2 patients with acute myeloblastic leukemia, 1 showed persistently elevated binding levels during the short period of observation (2 weeks). He had been treated for polycythemia vera for 10 years prior to the onset of leukemia. Leukocytes were in the 15,000/mm³ range, with 75 % myeloblasts. The white blood cell count of the second patient was in the area of 50,000/mm³ with 50 to 60 % myeloblasts. However, these 2 hematologic pictures were duplicated among the remaining 8 patients in this group whose sera showed normal binding capacity. Leukocytes in this latter group ranged from 13,000/mm³ to 103,000/mm³ with 5 % to 80 % myeloblasts and myelocytes. The degree of serum B_{12} binding in none of the patients with acute leukemia ever reached levels observed in subjects with untreated chronic myelocytic leukemia.

Chronic myelocytic leukemia. More than 200 determinations of serum B_{12} binding capacity were made on 15 patients, 13 of whom showed elevated values. Of the remaining 2, 1 was in hematologic and clinical remission as a result of previous therapy. Sera from the second patient were obtained while he was recieving myleran, which resulted in a subsequent good remission. B_{12} binding values in the latter case were at the borderline between normal and slightly elevated levels and fell to mean normal as treatment was continued. All remaining 13 cases were in varying degrees of relapse and showed moderately elevated to extremely high levels of serum B_{12} binding. Long term observation showed that with treatment, as total number of leukocytes fell and immaturity decreased,

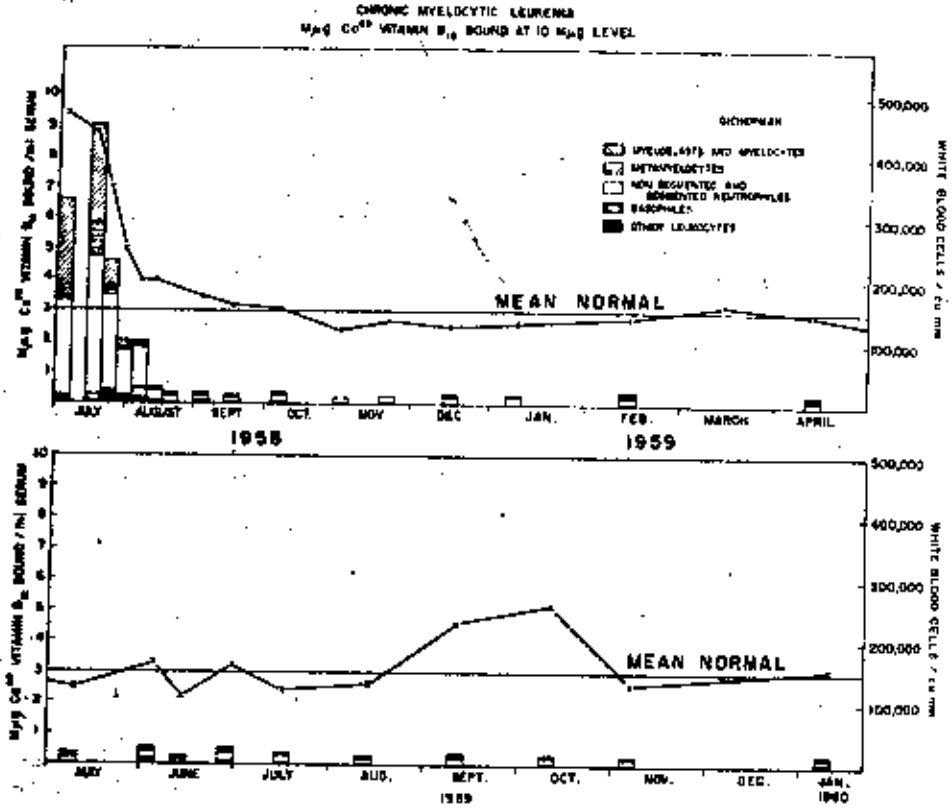

Fig. 2. Co^{60} B_{12} binding capacity of serum at 10 mμg. level in relation to leukocyte count in patient with chronic myelocytic leukemia.

serum binding capacity was usually reduced. Representative examples of these data are shown in figs 2, 3, 4, and 5.

Illustrative cases

G. I., adult white male, age 65 years, has been treated with myleran since July 1958. Clinical and hematologic responses have been excellent. As the total number of immature leukocytes dropped and blood picture reverted towards normal Co^{60} B_{12} binding capacity fell and stayed in the normal range for almost the entire period (fig. 2). There was one episode (September and October 1959) when binding capacity rose temporarily to almost twice the normal level with no apparent change in hematologic state.

D. A., adult white female, age 39 years, has been under treatment since April 1959. Clinical and hematologic responses to myleran have been highly satisfactory. B_{12} binding capacity fell from high levels to close to the mean normal as total number of leukocyte approached normal and immature cells disappeared (fig. 3).

V. O., adult white female, age 52 years, has been treated with myleran since July 1959. Fig. 4 shows that there was fair positive correlation most of the time between total leukocytes and abnormal white blood cells and binding capacity of serum. It is noteworthy that in September 1959 elevation of serum B_{12} binding capacity preceded a rise in total leukocytes by 2 to 3 weeks. Subsequent intensive treatment with myleran

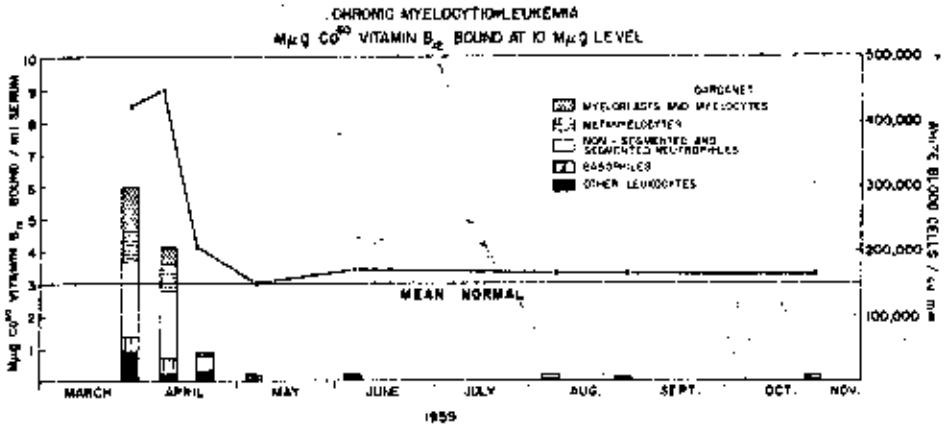

Fig. 3. Co^{60} B_{12} binding capacity of serum at 10 mμg. level in relation to leukocyte count in patient with chronic myelocytic leukemia.

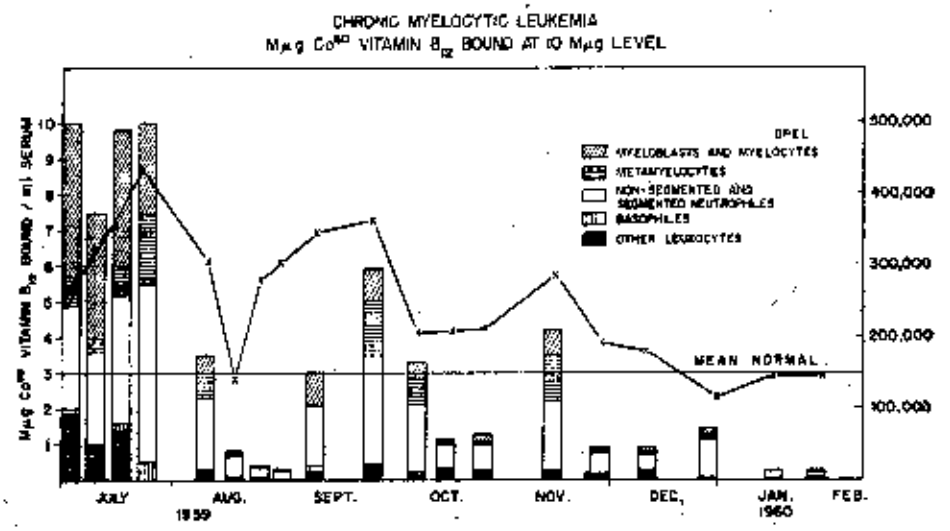

Fig. 4. Co^{60} B_{12} binding capacity of serum at 10 mμg. level in relation to leukocyte count in patient with chronic myelocytic leukemia.

was followed by a reduction in total leukocytes and serum B_{12} binding capacity to normal levels. It is pertinent, however, that in December 1959 when total leukocytes rose to 70,000/mm³, with 9 myelocytes, B_{12} binding capacity remained normal.

F. O., adult white female, age 47 years, had been treated with myleran since August 1958. Here again the pattern showed a tendency of serum B_{12} binding to follow the leukocyte count, with some exceptions (fig. 5). In October 1958 binding levels remained extremely high even though the leukocytes fell from over 300,000/mm³ to 100,000/mm³. It should be noted that 1 month later the leukocytes rose to a peak of 300,000/mm³, and until total white blood cells was reduced and maintained below 50,000/mm³ serum B_{12}

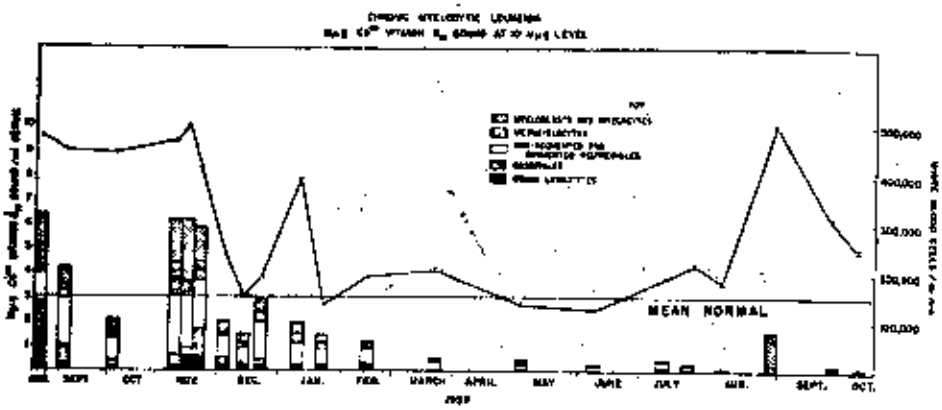

Fig. 5. Co^{60} B_{12} binding capacity of serum at 10 mμg. level in relation to leukocyte count in patient with chronic myelocytic leukemia.

Table I. Total leukocytes and differential counts in patients with myeloid metaplasia

Patient	Total WBC per mm³	Seg. neut.	Non-seg. neut.	Meta-my-elo-cytes	My-elo-cytes	My-elo-blasts
G ..	45,000	58	10	5	7	1
F ..	6,000	20	20	1	9	
Z ..	16,500	23	37	13	13	1
Ga ..	38,000	53	11	6	9	10
P[a] ..	12,600	43	10	12	12	
S[a] ..	12,700	70	4	4		
C[a] ..	26,800	63	23		4	1

[a] Elevated serum B_{12} binding.

binding did not fall to the normal range. Shortly before death, when white blood cells rose to 80,000/mm³ and the majority of cells were in the myelocytic stage, B_{12} binding rose to enormous levels. Following treatment with uracil mustard leukocytes were reduced to about 50,000/mm³, but serum B_{12} binding levels fell only slightly.

Chronic lymphocytic leukemia. In this group of 38 patients 8 showed elevated serum B_{12} binding capacity (fig. 1). Leukocytes ranged from 5,700 to 554,000/mm³ with absolute lymphocyte counts of 5,000 to 550,000/mm³. The degree of anemia was also variable with hematocrit values from 22 % to 43 %. The platelets ranged from 50,000 to 215,000/mm³. Among 30 patients with chronic lymphocytic leukemia whose serum binding was normal there was a similar spread in hematocrit, leukocyte and platelet levels. There were 2 patients in this group whose B_{12} binding capacity fell below the lower limit of normal.

Myeloid metaplasia. Of 7 patients studied 3 showed elevated serum B_{12} binding capacity (fig. 6). Antecedent history in the latter cases indicated previous diagnoses of polycythemia vera in 2 instances (S. N. and C. V.) and chronic myelocytic leukemia in the third (P. I.) There was however no suggestion of previous hematologic disorder in 4 subjects whose sera showed normal values (agnogenic myeloid metaplasia). Total leukocytes in the high binding group ranged from 12,600/mm³ to 260,000/mm³ with 11 % and 4 % myelocytes, respectively. In patients with normal binding the

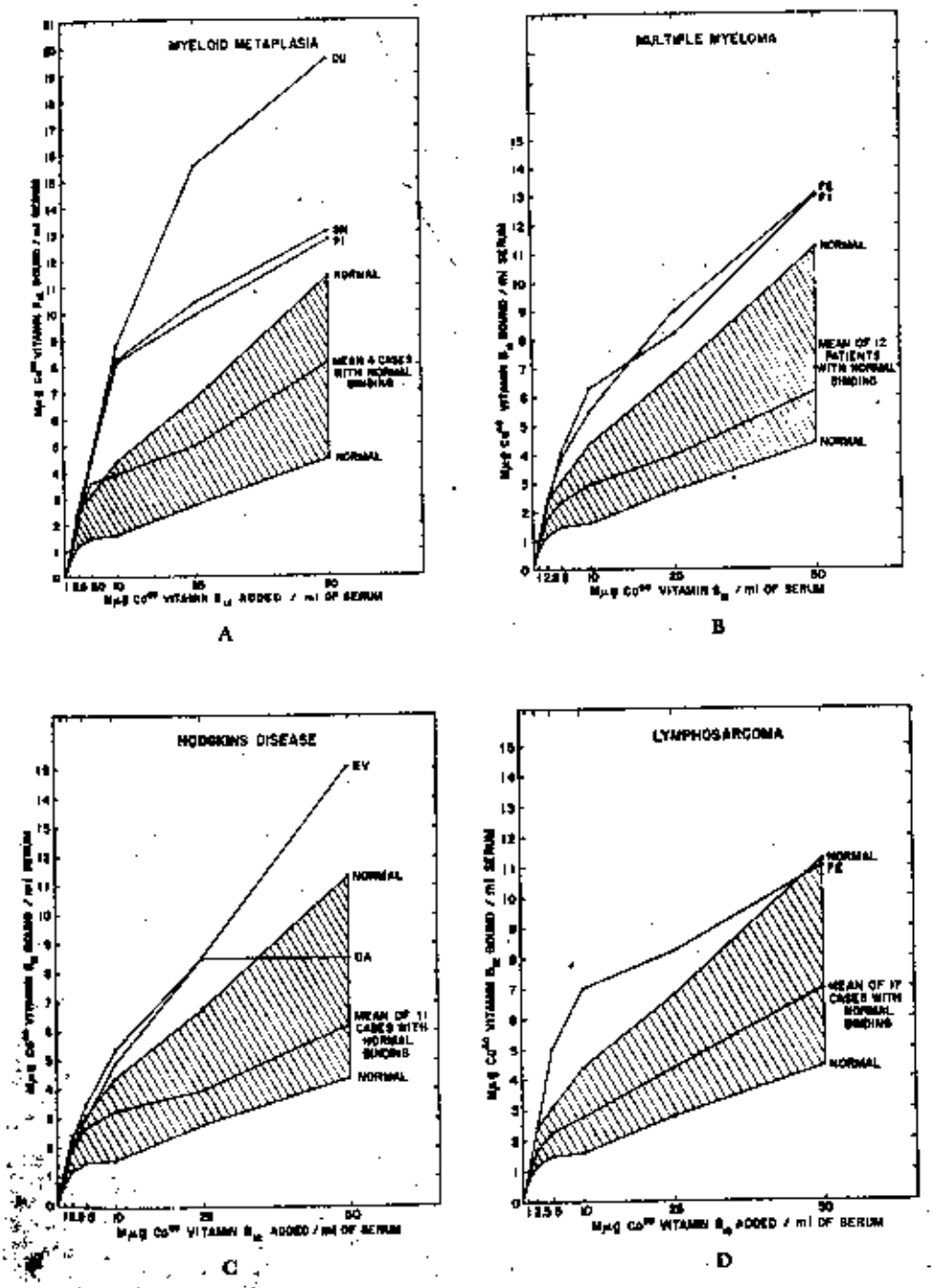

Fig. 6. Serum Co^{60} B_{12} binding capacity in (A) myeloid metaplasia, (B) multiple myeloma, (C) Hodgkin's disease, and (D) lymphosarcoma.

Table II. Leukocyte counts in patients with polycythemia vera, showing normal and increased Co^{60} B_{12} binding capacity

Total W. B. C./mm³

Patients with normal serum B_{12} binding capacity	Patients with elevated B_{12} binding capacity
7,500	7,800
8,600	8,400
8,900	10,500
9,000	12,700
9,000	15,000
9,400	15,000
9,500	15,000
9,800	16,000
13,000	16,400
14,700	16,500
25,800	20,000
	28,700
	31,000
	33,000
Mean 11,175	Mean 17,571
Median 9,200	Median 15,500

leukocytes ranged from 4,500/mm³ to 38,000/mm³ with 7 % and 9 % myelocytes, respectively (table I).

Multiple myeloma. There were 18 patients in this group, 2 of whom showed elevated serum B_{12} binding capacity (fig. 6). The diagnosis was based on the presence of immature plasma cells (myeloma cells) in the bone marrow. There were varying degrees of bone changes. Serum from one of the subjects with increased values contained cryoglobulin, but in all other respects the clinical course was the usual one observed in this disease. Leukocyte counts in these 2 cases were 7,150/mm³ with 72 % neutrophils and 12,500/mm³ with 80 % neutrophils, respectively. This was within the range observed in the other 14 patients in whom normal binding was found. There was no apparent relationship of binding capacity to serum globulin values.

Hodgkin's disease. There were 2 patients of a total of 14 whose serum binding B_{12} levels were elevated (fig. 6). The disease was of moderately long standing in both instances (about 3 years) and involved chiefly abdominal lymph nodes, spleen and liver. Serial studies in 1 case showed reduction to normal values following radiation to retroperitoneal nodes. The leukocytes at the time of elevated serum binding levels were 10,600/mm³ with 6 % neutrophils. There were never any other increases in B_{12} binding levels in this patient during subsequent periods of relapse. The second patient was in relatively good clinical condition with no anemia. The leukocyte count was 9,600/mm³ with 70 % neutrophils.

Lymphosarcoma. Included in this group of 18 patients was 1 case each of reticulum cell sarcoma and giant follicle lymphoblastoma. The single abnormally high value for serum binding capacity occurred in a subject with widespread lymphosarcoma who was in the late stage of the disease (fig. 6). This was an isolated rise during the clinical course since normal values were obtained prior to and following this determination. There was moderate anemia. The total leukocyte count was 8,750/mm³ with 69 % segmented neutrophils. This hemogram was in a similar range when normal B_{12} serum binding levels were found on prior and subsequent occasions.

Polycythemia vera. Of 26 cases 14 showed elevated serum B_{12} binding capacity (fig. 7). Patients were for the most part untreated at the time these examinations were made. From data on hand there does not appear to be any relationship

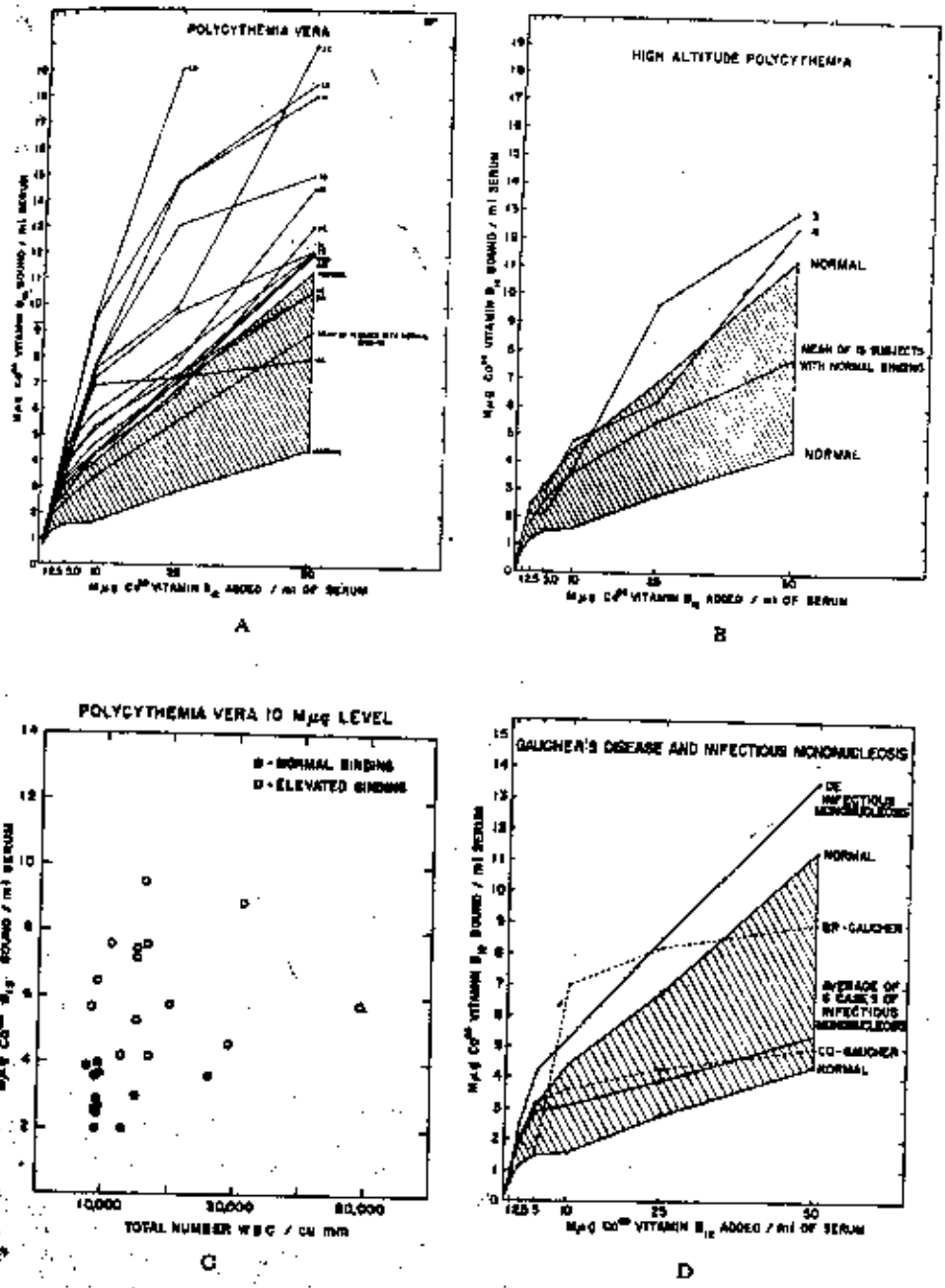

Fig. 7. Serum Co^{60} B_{12} binding capacity in (A) polycythemia vera, (B) high altitude polycythemia and (D) Gaucher's disease and infectious mononucleosis.
(C). Relationship of Co^{60} B_{12} binding capacity to leukocyte counts at 10 mμg. level in chronic myeloid leukemia.

Table III. Leukocyte counts in patients with high altitude polycythemia

Total white blood cells/mm³		
5,600		9,800
6,000		9,800
7,200		[1]11,300
8,000		11,300
8,300		11,500
8,400		11,600
9,200		12,000
9,500		
9,700	Mean	9,300
9,800	Median	9,700

[1] Elevated B_{12} serum binding capacity.

Table IV. Leukocyte and differential counts in patients with hyperleukocytosis

Pat.	Diagnosis	Total WBC	Seg. neut.	Non-seg. neut.	Meta-my-elo-cytes	My-elo-cytes
A	Peri-rectal abscess	54,000	19	21	23	20
K	F. U. O.	85,000	70	16		
S	G. I. bleeding	55,000	78	6		
H	Broncho-pneumonia	54,300	88	5		
R	Pyelone-phritis	45,000	85	11		
W	Coronary occlusion	45,000	76	12		
G	Cerebral apoplexy	45,000				

between serum binding capacity, hematocrit values, or treatment with P^{32}, myleran, or phlebotomy. Platelet counts ranged from 70,000 to 500,000/mm³ and were also unrelated to serum B_{12} binding capcity. If leukocyte counts of the normal and high binding groups are examined the medians are 9,200/mm³ and 15,500/mm³ respectively (table II). In the former there were only 3 patients with total white blood cells over 10,000/mm³, while in the latter all but 2 subjects had values greater than 10,000/mm³ (fig. 7).

Co^{60} B_{12} serum binding capacity of 17 patients with secondary polycythemia was examined. There was only one slightly elevated value and another at the upper limit of normal (fig. 7). The median leukocyte level in this group was 9,700/mm³. Hematocrit values ranged from 47 to 80 %. Platelet counts were all normal. Two cases of stress polycythemia showed normal serum binding capacity (table III).

Infectious mononucleosis. One case of a total of 8 showed elevated Co^{60} vitamin B_{12} serum binding capacity (fig. 7). This patient had clinical and laboratory evidence of hepatitis at the time of these studies. The leukocyte count was 7,500/mm³ with 75 % mononuclear cells, many of which were typical of this disease. Serum B_{12} binding levels fell slowly as the patient improved. However, there were other patients in this group who also showed clinical and laboratory signs of hepatic involvement in whom serum B_{12} binding capacity was normal.

Hyperleukocytosis and leukopenia. There were 6 cases in the former group and 2 in the latter, all of whom showed normal B_{12} binding levels. Leukocyte counts were between 45,000 to 85,000/mm³ in the first category and below 3,000/mm³ in the second. No abnormal leukocytes were present in the peripheral smears of hyperleukocytic patients. Diseases associated with high leukocyte counts are shown in table IV. Bone marrow in both groups of patients showed increased myeloid activity.

Gaucher's disease. Of 2 patients,

Table V. Hematologic disorders and serum Co^{60} binding capacity

Diagnosis	Total	Normal binding	High binding	Low binding
Acute lymphoblastic leukemia	11	8	3	0
Acute myeloblastic leukemia	9	7	2	0
Acute monoblastic leukemia	2	2	0	0
Chronic lymphocytic leukemia	38	28	8	2
Chronic myelocytic leukemia	15	2	13	0
Myeloid metaplasia	7	4	3	0
Multiple myeloma	18	16	2	0
Hodgkin's disease	14	12	2	0
Lymphosarcoma[1]	19	18	1	0
Polycythemia vera	26	12	14	0
Secondary polycythemia	17	16	1	0
Stress polycythemia	1	1	0	0
Infectious mononucleosis	8	7	1	0
Hyperleukocytosis	8	8	0	0
Leukopenia	4	4	0	0
Gaucher's disease	2	1	1	0
I. T. P.	13	13		
Pancytopenia, anemia in uremia, Cooley, secondary anemia, aplastic anemia, hemachromatosis, chronic hemolytic anemia, sickle cell anemia, pernicious anemia (relapse and treated) eosinophilia	21	21		
	233	178	53	2

[1] Includes 1 case each of reticulum cell sarcoma and giant follicle lymphoblastoma.

each showed normal and increased B_{12} binding levels (fig. 7). In the former splenectomy was successfully performed 2 years previously for thrombocytopenia purpura. The patient with elevated binding capacity expired shortly after admission to the hospital due to cerebral hemorrhage. Leukocytes varied from 14,100/mm³ with 44 % neutrophils to 16,400/mm³ with 73 % neutrophils, respectively.

Idiopathic thrombocytopenic purpura and serious anemias. Thirteen cases of idiopathic thrombocytopenic purpura and 20 patients with a variety of anemias were studied (table V). All showed normal Co^{60} B_{12} binding levels.

Various medical disorders

Table VI lists the various medical disorders and the number of patients in each group with normal, increased and decreased serum B_{12} binding capacity. In a total of 138 cases there were 10 instances of increased and 5 of reduced binding beyond the limits of 2 standard deviations. In view of the observations in normal subjects, these variations may not be significant. In the former group 4 of the subjects were classified as acute or subacute hepatitis, and 1 each was found in disseminated lupus erythematosis, dermatomyositis, gastric ulcer, uremia, and an apparently normal person more than 60 years old. Decreased binding was

Table VI. Listing of medical disorders in which serum Co^{60} vitamin B_{12} binding was studied

Diagnosis	Total	Binding		
		Normal	High	Low
Aged (60 years)	13	11	1	1
Acute and subacute hepatitis	20	16	4	0
Cirrhosis of liver	6	6	0	0
Diabetes mellitus	15	15	0	0
Uremia	5	3	1	1
Toxic goitre	6	6	0	0
Hypothyroidism	6	6	0	0
Gout	3	3	0	0
Pregnancy (at term)	5	4	0	1
Generalized arteriosclerosis	4	4	0	0
Hypertension	6	6	0	0
Sarcoidosis	2	2	0	0
Acute rheumatic fever	2	2	0	0
Disseminated lupus	3	2	1	0
Dermatomyositis	1	0	1	0
Acute glaucoma	1	1	0	0
Benign gastric ulcer	3	2	1	0
Ulcerative colitis	2	2	0	0
Sprue	2	2	0	0
Schistosomiasis	1	1	0	0
Acute gastritis	2	2	0	0
Chronic cholecystitis	10	10	0	0
Chronic bronchitis with lung abscess	2	2	0	0
Acute suppurative appendicitis	4	4	0	0
Acute cholecystitis	2	2	0	0
Acute lymphadenitis	1	1	0	0
Acute orchitis	1	1	0	0
Calculus pyelonephritis	4	3	1	0
Gangrene of foot	1	1	0	0
Pneumonia	1	1	0	0
Herpes zoster	1	1	0	0
Lateral sclerosis of spinal cord	3	1	0	2
	138	123	10	5

found in 2 patients with amyotrophic lateral sclerosis, and 1 each in uremia, pregnancy at term, and in a person more than 60 years of age.

Neoplasms. Of 71 subjects there are 3 with elevated and 6 with decreased B_{12} binding capacity (table VII). The increased values were found in 1 case each of basal cell epithelioma, uterine fibroids and carcinoma of breast with metastasis to regional lymph nodes. In the group of patients with decreased binding capacity there were 3 instances of lung, 2 of ovarian, and 1 of breast carcinoma. In all instances there was only moderately advanced disease with no evidence of widespread organ or tissue invasion.

Table VII. Listing of neoplastic diseases in which serum Co^{60} vitamin B_{12} binding was studied

Diagnosis	Total	Binding		
		Normal	High	Low
Carcinoma of breast	10	8	1	1
» » colon	11	11	0	0
» » lung	4	1	0	3
» » prostate	8	8	0	0
» » pancreas	2	2	0	0
» » esophagus	2	2	0	0
» » ovary	5	3	0	2
» » thyroid	2	2	0	0
» » urinary bladder	3	3	0	0
» » gall bladder	1	1	0	0
» » renal pelvis	1	1	0	0
» » uterus	2	2	0	0
Glioblastoma	1	1	0	0
Benign prostatic hypertrophy	4	4	0	0
Fibroadenoma of breast	4	4	0	0
Basal cell epithelium of skin	1	0	1	0
Fibroid uterus	4	3	1	0
Mixed tumor of parotid	1	1	0	0
Mycosis fungoides	1	1	0	0
Simple lipoma	1	1	0	0
Simple ovarian cyst	1	1	0	0
Chronic cystic mastitis	2	2	0	0
	71	62	3	6

Discussion

It has been demonstrated by electrophoresis that the substance which binds vitamin B_{12} endogenously migrates largely with alpha globulins and probably represents specific factor (11, 14, 23). When vitamin B_{12} is added in vitro this factor, and other proteins, can bind B_{12}. From our data it is apparent that any increase in vitamin B_{12} binding capacity occurs in the first phase of the curve and the second phase is graphically parallel to that of the normal (15, 16). The origin of B_{12} binding proteins is unknown.

Recently, the seromucoid which has an electrophoretic mobility similar to that of alpha 1 and alpha 2 globulins has been found to include the specific binding substance (19, 30). The total seromucoids are increased in human subjects with neoplastic disease, trauma, inflammation, degenerative tissue changes, and in certain tumor bearing animals, (1, 8, 22, 31). However, the data in the present study do not disclose any relationship of vitamin B_{12} serum binding capacity to diseases other than most cases of chronic myelocytic leukemia, about half of the patients with polycythemia vera, myeloid metaplasia with antecedent histories of polycythemia or chronic myelocytic leukemia, and some cases of acute myeloblastic and lymphoblastic leukemia. The occasional subjects with chronic lymphocytic leukemia, Hodgkin's disease, multiple myeloma, lymphosarcoma, Gaucher's dis-

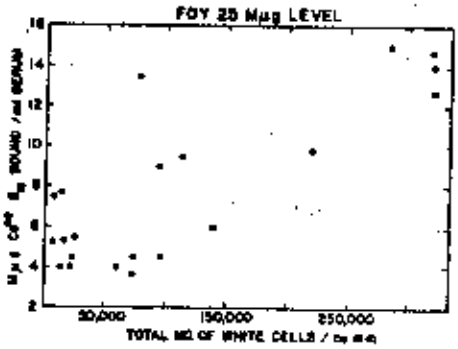

Fig. 8. Relationship of total number of leukocytes to serum Co^{60} B_{12} binding capacity at the 25 mμg. level in chronic myeloid leukemia.

case and infectious mononucleosis, showing elevated binding capacity may not be statistically significant. Since seromucoid fractions are increased in chronic myelocytic leukemia it appears likely that this substance is responsible for increased B_{12} binding capacity noted in this disease. However, this does not exclude the possibility that there is an increase in the number of binding sites on the molecules of specific binding substances (seromucoid?) or that there is another binding substance elaborated which is different from the naturally occurring one. Miller and Sullivan have shown that B_{12} bound in vitro in chronic myelocytic leukemia has a stronger bond than that found in normal serum and resembles in vitro bound B_{12} in that it must be heated before it becomes available for microbiologic assay (19). This again would suggest that naturally occurring specific binding substance may be present in increased amounts or have a greater avidity for vitamin B_{12}.

The role of leukocytes in the myeloid series in production of vitamin B_{12} specific binding substance is strongly suggested by some of the data in the present studies. The presence of elevated binding capacity in untreated chronic myelocytic leukemia and reduction to normal as total number of immature leukocytes fall indicates some correlation (fig. 8). That such correlation does not always exist may mean that binding capacity which in turn depends on specific binding substance, is related to turnover (survival time) of myeloid cells, and this turnover may not always be reflected by total number of leukocytes in the peripheral blood. There was less clear-cut correlation between binding capacity and total circulating metamyelocytes, mature leukocytes and basophils (fig. 9). Elevated B_{12} binding when leukocytes are falling or rising may reflect greater destruction than production of white blood cells, and hence an increased amount of available binding protein. Only when production of leukocytes is reduced in hematopoeitic sites and maintained at this level can binding capacity of serum be kept within a normal range. Mollin and Ross reported elevated B_{12} concentration in serum in chronic myelocytic leukemia for as long as 40 days after treatment was started and suggested that this was due to persistent presence of binding substance in serum for weeks (20). Precise measurements of half-life of binding protein in normal and abnormal sera have not been established.

The same explanation may be obtained in patients with polycythemia vera, since elevated binding capacity was seen almost entierely in those subjects with leukocyte levels greater than 10,000/mm³. Table II shows the median value in this group to be 15,000/mm³ as compared to 9,200/mm³ in patients showing normal B_{12} binding capacity. Higher white blood cell levels may be associated

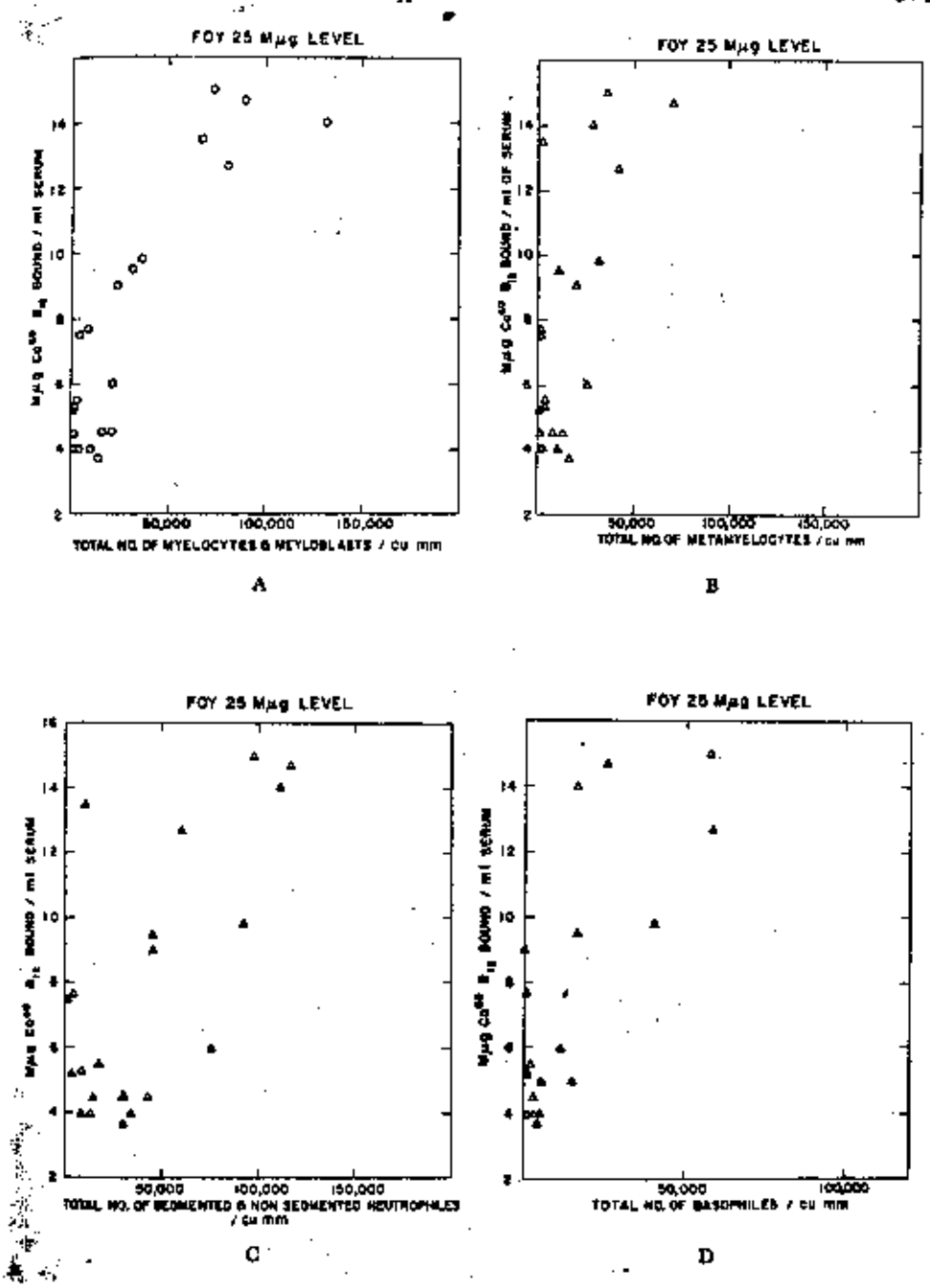

Fig. 9. Relationship of (A) total myelocytes and myeloblasts, (B) total metamyelocytes, (C) total segmented and non-segmented neutrophils, and (D) total basophils to serum Co^{60} vitamin B_{12} binding capacity at the 25 mμg. level in chronic myeloid leukemia.

with increased myeloid activity in hemätopoietic sites and reflected only by peripheral leukocytosis with infrequent immature forms. In high altitude polycythemia the total leukocytes was below 10,000/mm³ and only one case in 17 showed a slightly elevated B_{12} binding capacity.

Since cases of acute myeloblastic leukemia tend to show normal or only moderately elevated binding compared to untreated chronic myelocytic leukemia, it would appear that more binding substance is derived from products of myelocytes than from myeloblasts. Further evidence for this is seen in patients with hyperleukocytosis in whom maturation of leukocytes to the segmented and nonsegmented neutrophil stage was associated with normal serum binding capacity. These results differ with reports of elevated B_{12} content of serum in extreme leukocytosis (3, 20). All the patients with myeloid metaplasia exhibiting increased serum B_{12} binding had antecedent histories pointing either to polycythemia vera or to chronic myelocytic leukemia. This suggests a continuation of the original disease process which has reached an end stage. In contrast, patients with so-called "idiopathic" myeloid metaplasia show normal serum binding.

More difficult to explain, however, are instances of elevated serum binding capacity in some patients with acute lymphoblastic and chronic lymphocytic leukemia, in contrast to the report of Raccuglia and Sacks (24). Experiments in this laboratory indicate that all leukocytes have the capacity to bind vitamin B_{12} in vitro (34). When white blood cells, suspended in their own plasma are subjected to destruction by sonic vibration the products have a greater capacity to bind B_{12} than do intact cells. Myeloid cells and their products have a considerably higher capacity than lymphocytes and theirs. The sera of patients with acute or chronic leukemia contain substances liberated from disintegrated leukocytes. Since survival time of myeloid cells is shorter than that of lymphocytes, and more binding substance can be liberated to the serum, it is reasonable to expect elevated B_{12} binding capacity in myelocytic leukemia.

The possibility that binding substance (or substances) may be liberated from organs and tissues as a consequence of cellular invasion in malignant hematologic disorders cannot be discounted. However, this occurs rarely in patients with widespread non-hematologic malignancy in whom there is ample evidence of tissue invasion and destruction. This is shown in the section on neoplastic diseases (part III). The role of bone marrow activity in malignant hematologic disease but not epithelial or soft tissue neoplasm may be a factor in production of binding substance. Extreme variations in numbers of erythrocytes and platelets are not associated with abnormal B_{12} binding capacity and they are probably not concerned with formation of the binding proteins. Reduced serum B_{12} binding capacity noted in 2 patients with chronic lymphocytic leukemia was not associated with changes in serum protein.

The association of elevated B_{12} content of serum in hepatic disease has been reported from many laboratories (9, 10, 12, 13, 25, 28, 29). However, binding capacity has been reported as normal or increased (12, 25, 28, 29). Stevenson found B_{12} content of serum increased but binding capacity normal in acute and chronic liver disease (29). The same author reported normal disappearance from plasma of intravenously administered B_{12}

in severe liver disease (28). If the latter techniques may be regarded as an index of serum binding this would confirm the observation of normal serum binding in patients with severe liver damage. Rachmilewitz and his co-workers, on the other hand, noted an increase in binding capacity in those patients exhibiting evidence of hepatocellular damage and in whom B_{12} content of serum was elevated (25). Jones et al. reported a limited increase in serum B_{12} binding capacity in some cases of Laennec's cirrhosis, but this was an inconstant finding (12). Results from the present study show increased B_{12} binding capacity in acute and subacute hepatocellular disease in 4 of 20 cases. In 6 patients with established, and in some instances advanced, cirrhosis of the liver the binding capacity was consistently normal. Laboratory tests of hepatic function including serum protein examinations disclosed no differences between subjects with elevated or normal B_{12} binding capacity. Although no microbiological assays for vitamin B_{12} were done in this study, previous reports indicate that increased content in patients with acute hepatic insufficiency due to the "free" form implies no augmentation of serum binding protein (25). The source of this substance in the patients reported may be damaged liver cells.

The decreased B_{12} binding capacity noted in the few cases of carcinoma of lung and ovaries warrants further investigation in these groups. There is no apparent explanation for the occasional alteration noted in the above series.

Summary

1. Serum vitamin B_{12} binding capacity is uniformly increased in patients with chronic myelocytic leukemia, in about 50 % of subjects with polycythemia vera, in myeloid metaplasia, and in occasional persons with acute myeloblastic and lymphoblastic leukemia.

2. The data in most cases of chronic myelocytic leukemia suggest a positive correlation between total number of circulating myelocytes and serum B_{12} binding capacity.

3. The increased binding substance in chronic myelocytic leukemia may reflect augmented catabolism of myelocytes not necessarily associated with leukocytosis.

4. Normal serum B_{12} binding capacity in hyperleukocytosis further suggests that the specific protein is derived from products of younger leukocytes (myelocytes) rather than more mature forms.

5. In polycythemia vera elevated serum B_{12} binding capacity is usually associated with total leukocyte levels greater than 10,000/mm³. In secondary polycythemia the binding values are almost all within the normal range. In these patients the mean leukocyte level was below 10,000/mm³.

6. Increased serum B_{12} binding capacity in myeloid metaplasia was found only in patients with antecedent history of polycythemia vera or chronic myelocytic leukemia, 2 diseases frequently associated with elevated values.

7. Serum B_{12} binding capacity was unrelated to level of circulating erythrocytes or platelets.

8. Elevated serum B_{12} binding capacity was noted in some cases of acute and subacute hepatitis.

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References

1. Baldwin, R. W. & Harris, H. J.: Serum protein and glycoprotein changes during growth of experimental tumors in rats. Brit. J. Cancer *12:* 99, 1958.
2. Beard, M. F., Pitney, W. R. & Sanneman, E. H.: Serum concentrates of vitamin B_{12} in patients suffering from leukemia. Blood *9:* 789, 1954.
3. Beard, M. F., Pitney, W. R., Sanneman, E. H., Sakel, M. J. & Moorhead, H. H.: Serum concentrates of vitamin B_{12} in acute leukemia. Ann. int. Med. *41:* 323, 1954.
4. Bertcher, R. W. & Meyer, L. M.: Co^{60} vitamin B_{12} binding capacity of normal human serum. Proc. Soc. exp. Biol. *94:* 169, 1957.
5. Bertcher, R. W., Meyer, L. M., Varnus, H. & Mulzac, C.: Some characteristics of binding of vitamin B_{12} by normal human serum. Acta Hemat. *23:* 287, 1960.
6. Cronkite, E. P., Henley, E., Driscoll, D. H., Meyer, L. M., Dohan, F. C., Rubini, J. R. & Wolins, W.: Studies on the kinetics of intravenously injected Co^{60} labelled vitamin B_{12} in man. Hematolog. Latina *2:* 265, 1959.
7. Dunn, A. L., Walsh, J. R. & Holthaus, J. M.: Radioactive cyanocobalamin (vitamin B_{12}) in renal disease. Arch. int. Med. *101:* 927, 1958.
8. Greenspan, E. M.: Survey of clinical significance of serum mucoprotein level. Arch. int. Med. *93:* 863, 1954.
9. Grossowicz, N., Hochman, A., Aronovitch, J., Izak, G. & Rachmilewitz, M.: Malignant growth in the liver and serum vitamin B_{12} levels. Lancet *I:* 1116, 1957.
10. Halstead, J. A., Carroll, J. & Rubert, S.: Serum and tissue concentration of vitamin B_{12} in certain pathologic states. New Engl. J. Med. *260:* 575, 1959.
11. Heinrich, H. C. & Erdmann-Oehlecher, S.: Der Vitamin B_{12} Stoffwechsel bei Hämoblastosen. II. Clin. Chim. Acta. *1:* 911, 1956.
12. Jones, P. N., Mills, E. H. & Capps, R. B.: The effect of liver disease on serum vitamin B_{12} concentrates. J. Lab. Clin. Med. *49:* 910, 1957.
13. Lear, A. A., Harris, J. W., Castle, W. B. & Fleming, E. M.: The serum vitamin B_{12} concentration in pernicious anemia. J. Lab. Clin. Med. *44:* 715, 1954.
14. Mendelsohn, R. S., Watkin, D. M., Horrett, A. P. & Fahey, J. L.: Identification of the vitamin B_{12} binding protein in the serum of normals and of patients with chronic myelocytic leukemia. Blood *13:* 740, 1958.
15. Meyer, L. M., Bertcher, R. W. & Cronkite, E. P.: Serum Co^{60} vitamin B_{12} binding capacity in some hematologic disorders. Proc. Soc. exp. Biol. *96:* 360, 1957.
16. Meyer, L. M., Bertcher, R. W., Cronkite, E. P., Miller, I. F. & Mulzac, C.: Co^{60} vitamin B_{12} binding capacity of serum in benign and malignant disorders of the hematopoietic system. Cancer Res. *3:* 42, 1959.
17. Miller, A. & Sullivan, J. F.: The in vitro binding of Co^{60} labelled vitamin B_{12} by normal and leukemic sera. J. Clin. Invest. *37:* 556, 1958.
18. Miller, A., Corbin, H. F. & Sullivan, J. F.: The plasma disappearance, excretion and tissue distribution of Co^{60} labelled vitamin B_{12} in human beings. J. Clin. Invest. *36:* 18, 1957.
19. Miller, A. & Sullivan, J. F.: Some physicochemical properties of the vitamin B_{12} binding substances of normal and chronic myelogenous leukemic sera. J. Lab. Clin. Med. *53:* 607, 1959.
20. Mollin, D. L. & Ross, G. I. M.: Serum vitamin B_{12} concentrations in leukemia and in some other hematological conditions. Brit. J. Hemat. *1:* 155, 1955.
21. Mollin, D. L., Pitney, W. R., Baker, S. J. & Bradley, J. E.: The plasma clearance and urinary excretion of parenterally administered Co^{60} B_{12}. Blood *11:* 31, 1956.
22. Mochides, E., Stefanini, M., Magalini, S. I. & Kistner, S. A.: Content and composition of the mucoprotein fraction of human serum (serumucoid) in disease, with special reference to hematologic disorders. J. Clin. Invest. *37:* 127, 1958.
23. Pitney, W. R., Beard, M. F. & Van Loon, E. J.: Observations on the bound form of vitamin B_{12} in human serum. J. Biol. Chem. *207:* 143, 1954.
24. Racciolla, G. & Sacks, M. S.: Vitamin B_{12}

binding capacity of normal and leukemic sera. J. Lab. Clin. Med. *50:* 69, 1957.
25. Rachmilewitz, M., Aronovitch, J. & Grossowicz, M.: Serum concentrations of vitamin B_{12} in acute and chronic liver disease. J. Lab. Clin. Med. *48:* 339, 1956.
26. Ritz, N. D. & Meyer, L. M.: Clearance of intravenously injected Co^{60} labelled B_{12} in chronic myelocytic leukemia and other conditions. Cancer. In press.
27. Rosenthal, H. R. & Sarett, H. P.: The determination of vitamin B_{12} activity in human serum. J. Biol. Chem. *199:* 433, 1952.
28. Stevenson, T. D.: Vitamin B_{12} metabolism in liver disease. Clin. Res. Proc. *5:* 112, 1957.
29. Stevenson, T. D. & Beard, M. F.: Serum vitamin B_{12} content in liver disease. J. Med. *260:* 206, 1959.
30. Weinstein, I. B., Weissman, S. M. & Watkin, D. M.: The plasma vitamin B_{12} binding substances: I. Its detection in the seromucoid fraction of plasma from normal subjects and patients with chronic myelocytic leukemia. J. Clin. Invest. *38:* 1904, 1959.
31. Winzler, R. J. & Smyth, I. M.: Studies on the mucoproteins of human plasma. II. Plasma mucoprotein levels in cancer patients. J. Clin. Invest. *27:* 617, 1948.
32. Unpublished data.

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~~Acta Med. Scand. 164: 157-175~~

Co^{60} Vit. B_{12} Binding Capacity of Normal Human Cerebrospinal Fluid.*†
(24715)

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As extension of continuing investigations in this laboratory on Co^{60} Vit. B_{12} combining capacity of serum and salive(1,2,3), the following study was made on ability of normal human cerebrospinal fluid to bind radiovitamin. Utilizing microbiologic assay methods, Sobotka, Christoff and Baker recently reported Vit. B_{12} content of cerebrospinal fluid from normal subjects to range from 0-30 μg/ml(4). These authors found elevation of Vit. B_{12} in the cerebrospinal fluid and serum of about one-third of patients with multiple sclerosis.

Material and methods. Cerebrospinal fluid was obtained from 22 normal subjects with no apparent neurologic disorder, who were subjected to spinal puncture for injecting an anesthetic agent. The Co^{60} Vit. B_{12} had a specific activity of 0.823 mc/mg. To successive 1 ml aliquots of cerebrospinal fluid in Visking bags were added 1, 2, 5, 5, 10, 25, 50 and 100 mμg of radiovitamin. After incubation at room temperature for 2 hours, the mixture was subjected to exhaustive dialysis in cold, running tap water for 48 hours. All specimens were set up in duplicate, one to serve as a standard. Standards and dialyzed samples were placed each in 4 ml vials, filled with concentrated sulfuric acid to dissolve the casing, and radioactivity measured in a well-type scintillation counter. "Bound" Vit. B_{12} was calculated as activity retained in the bag after dialysis. Similar studies were made in 5 instances in which 1 ml of normal saline was substituted for cerebrospinal fluid, Co^{60} Vit. B_{12} added, and dialysis performed as de-

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TABLE I. Co^{60} Vit. B_{12} Binding Capacity of Normal Human Cerebrospinal Fluid.

mμg Co^{60} B_{12} added/ml spinal fluid	1	2.5	5	10	25	50	100
Mean mμg Co^{60} B_{12} bound	.47	.57	.71	1.0	1.6	3.4	4.2

scribed above. Protein content of 10 samples of cerebrospinal fluid was determined by the method of Looney and Walsh(5).

Results. Table 1 shows mean values for Co^{60} Vit. B_{12} combining capacity of cerebrospinal fluid from 22 human subjects with no clinical evidence of neurologic disorder. Mean CSF protein value was 34.8 mg %. Table II summarizes mean results of dialysis in 5

TABLE II. Co^{60} Vit. B_{12} in Saline Retained after Dialysis.

mμg Co^{60} B_{12} added/ml saline	1	2.5	5	10	25	50	100
Mean mμg Co^{60} B_{12} retained	.07	.09	.12	.15	.25	.5	1.0

cases in which saline was substituted for cerebrospinal fluid. Fig. 1 depicts mean spinal fluid binding capacity minus "background" of mean saline values described in Table II. For comparative purposes mean Co^{60} Vit. B_{12} binding capacity of normal human serum is shown in Fig. 1.

Discussion. Previous studies suggest a 2-phase Co^{60} Vit. B_{12} binding capacity of normal human serum, with an increase of first component in chronic myelocytic leukemia, and some patients with myeloid metaplasia and polycythemia.(1,2). This phenomenon appears to be lacking in normal cerebrospinal fluid (Fig. 1). Furthermore, slope of the line describing spinal fluid binding capacity does not parallel either of the serum components. Serum binding capacity at the 1 mμg level is 2.5 times that of the spinal fluid and more than 3.5 times higher at the 50 mμg point.

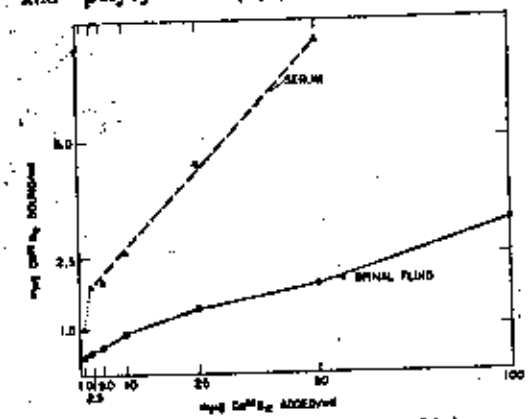

FIG. 1. Comparison of Co^{60} vit. B_{12} combining capacity of normal human serum and cerebrospinal fluid (saline values deducted).

Pitney found serum Vit. B_{12} bound to alpha globulin *in vivo* and to beta globulin when added *in vitro*(6). Pert and Kutt have recently investigated the distribution of protein fractions in cerebrospinal fluid using starch gel electrophoresis(7). Electrophoretic patterns of normal serum and concentrated spinal fluid show alpha and beta globulin peaks that are essentially the same. Consequently small amounts of protein in spinal fluid are probably not responsible for B_{12} binding in the spinal fluid unless they are in some fashion altered and have a strikingly increased number of binding sites or are potentiated by some other substance or substances present. However many other materials have the capacity to bind Vit. B_{12} and their presence or absence in spinal fluid have not been demonstrated(8).

Conclusions. 1. Normal human cerebrospinal fluid has the capacity to bind Co^{60} Vit. B_{12}. 2. This capacity to bind Vit. B_{12} increases as concentration of radiovitamin is increased. 3. Relationship of Co^{60} B_{12} binding capacity to protein content is higher for normal cerebrospinal fluid than for normal human serum.

1. Bertcher, R. W., Meyer, L. M., Proc. Soc. Exp. Biol. and Med., 1957, v94, 169.

2. Meyer, L. M., Bertcher, R. W., Cronkite, E. P., *ibid.*, 1957, v96, 360.

3. Bertcher, R. W., Meyer, L. M., Miller, I. F., *ibid.*, 1958, v99, 513.

4. Sobotka, H., Christoff, N., Baker, H., *ibid.*, 1958, v98, 934.

5. Looney, J. M., Walsh, A. L., *J. Biol. Chem.*, 1939, v127, 117.

6. Pitney, W. R., Beard, M. F., Van Loon, E. J., *ibid.*, 1954, v207, 143.

7. Pert, J. H., Kutt, H., Proc. Soc. Exp. Biol. and Med., 1958, v99, 181.

8. Grasback, R., *Acta Med. Scand.*, 1956, suppl. 314, v154, 3.

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Relationship between Faecal Bile Acids, Absorption of Fat and Vitamin B_{12}, and Serum Lipids in Patients with Ileal Resections

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Abstract. Faecal bile salt, neutral steroid and fat excretion, vitamin B_{12} absorption, and jejunal lipids, bile acids and cholesterol during fat digestion, serum cholesterol and cholesterol synthesis were measured in 3 patients with incomplete (IIR) and 9 patients with complete (CIR) resection of the terminal ileum. These parameters were virtually normal in IIR and in one case with CIR while in other cases they were frequently abnormal, bile salt excretion being increased upto 12-fold. The finding that faecal bile salts correlated positively with faecal fat and cholesterol synthesis, and negatively with jejunal constituents and serum cholesterol, supports the previously unproved concept that augmented elimination of cholesterol into faeces as bile acids proportionately depletes serum cholesterol despite increased cholesterol synthesis, proportionately decreases jejunal bile salts despite markedly augmented bile acid production and proportionately impairs micellar solubilization of lipids during fat absorption, leading to steatorrhoea. Furthermore, faecal bile salts showed a positive correlation with faecal water and a negative one with the Schilling test values. Faecal bile salts are suggested to be a sensitive indicator of ileal dysfunction.

Key-words: Ileal resection, bile acid excretion, micelle formation, malabsorption, steatorrhoea, diarrhoea, vitamin B_{12} absorption, serum cholesterol, cholesterol synthesis, methyl sterols.

Since the first report [1] that in a patient with resection of the terminal ileum wastage of bile salts into faeces was markedly augmented, the finding has been confirmed, mostly with indirect methods, in a great number of studies [2–7]. These investigations have shown that in ileal dysfunction of varied origin the bile salt pool is markedly reduced and that despite enhanced bile acid synthesis, intestinal bile salt concentration and hence micelle formation are lowered, with resulting steatorrhoea. Augmented bile salt flux into the colon exacerbates the diarrhoea by inhibiting water absorption and stimulating water and electrolyte secretion into the colon [8–11].

In patients with ileal resection bile salt loss has only been quantified directly by faecal analysis [1, 13–15] in a few cases, even though faecal bile acids could be a most sensitive index of ileal dysfunction. In the present investigation faecal bile salt elimination and sterol balance were measured after ileal resections. The values were related to the clinical condition, faecal constituents, vitamin B_{12} absorption, ability to maintain the intestinal micellar phase during fat digestion, serum lipids, and ability to augment cholesterol synthesis to balance increased elimination into the faeces. Some preliminary results have been presented earlier [13, 14].

Material and Methods

Patients. The series comprises 13 subjects, for whom clinical observations and some pertinent laboratory data are listed in Table 1. Length of the remaining intact bowel was estimated by the surgeon during the operation for intestinal resection. Patients 1–3 still had from 35 to 50 cm of intact terminal ileum (called incomplete ileal resection; IIR), 170–220 cm of the more proximal ileum being removed, while subjects 4–11 had undergone complete resection (75 to 180 cm) of the terminal ileum (CIR) and cases 7 and 11 had lost the right colon, in addition to the terminal ileum; these two patients were suffering temporarily from diarrhoea. Patients 12 and 13 (included in CIR) had undergone a massive intestinal resection for intestinal gangrene occurring in connection with bleeding gastric ulcer and for intestinal involvement in Hodgkin's disease, respectively. The general condition of patients 1–11 was good and they had been maintaining their weight; patients 2, 4, and 6 were underweight, and patients 3, 5, and 7 were slightly obese. Subjects 12 and 13 were severely malnourished, had a marked diarrhoea and tended to lose weight continuously. One of them died 4 weeks after the examination from sepsis and the other in 3 weeks from her underlying Hodgkin's disease.

Studies. All the patients were put in the hospital clinical research ward and fed on a low-cholesterol solid food diet containing 80 g of fat (from meat, lard and soya oil) /2400 kcal. The patients were given 600 mg of both sitosterol and Cr_2O_3 (Orion Oy, Helsinki), divided into three equal doses, as internal markers for correction of sterol degradation and faecal flow [16], respectively. On gas-liquid chromatography (GLC), the sitosterol contained 85% of β-sitosterol and 15% of other plant sterols. Analysis of the diet indicated that the total β-sitosterol intake was on average 624 mg/2400 kcal. Three-day stool collections were performed after the subjects had been consuming the diet, Cr_2O_3 and sitosterol for one week. The

Table 1. *Clinical observations and laboratory data of patients studied*

Case No.	Age and Sex	Weight kg	Weight Relat.	Post-operative period	Diagnosis and resection	Surgical indication	Schilling[a] test, %/48 h	Xylose[b] excretion, g/5 h	Faecal[c] fat, g/day	Serum lipids mg/100 ml chol.	Serum lipids mg/100 ml trigl.
					Incomplete ileal resection						
1.	51, ♀	57	1.07	1 y	Jejuno-ileostomy, 220 cm of ileum resected, 50 cm of terminal ileum intact	Intestinal volvulus	37.9	3.8	2.0	199	81
2.	54, ♀	50	0.85	2 y	Jejuno-ileostomy, 200 cm of ileum resected, 40 cm of terminal ileum intact. IHD	Thrombosis of mesenteric artery	18.0	7.7	4.8	312	115
3.	65, ♂	88	1.24	3 y	Ileal-ileostomy, 120 cm of ileum resected, 35 cm of terminal ileum intact. IHD	Thrombosis of mesenteric artery	40.0	5.5	4.1	214	120
					Complete ileal resection						
4.	55, ♀	50	0.85	3 y	Ileal-ascending colostomy, 1 m of distal ileum and 16 cm of ascending colon resected	Intestinal obstruction	34.0	7.0	2.8	211	123
5.	57, ♀	68	1.36	3 y	Ileal caecostomy, 1 m of terminal ileum and 60 cm of jejunum resected	Intestinal obstruction	8.4	6.5	5.6	182	196
6.	19, ♂	49	0.79	19 y	Ileal-ascending colostomy, length of resection unknown. Operated on when 5 months old	Intestinal obstruction	15.0	5.7	5.9	158 (128)	106
7.	43, ♂	80	1.19	1 y	Ileal-transverse colostomy, 110 cm of terminal ileum and right colon resected	Regional enteritis	11.0	6.1	6.0	200	276
8.	68, ♀	46	0.94	12 y	Ileal-caecostomy, 75 cm of terminal ileum resected	Intestinal obstruction	5.7	2.6	8.2	144	116
9.	51, ♂	59	0.95	1 mo	Ileal-ileostomy, 5 cm of terminal ileum intact. 180 cm of distal ileum resected	Paraduodenal strangulation	4.2	4.2	8.4	129 (100)	114
10.	59, ♀	55	0.98	10 y	Ileal-caecostomy, 85 cm of terminal ileum removed	Intestinal obstruction	—	—	10.0	180	146
11.	56, ♂	61	0.98	15 y	Ileal-transverse colostomy, 1.5 m of distal ileum and right colon resected	Intestinal obstruction Intestinal tbc 15 years earlier	2.1	5.2	9.6	109	97
12.	68, ♀	40	0.74	7 mo	Jejuno-transverse colostomy, 50 cm of jejunum intact, the rest of small intestine and the right colon resected	Hodgkin's disease	—	—	16.7	101	70
13.	35, ♀	47	0.87	3 mo	Jejuno-descending colostomy, 50 cm of jejunum, and half of the descending colon intact, ileum and the rest of the colon and jejunum resected	Intestinal necrosis after bleeding gastric ulcer	—	—	21.0	66	65

[a] Normal values >7 %.
[b] Normally >5 g/h.
[c] Normally >6.0 day/g.
IHD = Ischaemic heart disease. Cholesterol values in parentheses were obtained during cholestyramine treatment.

Schilling test (with intrinsic factor) and xylose absorption test were performed during stool collections.

Serum free methyl sterols were measured in 12 subjects so as to obtain some information on the compensatory increase of cholesterol synthesis [17]. The latter was studied in patients 8 and 10 with the acetate-mevalonate test too. For this purpose a mixture of ^{14}C-acetate and ^{3}H-mevalonate (25 μCi and 12.5 μCi, respectively) was administered intravenously, and the $^{14}C/^{3}H$ ratio in serum cholesterol was used as an index of cholesterol synthesis between acetate and mevalonate [17]. Activation of synthesis increases and inhibition decreases the ratio. Though the latter can be affected nonspecifically by changes in the acetate pool, the validity of this test of assessing cholesterol synthesis has been indicated by simultaneous sterol balance studies, serum methyl sterol determinations and sometimes incubations in vitro of liver biopsy specimens (cf. [17]). A clear-cut parallelism was found between these different methods in many clinical conditions associated with increased (obesity, hyperglyceridaemia, ileal by-pass and cholestyramine treatment) and decreased (hypercholesterolaemia and total fast) cholesterol production. Thus, though the acetate-mevalonate test is not measuring synthesis in terms of mg/day, it appears to be suitable for qualitative detection of changes in cholesterol synthesis.

After the stool had been collected, the jejunum was intubated as described in detail elsewhere in connexion with studies on the formation of the micellar phase [18]. This was done by sucking the intestinal contents with a double-lumen tube for $1^1/_2$ h after instillation of a standardized test meal through the other lumen of the tube into the stomach. The study was performed successfully in patients 1–5, 7–9, and 10. The test meal contained 17.6 g of olive oil mixed with ^{3}H-tristearin (15 μCi), 136 mg of β-sitosterol and 72 mg of cholesterol. β-sitosterol was used as a rough marker for measuring the dilution of the intestinal contents [18]. Since in cases with poor micelle formation most of the β-sitosterol stays in the oil phase and is partially precipitated, it may not be evenly dispersed in the intestinal contents during sampling, and in consequence the degree of dilution is occasionally overestimated [19].

Chemical Analysis. Dietary sterols, faecal neutral steroids derived from cholesterol and β-sitosterol (the terms faecal cholesterol and β-sitosterol include, in addition to the parent compounds, their respective bacterial conversion products coprostanol and coprostanone), and bile acids were determined with the combined thin-layer chromatographic (TLC) and GLC methods [20, 21]. Since recovery of the ingested β-sitosterol from the faeces was practically complete in every patient, all the faecal values are expressed in relation to Cr_2O_3. Faecal Cr_2O_3 was measured as presented by Bolin *et al.* [22] and faecal fat according to van de Kamer *et al.* [23].

The oil and micellar phases of the intestinal contents obtained during the $1^1/_2$ h period were separated according to Hofmann and Borgström [24], using 2 h ultracentrifugation. Analyses of lipid, sterols and bile acids are presented in detail elsewhere [18]. Outlines of the methodology are as follows: Total lipids and sterols, and free bile acids were determined from the pooled $1^1/_2$ h collection. The different lipid classes of the oil phase were separated by TLC. Sterols and sterol esters were measured by GLC, and triglycerides (TG), free fatty acids (FFA), 1.3- and 1.2-diglycerides, and monoglycerides (MG) by radioactivity (using the specific activity of fed triglycerides) and in some cases chemically. Total and fractionated micellar lipids were analysed similarly. The difference between the total intestinal lipids and total micellar lipids is due to the oil phase lipids (and sterols). Total bile salts were measured from the micellar phase, but the fraction precipitated by centrifugation was not measured. For determination of individual bile acids, a 1% NGS (Neopentyl Glycol Succinate) column was used in GLC. The lipids and sterols which are sedimented during ultracentrifugation were not analysed and were thus included in the oil phase. pH of the intestinal contents was measured only in three cases with CIR and ranged from 6.4–6.7.

Free methyl sterols in serum were measured by GLC [25] after the unesterified sterol mixture had been separated by TLC from the chloroform-methanol extract of serum lipids. Determination of the $^{14}C/^{3}H$ ratio in serum cholesterol was performed as described earlier [26].

Results

Table 1 shows that ileal dysfunction, as judged from the Schilling test, was not present in the patients with IIR. Three of the seven studies on the patients with CIR indicated reduced vitamin B_{12} absorption. Xylose excretion was abnormal in one case of the former and two of the latter group.

Faecal Fat. The mean fat excretion was higher after CIR (9.4 ± 1.7 g/day) than after IIR (3.6 ± 0.8 g/day) or in the controls (<6 g/day). Seven of the patients had abnormally high faecal fat, the highest value being 21.0 g/day (Table 1).

Faecal Steroids. Faecal bile salt excretion was normal in all patients with IIR. One of the patients with CIR, despite total removal of the terminal ileum, exhibited normal faecal elimination of bile acids (Fig. 1). In the others, including the two patients with extensive resection, the values were 2–12 times as high as the normal average. The mean bile salt output in CIR was 1765 mg/day, in IIR 215 mg/day and in controls 238 mg/day (Table 2). In these patients, in contrast to normal subjects (r = 0.85), bile acid excretion was not dependent on body weight (Table 3). Cholestyramine, administered to two patients, further augmented faecal bile salt elimination by 0.9–1.5 g, so that the final output was up to 15 times the normal level (3.6 g/day or 61 mg/kg).

As shown in Fig. 1, the length of the ileal resection bore no relation to the faecal bile salt loss. The latter, however, was correlated with faecal mass, faecal fat and vitamin B_{12} absorption (Table 3, Fig. 2). Since the Schilling test was abnormal in 57%, faecal fat in 70% and bile salt output in 90% of the patients with CIR, and since bile salt elimination was usually grossly increased, faecal bile acids appear to be a sensitive index of ileal dysfunction in patients with CIR; no such abnormality was present in patients with IIR.

Faecal neutral sterol excretion was within normal limits, an abnormally negative sterol balance being due exclusively to bile salt loss. As in normal subjects ($r = 0.59$), neutral sterol output was correlated with body weight (Table 3). No coprostanol or coprostanone derivatives were found in the faecal steroids of patients 6–13 (Table 4). In the others, bacterial conversion of cholesterol and β-sitosterol to secondary products was normal, no loss of β-sitosterol being detectable.

Micellar and Oil Phases of Intestinal Contents. The low β-sitosterol concentration in the jejunal content of patients with IIR (Fig. 3) suggests that the intestinal contents were diluted. This explains the subnormal

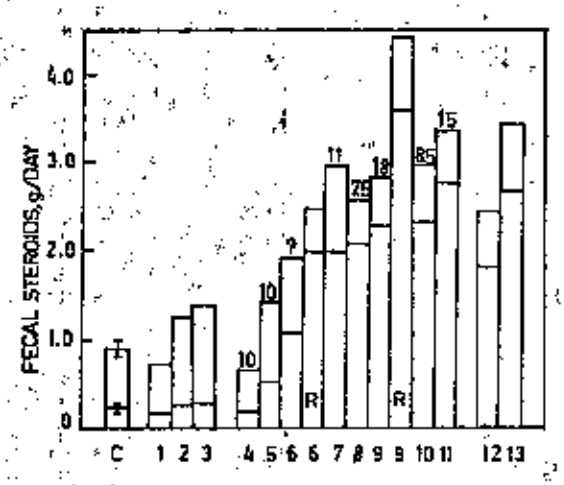

Fig. 1. Faecal steroid excretion of control subjects (*C* standard errors of bile acids and total steroids indicated by vertical lines) and patients with incomplete [1–3] and complete [4–13] ileal resection. Bile acids are indicated in the lower and neutral steroids in the upper part of the bars. Figures at the base of each bar refer to the number of the patient in Table 1. Cholestyramine treatment of cases 6 and 9 indicated by R. Figures on the top of each bar show the length (*dm*) of the resection of the terminal ileum

Table 2. *Serum lipids, faecal bile salts and sterol balance in control subjects and patients with incomplete (IIR) and complete (CIR) ileal resection*

Group	Serum lipids (mg/100 ml)		Bile acids		Sterol balance	
	chol.	trigl.	mg/24 h	mg/kg/24 h	mg/day	mg/kg
Controls	203±11	87±8	238±25	3.8±0.2	797±74	12.7±0.8
IIR	212±36	105±12	215±36	3.1±0.3	1009±195	16.1±3.6
CIR	148±14*	131±17*	1765±262*	32.9±5.7*	2371±280*	44.2±5.9*

Statistically significant differences ($P<0.05$) from controls or IIR are indicated by *. Control values (13 subjects) are from our unpublished sterol balance studies. Mean ± SE.

Table 3. *Correlation coefficients between different faecal, intestinal and serum parameters recorded in ileectomized patients (cases 1–13)*

Factor	Faecal fat	Faecal bile acids	Faecal neutral steroids	Sterol balance	Schilling test	Serum chol.	Serum[a] methyl sterol
Body weight	−0.43	−0.20	0.57*	−0.09	0.21	−0.90	0.31
Faecal mass	0.91*	0.81*	−0.16	0.82*	−0.75*	−0.78*	0.72*
Faecal fat	—	0.72*	−0.11	0.74*	−0.89*	−0.76*	0.59
Faecal bile acids	0.72*	—	−0.28	−0.96*	−0.80*	−0.74*	0.80*
Sterol balance	0.74*	0.98*	−0.09	—	−0.81*	−0.69*	0.85*
Serum cholesterol	−0.76*	−0.74*	0.45	−0.69*	0.51	—	−0.38
Micellar bile acids[b]	−0.87*	−0.70*	0.44	−0.67*	0.70*	0.67*	−0.01
Micellar lipids[b]	−0.69*	−0.72*	0.14	−0.72*	0.51	0.29	−0.43
Oil phase lipids[c]	0.81*	0.77*	0.19	0.85*	−0.78*	−0.39	0.45
Micellar cholesterol[b]	−0.78*	−0.67*	0.01	−0.70*	0.52	0.35	−0.22
Total cholesterol[b]	−0.77*	−0.67*	0.31	−0.67*	0.50	0.47	−0.01

[a] Calculated for fraction V in Fig. 2 containing a diunsaturated dimethylsterol.
[b] Calculated from amounts found in the intestinal pool in which the 136 mg of β-sitosterol administered was dispersed.
[c] Calculated from relative values (percent of total lipids). Statistically significant ($P<0.05$) correlation coefficients indicated by *.

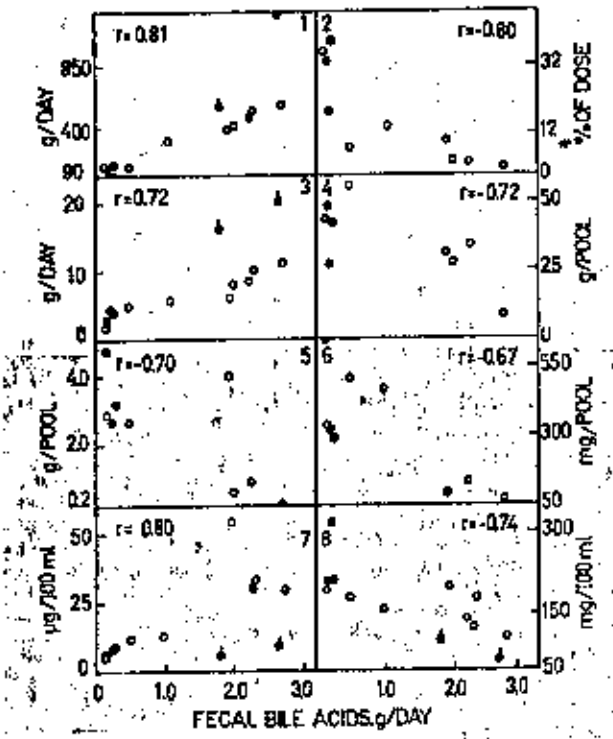

Fig. 2. Correlation of faecal bile acids with: *1*, faecal mass, *2*, Schilling test, *3*, faecal fat, *4*, *5* and *6*, jejunal micellar fat and bile salts, and jejunal total cholesterol, respectively; calculated for amounts found in the intestinal pool in which 136 mg of β-sitosterol administered in the test meal was dispersed, *7*, serum methyl sterol (fraction V ind Fig. 5) and *8*, serum cholesterol. • incomplete ileal resection, ○ complete ileal resection, ◆ with a small vertical line = extensive intestinal resection (not used in calculation of correlation coefficient for methyl sterol)

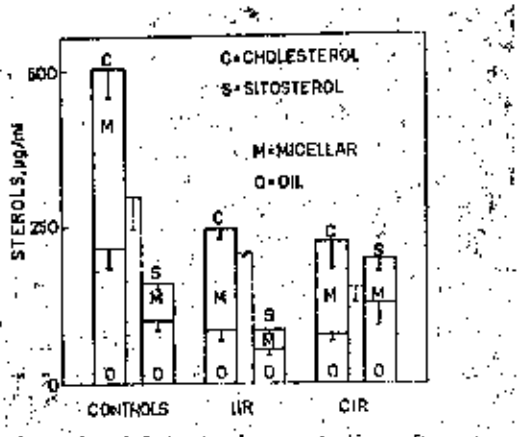

Fig. 3. Cholesterol and β-sitosterol concentrations after a test meal in the micellar and oil phases of the jejunal contents in controls [18] and in patients with incomplete (*IIR*) and complete (*CIR*) ileal resections. Bile acids (×10³ μg) are illustrated with broken lines. The vertical line in the top of each fraction shows standard error of mean

Table 4. *Presence of bacterial secondary products (coprostanols and coprostanones) of neutral sterols in faeces of control subjects and patients with ileal resection (cases 1–13)*

Case No.	Cholesterol (%)		Plant sterols (%)	
	Intact	Secondary products	Intact	Secondary products
Controls[a] Mean±SE	12±2	88±2	46±5	54±5
1	11	89	49	50
2	12	88	30	70
3	7	93	51	49
4	7	93	43	53
5	8	92	49	51
6–13	100	0	100	0

[a] Data from our unpublished sterol balance studies.

levels of cholesterol (Fig. 3) and total lipids (Fig. 4). The relative micellar solubilization of the two sterols is normal, despite the low bile salt concentration.

In CIR, the mean β-sitosterol concentration was normal, that of cholesterol being markedly reduced, probably owing to decreased biliary excretion. The average bile salt concentration was also reduced, subnormal values (<4 mM) being found in two of the six studies. The absolute amount of micellar cholesterol was low, yet the relative micellar solubilization of both sterols was normal.

In IIR, micellar solubilization of lipids was normal, provided the dilution was taken into consideration. Despite the low bile salt concentration, average micellar lipids were normal in CIR, only one value being subnormal (<1.5 mg/ml). The oil phase, however, was markedly increased and contained large amounts of digestion products, FFA and MG (Fig. 4). Thus, impaired lipolysis was not responsible for the relative decrease of the micellar phase; micellar lipids comprised 20±4, 40±6, and 31±4% of total jejunal lipids in CIR, IIR and controls, respectively.

The concentrations of micellar lipids and sterols were not correlated with those of bile salts. However, the molar sum of bile acids, FFA and MG was correlated with both micellar cholesterol ($r = 0.69$) and sitosterol ($r = 0.72$), suggesting that the digestion products of triglycerides did facilitate micellar solubilization of sterols. No significant association was found between the concentrations of intestinal constituents and faecal data. However, the micellar lipids, bile acids and cholesterol found in the intestinal pool in which the administered β-sitosterol was dispersed were correlated negatively with faecal bile salts and fat, while the oil phase lipids exhibited a positive correlation with the faecal constituents (Table 3, Fig. 2).

Intestinal Bile Acid Pattern. Both the absolute and relative amounts of free bile salts were higher in IIR and CIR than in the controls (Table 5). In cases 4 and 11 the free bile salts comprised 15% and 50%, respectively, of the total, indicating bacterial deconjugation,

Table 5. *Intestinal bile salt patterns during fat digestion in control subjects and in patients with incomplete (IIR) and complete (CIR) ileal resection*

Group	Bile acid fraction	µg/ml	Relative distribution % of total		
			Cholic	Deoxy	Cheno
Controls	Total	2997±525	54±12	15±6	32±7
	Free	12± 4	45±14	15±8	40±9
IIR	Total	2098±47*	61±10	11±2	28±6
	Free	42± 5*	59± 9	12±1	29±9
CIR	Total	1520±232*	51± 4	13±7	36±4
	Free	110± 43*	39± 6	16±7	45±6

Statistically significant differences ($P<0.05$) from controls [18] are indicated by *. Mean±SE.

Table 6. *The $^{14}C/^{3}H$ ratio in serum free cholesterol during the first day after intravenous administration of a ^{14}C-acetate-^{3}H-mevalonate mixture to control subjects and to two patients with ileal resection*

Subject	Ratio $^{14}C/^{3}H \times 100$ in serum free chol.		
	1 h	4 h	24 h
Controls	1.3–7.3	0.3–6.6	0.8–7.0
Case 9	21.7	16.1	15.8
Case 10	26.0	16.5	17.6

* Data of 10 control subjects [17]; 95% confidence limits.

Among the individual bile acids, the proportion of free chenodeoxycholic acid exceeded the corresponding value of the total fraction in CIR and in the controls, but not in IIR. On the whole the individual bile acids were present in normal proportions after ileal resection. The deoxycholic acid concentration was normal in patients 3, 5, and 10, and subnormal or absent in others. Faecal specimens from patients 8, 12, and 13, who were not on antibiotics, contained only primary bile acids.

Serum Lipids. Serum cholesterol (free and total) was subnormal in CIR, strikingly low values being found in patients 12–13 who had undergone extensive intestinal resection (Table 1 and 2). Cholestyramine, which increased faecal bile salt elimination, also caused a further significant reduction of serum cholesterol in the two patients studied. Triglycerides, however, were not low in CIR as compared with IIR, being actually higher than in the controls. Serum cholesterol was correlated positively with micellar bile acids and negatively with the sterol balance or faecal bile salts and fat (Table 3, Fig. 2).

That cholesterol synthesis was increased after ileal resection was indicated not only by the sterol balance data but also by the augmented serum methyl sterol concentrations (Fig. 5). In the cases in which the nutritional state was normal, a significant positive correlation existed between sterol balance or faecal bile salts and serum methyl sterols (Table 3, Fig. 2). But in the two cases with extensive resection and severe malnutrition methyl sterols were normal (Fig. 5). Thus in these cases the compensatory increase of cholesterol synthesis was probably low,

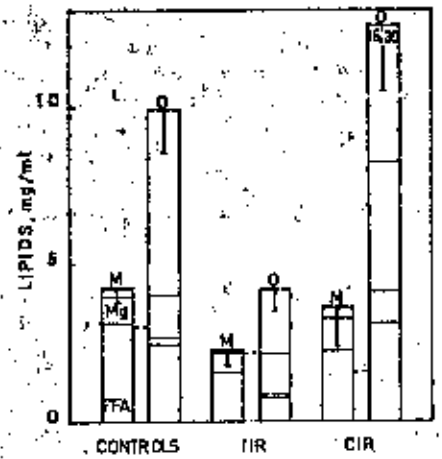

Fig. 4. Lipid concentrations in the micellar (*M*) and oil (*O*) phases of the jejunal contents in the same patients as in Fig. 3. The order of different fractions from the bottom to the top: free fatty acids (*FFA*), monoglycerides (*MG*), sum of 1,3- and 1,2-diglycerides and intact triglycerides. Bile acids are illustrated with horizontal broken lines. The vertical line in the top of each bar indicates standard error of total lipids in each phase

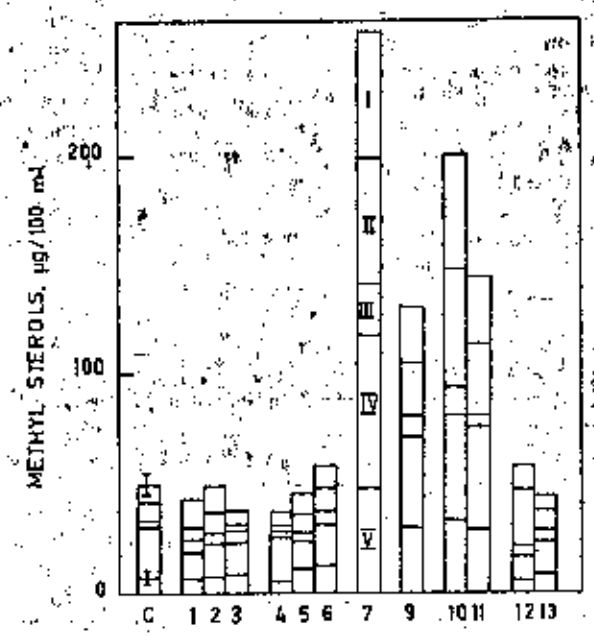

Fig. 5. Unesterified serum methyl sterols in the same patients as in Fig. 1. Fractions from the bottom to the top each bar are: *V* diunsaturated dimethylsterol, *IV* lanosterol, *III* monounsaturated monomethylsterol, monounsaturated trimethylsterol and diunsaturated dimethylsterol, *II* monounsaturated dimethylsterol and *I* dihydrolanosterol and two methostenols

faecal elimination of cholesterol being balanced primarily by mobilization of tissue cholesterol.

Finally, the ^{14}C-acetate-^{3}H-mevalonate test (Table 6) indicates that, as compared the values found in normal subjects, the $^{14}C/^{3}H$ ratio in serum cholesterol in the ileotomized patients 8 and 10 was markedly increased. This suggests that cholesterol synthesis was augmented between acetate and mevalonate.

Discussion

During the normal enterohepatic circulation, bile acid conjugates are effectively reabsorbed by the terminal ileum [4, 5, 27, 28]. Direct measurement of faecal bile acids in the present series revealed that the faecal bile salt wastage was increased up to 12-fold after resection of the terminal ileum. This wastage was demonstrated to be associated via the bile salt deficiency with impaired intestinal micelle formation and, further, steatorrhoea, and via augmented bile salt synthesis with proportional depletion of serum cholesterol and, further, stimulated cholesterol synthesis. However, retention of a relatively short segment, 35–50 cm, of the very terminal ileum was enough to prevent bile salt loss and vitamin B_{12} malabsorption, even if the rest of the more proximal ileum had been removed. Four alternative interpretations are possible: reabsorption is very effective in the terminal ileum, the ileocaecal valve performs an important function, colonic absorption is increased or the remaining small intestine adapts to absorb bile acids and vitamin B_{12}.

Patient 8, in whom the ileocaecal valve was intact, lost excessive amounts of bile salts while patient 4, who had no ileocaecal valve, had no wastage of bile salts. This suggests that the remaining small intestine can adapt to reabsorb bile salts especially because colonic bile acid absorption appears to be quantitatively less important [29]. This adaptation could be due to changes in mucosal function or be the result of the altered intestinal bile salt pattern. Glycochenodeoxycholate actually predominates among the intestinal bile salts in ileal dysfunction [5, 7, 30, 31]. This change of the pattern may reduce faecal bile salt loss in ileopathy because glycine conjugates of bile acids are absorbed effectively from the jejunum, too [28]. The absence of the ileocaecal valve permits bacteria to invade the contents of the upper intestine, where they can deconjugate bile salts and transform free bile acids to secondary products, processes taking place normally primarily in the colon [32, 33]. Since deconjugated bile salts are rapidly absorbed in the upper intestine [28], the bacterial overgrowth may improve bile salt absorption and decrease loss into the faeces. Patient 4 actually had 15% of her jejunal bile salts in deconjugated form. On the other hand, about 50% of the jejunal bile salts in patient 11 were in free form, indicating that in this case, at least, bacterial overgrowth was unable to prevent excessive faecal loss of bile salts.

The jejunal deoxycholate concentration is usually, but not always [7, 32], low in ileopathy probably due to a compromised reabsorption of bile acids [30, 34]. Patients with large ileal resection and severe steatorrhoea have been reported to excrete large amounts of deoxycholic and lithocholic acids [35]. Since deoxycholate is formed from cholic acid by bacterial enzymes mainly in the colon its jejunal concentration depends on reabsorption and bacterial action. Lack of secondary products of neutral sterols or bile acids in the faeces of patients with severe ileal dysfunction in the present series indicated that overall bacterial action on steroids was actually decreased, a conclusion also reached by others for faecal bile acids in ileopathy [12]. The significance of this finding on bile acid and overall cholesterol metabolism is unknown. In germ-free rats the absence of microbial action is associated with a reduced turnover of bile acids [33].

In the present series faecal bile salt excretion was a good index of ileal dysfunction. Patients 6, 8 and 10 had no gastro-intestinal symptoms or signs, yet faecal bile acid determination revealed ileopathy, iron-deficiency anaemia being the reason for hospitalization. In our patients with ileal by-pass, faecal bile salt excretion has always been markedly increased, although the Schilling test and faecal fat have occasionally been normal [36]. However, though the bile salt output is usually grossly increased, so that one three-day stool collection appears to be sufficient for analysis, even without a special diet or Cr_2O_3 marker, the clinical use of faecal bile salts for diagnosis of ileopathy and differential diagnosis of malabsorption is limited by methodological difficulties.

The maximum capacity of the human subject to produce bile salts is unknown. The results of the present study and those obtained in patients with bile fistula [38], ileal by-pass [36, 37] or ileostomy [12] indicate that production can increase up to 3–5 g/day. The finding that faecal bile salt excretion was not correlated with the length of the resection (Fig. 1) is presumably due to variable adaptation of reabsorption and differences in the capacity of the liver to augment its bile salt synthesis. Extensive intestinal resection, for instance, usually results in severe malnutrition (as seen in patients 12 and 13), which in turn limits hepatic bile acid synthesis [17].

It appears likely that absence of the terminal ileum explains the malabsorption of both vitamin B_{12} and bile acids, and the close correlation between the two. Bacterial overgrowth of the small intestine may have contributed to the vitamin B_{12} malabsorption [32], though deconjugated jejunal bile salts, indicators of bacterial overgrowth [32], were not correlated with the Schilling test values. The faecal wastage of bile acids resulted in proportional malabsorption of fat and water, the reduction of the absorptive area being an additional factor. The correlation between vitamin B_{12} and fat absorption, found earlier in idiopathic steatorrhoea [39] and after

ileostomy [29], is thus ultimately related to ileal dysfunction.

Reduced intestinal bile salt concentration, which might have been even more marked after successive test meals [40], indicated that the compensatory increase of bile salt synthesis, revealed by high faecal bile acid excretion, was not high enough to offset the faecal wastage. A low intraluminal bile acid concentration and poor micellar solubilization of lipids have been frequently found in patients with ileal dysfunction [1, 2, 6, 30, 40, 41], and the serum cholesterol level has been shown to decrease linearly with the length of the ileal resection [42]. Hepatic channelling of cholesterol to bile acid synthesis depleted serum cholesterol and secondarily reduced biliary cholesterol excretion, resulting in decreased intestinal cholesterol (Fig. 2). This, in turn, despite apparently decreased cholesterol absorption, which may have been proportional to the degree of bile salt deficiency, would explain the unchanged faecal neutral sterol excretion.

The ultimate reason for the development of bile salt deficiency in ileopathy is either depletion of cholesterol within a bile acid precursor pool due to limitation in the increase of cholesterol production or limited hepatic capacity to synthesize bile acids. There is some evidence that in hypercholesterolaemic patients limited bile salt synthesis rather than lack of cholesterol is the primary factor [17, 43]. In the present series the patients with extensive resections excreted surprisingly large amounts of bile acids, despite malnutrition, but might have produced even more if serum cholesterol had been maintained higher by adequately increased cholesterol synthesis.

The reason for the relatively high serum triglyceride levels in the patients with complete ileal resection is not apparent. It may be related to a dietary carbohydrate effect in patients with fat malabsorption. An increase of serum triglycerides is seen occasionally in patients whose enterohepatic circulation of bile acids is interrupted by cholestyramine [44] or ileal by-pass [45].

The increase of faecal cholesterol elimination induced by cholestyramine or by ileal by-pass was not correlated with the decrease of serum cholesterol in hypercholesterolaemic patients [30, 43], probably owing to a variable compensatory increase in synthesis. In the present series a negative correlation was found between the sterol balance data and the serum cholesterol level. That cholesterol synthesis was actually increased was indicated by serum methyl sterols and the acetate-mevalonate test, both of which have recently been used to detect changes in cholesterol synthesis in man [17]. However, the overall stimulation of cholesterol synthesis was insufficient to maintain a normal level of serum cholesterol. Thus, a new steady state was obtained in lower serum cholesterol concentration. Under these conditions, mobilization of tissue cholesterol apparently contributes to serum cholesterol and faecal steroids, in the cases, at least, in which malnutrition prevents any marked increase in synthesis. Thus, in some patients with ileal resection, the sterol balance values may overestimate cholesterol production.

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References

1. Hofmann, A. F., Grundy, S. M.: Abnormal bile salt metabolism in a patient with extensive lower intestinal resection. Clin. Res. 13, 254 (1965), abstract.
2. Hardison, W. G. M., Rosenberg, I. H.: Bile-salt deficiency in the steatorrhea following resection of the ileum and proximal colon. New Engl. J. Med. 277, 337 (1967).
3. Stanley, M. M., Nemchausky, B.: Fecal C^{14}-bile acid excretion in normal subjects and patients with steroid-wasting syndromes secondary to ileal dysfunction. J. Lab. clin. Med. 70, 627 (1967).
4. Austad, W. I., Lack, L., Tyor, M. P.: Importance of bile acids and of an intact distal small intestine for fat absorption. Gastroenterology 52, 638 (1967).
5. Heaton, K. W., Austad, W. I., Lack, L., Tyor, M. P.: Enterohepatic circulation of C^{14}-labeled bile salts in disorders of the distal small bowel. Gastroenterology 55, 5 (1968).
6. Meihoff, W. E., Kern, F., Jr.: Bile salt malabsorption in regional ileitis, ileal resection, and mannitol-induced diarrhea. J. clin. Invest. 47, 261 (1968).
7. Abaurre, R., Gordon, S. G., Mann, J. G., Kern, F., Jr.: Fasting bile salt pool size and composition after ileal resection. Gastroenterology 57, 679 (1969).
8. Forth, W., Rummel, W., Glasmer, H.: Zur resorptionshemmenden Wirkung von Gallensäuren. Naunyn-Schmiedebergs Arch. exp. Path. Pharmak. 254, 364 (1966).
9. Hofmann, A. F.: The syndrome of ileal disease and the broken enterohepatic circulation: cholerheic enteropathy. Gastroenterology 52, 752 (1967).
10. Mekhjian, H. S., Phillips, S. F., Hofmann, A. F.: Conjugated bile salts block water and electrolyte transport by the human colon. Gastroenterology 54, 1256 (1968), abstract.
11. Phillips, S. F., Mekhjian, H. S., Hofmann, A. F.: Effect of free and conjugated bile acids on electrolyte and water absorption by the human colon. In: 4th World Congr. on Gastroenterology, Abstracts, P. Riis, P. Anthonisen, and H. Baden (Eds.), p. 563 (abstract). Copenhagen: The Danish Gastroenterological Association 1970.
12. Fiasse, R., Eyssen, R., Dive, C., Harvengt, C., Kestens, P. J., Nagent de Deuxchaisnes, C.: Metabolism of bile acids, lipids and calcium in Crohn's disease with and without resection. In: 4th World Congr. of Gastroenterology, Abstracts, P. Riis, P. Anthonisen, and H. Baden (Eds.), p. 354 (abstract). Copenhagen: The Danish Gastroenterological Association 1970.
13. Usenius, R. J., Miettinen, T. A.: Excretion of faecal cholesterol and bile acids by patients with ileal resection and by rats with ileal bypass. Scand. J. clin. Lab. Invest. 21, Suppl. 101, 78 (1968), abstract.
14. Miettinen, T. A., Usenius, R.: Faecal steroids, micellar solubilization of intestinal lipids and serum methyl sterols in patients with ileal resections. Europ. J. clin. Invest. 1, 139 (1970), abstract.
15. Montatis, C. D., Myant, N. B., Tabaqchali, S.: The metabolism of cholesterol after resection or by-pass of the lower small intestine. Clin. Sci. 35, 537 (1968).

16. Grundy, S. M., Ahrens, E. H., Jr., Salen, G.: Dietary β-sitosterol as an internal standard to correct for cholesterol losses in sterol balance studies. J. Lipid Res. 9, 374 (1968).
17. Miettinen, T. A.: Detection of changes in human cholesterol metabolism. Ann. clin. Res. 2, 300 (1970).
18. — Siurala, M.: Bile salts, sterols, sterol esters, glycerides and fatty acids in micellar and oil phases of intestinal contents during fat digestion in man. Z. klin. Chem. 9, 47 (1971).
19. — — Micellar solubilisation of intestinal lipids and sterols in gluten enteropathy and liver cirrhosis. Scand. J. Gastroent. (1971), in press.
20. Grundy, S. M., Ahrens, E. H., Jr., Miettinen, T. A.: Quantitative isolation and gas-liquid chromatographic analysis of total fecal bile acids. J. Lipid Res. 6, 397 (1965).
21. Miettinen, T. A., Ahrens, E. H., Jr., Grundy, S. M.: Quantitative isolation and gas-liquid chromatographic analysis of total dietary and fecal neutral steroids. J. Lipid Res. 6, 411 (1965).
22. Bolin, D. W., King, R. P., Klosterman, E. W.: A simplified method for the determination of chromic oxide (Cr_2O_3) when used as an index substance. Science 116, 634 (1952).
23. Kamer, J. H. van de, ten Bokkel Huinink, H., Weyers, H. A.: Rapid method for the determination of fat in feces. J. biol. Chem. 177, 347 (1949).
24. Hofmann, A. F., Borgström, B.: The intraluminal phase of fat digestion in man: The lipid content of the micellar and oil phases of intestinal content obtained during fat digestion and absorption. J. clin. Invest. 43, 247 (1964).
25. Miettinen, T. A.: Lanosterol and other methyl sterols in serum of fed and fasted human subjects. Ann. Med. exp. Fenn. 46, 172 (1968).
26. — Effect of dietary cholesterol and cholic acid on cholesterol synthesis in rat and man. Progr. biochem. Pharmacol. 4, 68 (1968).
27. Borgström, B., Lundh, G., Hofmann, A.: The site of absorption of conjugated bile salts in man. Gastroenterology 45, 220 (1963).
28. Hislop, I. G., Hofmann, A. F., Schoenfield, L. J.: Determinants of the rate and site of bile acid absorption in man. J. clin. Invest. 46, 1070 (1967), abstract.
29. Miettinen, T. A., Paltoniemi, P.: Bile salt, fat, water and vitamin B_{12} excretion after ileostomy. Scand. J. Gastroent. (1971), in press.
30. McLeod, G. M., Wiggins, H. S.: Bile-salts in small intestinal contents after ileal resection and in other malabsorption syndromes. Lancet 1968 I, 873.
31. Garbutt, J. T., Heaton, K. W., Lack, L., Tyor, M. P.: Increased ratio of glycine- to taurine-conjugated bile salts in patients with ileal disorders. Gastroenterology 56, 711 (1969).
32. Tabaqchali, S.: The pathophysiological role of small intestinal bacterial flora. Scand. J. Gastroent. 5, Suppl. 6, 139 (1970).
33. Danielsson, H., Einarsson, K.: Formation and metabolism of bile acids. In: The biological basis of medicine, E. E. Bittar and N. Bittar (Eds.), vol. 5, p. 279. London and New York: Academic Press 1969.
34. Hess Thaysen, E., Brunsgaard, A., Eriksen, B.: Evaluation of the efficiency of bile salt recirculation in patients with terminal ileopathies by means of deoxycholate determination in duodenal aspirates. Scand. J. Gastroent. 5, 39 (1970).
35. Hofmann, A. F., Poley, J. R., Mekhjian, H. S., Phillips, S. F.: Hydroxy fatty acid: An apparent cause of diarrhea in patients with ileal resection and steatorrhea. J. clin. Invest. 49, 44a (1970), abstract.
36. Miettinen, T. A., Lempinen, M.: Ileal by-pass operation in familial hypercholesterolemia. Scand. J. clin. Lab. Invest. 25, Suppl. 113, 55 (1970).
37. Moore, R. B., Frantz, I. D., Jr., Varco, R. L., Buchwald, H.: Cholesterol dynamics after partial ileal bypass. In: Atherosclerosis: Proceedings of the Second International Symposium, R. J. Jones (ed.), p. 295. Berlin-Heidelberg-New York: Springer 1970.
38. Carey, J. B., Jr., Williams, G.: Maximum primary bile salt synthesis rates in man. Gastroenterology 56, 1249 (1969), abstract.
39. Stewart, J. S., Pollock, D. J., Hoffbrand, A. V., Mollin, D. L., Booth, C. C.: A study of proximal and distal intestinal structure and absorptive function in idiopathic steatorrhoea. Quart. J. Med. 36, 425 (1967).
40. Deest, B. W. van, Fordtran, J. S., Morawski, S. G., Wilson, J. D.: Bile salt and micellar fat concentration in proximal small bowel contents of ileectomy patients. J. clin. Invest. 47, 1314 (1968).
41. Krone, C. L., Theodor, E., Sleisenger, M. H., Jeffries, G. H.: Studies on the pathogenesis of malabsorption. Lipid hydrolysis and micelle formation in the intestinal lumen. Medicine (Baltimore) 47, 89 (1968).
42. Hallberg, D., Carlson, L. A.: Small intestinal resection and plasma lipids. 3rd Annual Meeting of European Society for clinical Investigation, Scheveningen (1969). Abstracts of papers, p. 37.
43. Miettinen, T. A.: Effect of cholestyramine on fecal steroid excretion and cholesterol synthesis in patients with hypercholesterolemia. In: Atherosclerosis: Proceedings of the Second International Symposium, R. J. Jones (ed.), p. 558. Berlin-Heidelberg-New York: Springer 1970.
44. Jones, R. J., Dobrilovic, L.: Lipoprotein lipid alterations with cholestyramine administration. J. Lab. clin. Med. 75, 953 (1970).
45. Buchwald, H., Moore, R. B., Frantz, I. D., Jr., Varco, R. L.: Clinical experience with partial ileal bypass in treatment of the hyperlipidemias. In: Atherosclerosis: Proceedings of the Second International Symposium, R. J. Jones, (ed.), p. 464. Berlin-Heidelberg-New York: Springer 1970.

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A Diagnostic Test for Chronic Myelogenous Leukemia Based on Abnormalities of B_{12}-binding

By

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The native vitamin B_{12} in human plasma or serum is bound to a protein with α_1-globulin mobility (13, 14, 16, 18) or transcobalamin (TC I) by our nomenclature (6). Shortly after either parenteral or oral intake and in the early stage of normal transport, B_{12} is bound to protein with β-globulin mobility, transcobalamin II (TC II) (7, 10). The binding pattern is similar when B_{12} is added in vitro (7).

In chronic myelogenous leukemia (CML) serum the B_{12} concentration is higher than normal (11) and the total B_{12}-binding capacity is increased (2). The increased binding capacity in CML is mainly due to increased TC I (9, 13, 14). TC II is decreased (9). Changes of the same type are seen in acute myelogenous leukemia (AML), but are not as pronounced (9). TC II is decreased in pernicious anemia (PA) and the binding to TC I is increased (9, 12), probably because the TC I-binding capacity is unsaturated. The plasma B_{12}-concentration is sometimes increased in polycythemia vera (15, 17) and may be elevated in myeloid metaplasia (15, 17) and in DiGuglielmo's syndrome (1). Although it is generally normal in undifferentiated acute leukemia (2, 17), high levels are sometimes seen (3). Plasma B_{12} is normal in chronic lymphocytic leukemia (2, 11), acute lymphocytic leukemia (11) and lymphoma (3).

In 1961 we reported the rate of disappearance of injected vitamin B_{12} from the plasma of 35 patients with various diseases of the blood (5). A slow rate of removal distinguished the patients with myeloproliferative disease from those with other disorders and from normal persons. These findings suggested that a diagnostic test for myeloproliferative disease could be devised, but a simplification of technique was needed. Subsequent studies showed that the rate of plasma disappearance of injected

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vitamin B_{12} was a function of the pattern of B_{12}-binding protein in plasma (8). With closer delineation of the abnormalities of B_{12} transport protein in leukemia stated above, the information needed to devise a suitable diagnostic test became available.

The technique of separation of TC I and II by column chromatography used in this laboratory (7) is too complex and time-consuming for diagnostic use. Even abbreviated column techniques are not likely to be accepted by routine hospital laboratories. An ammonium sulfate precipitation procedure used previously does not give a clean separation of the important proteins (8). Some form of electrophoresis appeared to be the most suitable method for a simple procedure likely to be accepted for routine testing.

Material and methods

In the present work these B_{12}-binding abnormalities were studied with use of paper electrophoresis.

The material of this study consisted of serum samples taken from patients with a variety of blood diseases and other diseases and from healthy personnel. Heparinized plasma was used in some cases. Serum samples were stored at —20 C if not tested immediately. One ml of serum was labeled with 1,500 pg of Co^{57}-B_{12} with an initial specific activity of 70 $\mu c/\mu g$ of cyanocobalamin (CT2P cyanocobalamin ^{57}Co, BP Lot 31, obtained from Radiochemical Centre, Amersham, England, through the courtesy of Merck Chemical Div., Merck & Co., Inc., Rahway, N. J.). This amount was selected since it will saturate sera that are normal with respect to TC II (TC I is usually already saturated with B_{12}) with little binding to other binders (9). Therefore the amount bound to TC II in relation to the amount bound to TC I will be at a maximum. In CML serum 1,500 pg can easily be added to TC I (9) and therefore the amount bound to TC I will be high in relation to the amount bound to TC II. Serum samples were incubated in a 37°C water-bath for one half-hour. Each incubated sample was subjected to electrophoresis on analytical filter paper (Schleicher & Schuell & Co., Keene, N. H., N2043-B, 40×40 mm) at pH 8.6 in barbiturate buffer (μ = 0.05) in an LKB electrophoresis apparatus at 110 V, 0.25 mA/cm. After electrophoresis serum proteins were stained with light green (Light green SF, yellowish, L-176, Fisher Scientific Co.) and a drawing was made from each strip in order to localize the proteins. The strips were cut into 1 cm sections and were counted for radioactivity in a well-type scintillation spectrometer. The background was from 24 to 29 cts/min. The automatic counting system stopped each sample count at either 300 cts or 5 min. The counting error (probable error) was in the range of 4—5% for all samples. Whilst all 15—16 sections of the paper were counted, it would be necessary to count only the α_1- and β-globulin strips in routine work.

Results

The results are given in fig. 1. The data are expressed as a ratio of cts/min. of the α_1-area to cts/min. of the β-area. Essentially this is a measurement of Co^{57}-B_{12}-TC I/CO^{57}-B_{12}-TC II. However, for neither protein was saturation proved, and the ratio does not necessarily reflect the total amounts of either substance. The mean ratio for the control group was 0.045 ± 0.026.

There was a wide range of individual values in chronic myelogenous leukemia, but the ratio was always higher than normal. The ratio did not seem to have a relationship to peripheral WBC (fig. 2). The highest ratio, 20.6 was seen in a

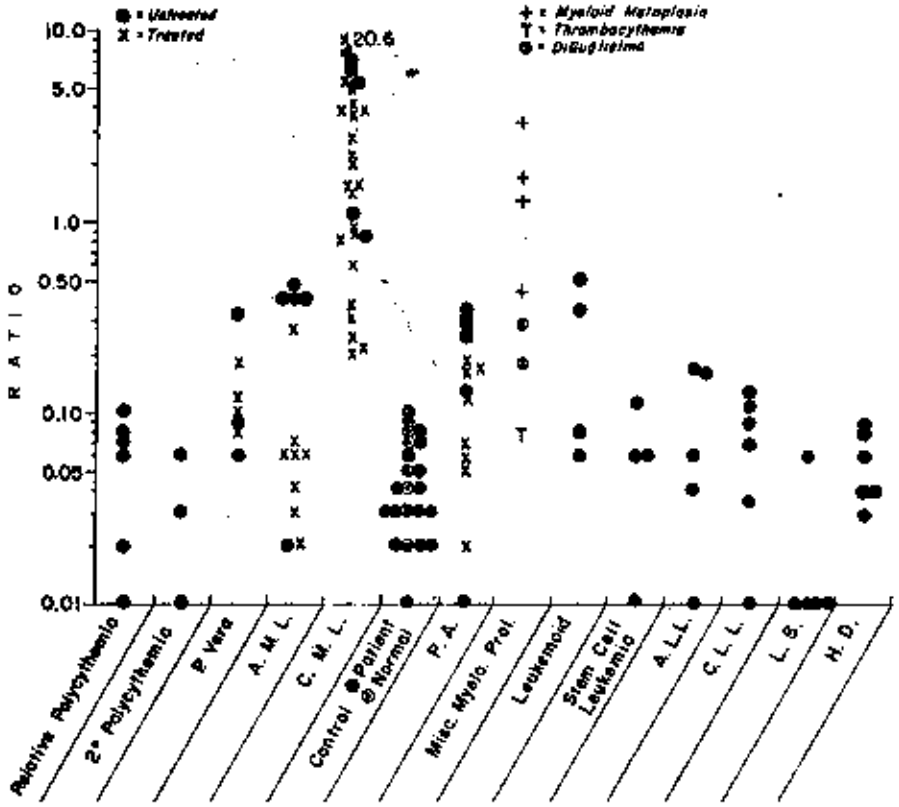

Fig. 1. Co^{60}-B_{12} bound to areas of electrophoresis strip: ratio for areas α_1/β (TCI/TCII). P. vera = polycythemia vera. AML = acute myelogenous leukemia. CML = chronic myelogenous leukemia. PA = pernicious anemia. Myelo. Prol. = myeloproliferative disorder. ALL = acute lymphoblastic leukemia. CLL = chronic lymphocytic leukemia. LS = lymphosarcoma. HD = Hodgkins disease.

patient with WBC of 6,800/mm³, but 50% blasts in peripheral blood. It tended to be lower in treated patients (fig. 3). In five patients in remission (WBC<10,000; Hb>10 g %, myelocytes 0—1% in peripheral blood) the ratio was from 0.2 to 0.8, mean 0.4.

Discussion

In normal serum most of the in vitro added B_{12}, when amounts are 1,500 pg/ml or under, is bound to TC II. The ratio for TC I:TC II is therefore low (<0.1). In chronic myelogenous leukemia TC I is increased and TC II decreased, thus the ratio is high. From previous work (9) it is known that when the patient is approaching remission TC I is decreasing and the TC II is increasing. Patients in remission had a lower ratio for TC I:TC II than those in relapse. It might be of value to do this test during treatment of the patient to see if the changes in B_{12}-binding could be used in prognosis.

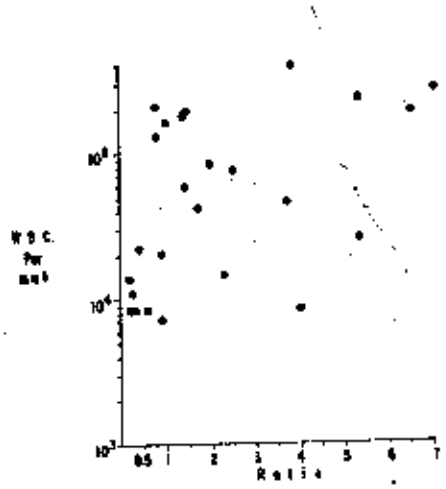

Fig. 2. The ratio compared to white cell count in patients with CML.

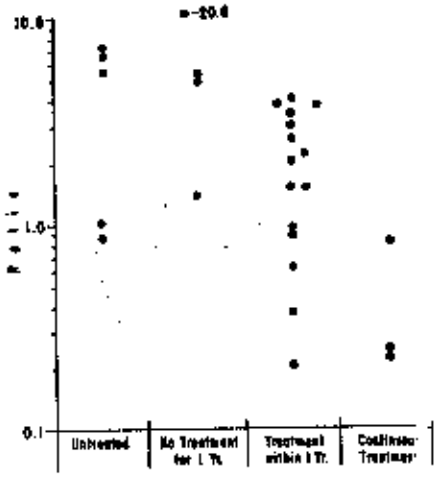

Fig. 3. The ratios in CML grouped according to stage of treatment.

This test is considered to be also of value in differential diagnosis of polycythemia vera and chronic myelogenous leukemia. There was no overlapping of the ratio between polycythemia vera and untreated chronic myelogenous leukemia patients. It cannot, however, be used to separate different types of polycythemia. Differential diagnosis between chronic myelogenous leukemia and myeloid metaplasia was not possible with this test because the ratios were within the same range. In acute leukemias, high ratios were found in acute myelogenous leukemias, but low ratios did not have differential diagnostic significance. Two patients with DiGuglielmo's syndrome had somewhat elevated ratios.

Two patients with leukemoid reactions had ratios of 0.34 and 0.5. Both had epidermoid carcinoma. Leukemia could not be completely excluded in one case. The reaction in the other came during recovery from severe depression of the bone marrow due to chemotherapy. A patient with breast cancer in the marrow had a normal ratio, as did a patient with leukemoid reaction due to acute cirrhosis and a third with marked eosinophilia.

The low ratios in polychythemia vera (PV) were puzzling. The plasma disappearance of injected B_{12} is as abnormal in this disease as in CML (5). Therefore a high ratio was expected in PV, but was not found. We applied (8) to the problem a previously used method for the separation of TC I and II by ammonium sulfate precipitation. In normal sera more than 70% of added B_{12} is in the A fraction and no more than 25% in the B fraction. In sera from 6 PV patients in relapse, the A fraction contained 24—52% and the D fraction 36—59%. There appears, therefore, to be some abnormality of B_{12}-binding in polycythemia vera which has not been identified.

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The method used is simple and suitable for clinical laboratories. Other types of electrophoresis medium were tried but paper was found to be most suitable. For instance, the application volume can be higher than with cellulose acetate.

The ratio seemed to be the best way to bring out the two abnormalities of CML. With this calculation there is no comparison of one strip with another and therefore the serum need not be measured when applied to the strip. Recently Gottlieb et al. (4) published the results of a similar study. Their objective was also the evaluation of a diagnostic test for CML but they used a brief, two step DEAE-cellulose column system for the separation of plasma. Their results were very similar to ours. We chose an electrophoretic system because general hospital laboratories are more familiar with paper electrophoresis than with column chromatography. The greatest disadvantage of our method is the requirement of Co^{57}-B_{12} of a higher specific activity than is now commercially available in some countries.

Summary

A diagnostic test for certain leukemias and related syndromes was evaluated. The test was based on the ratio of the amounts of Co^{57}-B_{12} bound to the two plasma B_{12} transcobalamins, TC I and TC II. High ratios were found in chronic myelogenous leukemia, in some patients with the acute form, and in myeloid metaplasia. Ratios were not elevated in the lymphocytic leukemias, malignant lymphoma or the polycythemias.

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References

1. Baldini, M., Fohenberg, H. H., Fukutake, K. & Damashek, W.: The anemia of the DiGuglielmo syndrome. Blood *14:* 334, 1959.
2. Beard, M. F., Putney, W. R. & Saxneman, E. H.: Serum concentrations of vitamin B_{12} in patients suffering from leukemia. Blood *9:* 789, 1954.
3. Erdman-Oehlecker, S. & Heinrich, H. C.: Der Vitamin B_{12}-Stoffwechsel bei Hämoblastosen. Clin. chim. Acta *1:* 269, 1956.
4. Gottlieb, C. S., Retief, F. P., Pratt, P. W. & Herbert, V.: Correlation of B_{12} binding proteins with disorders of B_{12} metabolism. Relation to hypo- and hyperleukocytic states and leukocyte turnover. J. clin. Invest *45:* 1016, 1966.
5. Hall, C. A.: The plasma disappearance of radioactive vitamin B_{12} in myeloproliferative diseases and other blood disorders. Blood *18:* 717, 1961.
6. Hall, C. A. & Finkler, A. E.: Abnormal transport of vitamin B_{12} in plasma in chronic myelogenous leukemia. Nature *204:* 1207, 1964.
7. Hall, C. A. & Finkler, A. E.: The dynamics of transcobalamin II. A vitamin B_{12}-binding substance in plasma. J. Lab. clin. Med. *65:* 459, 1965.
8. Hall, C. A. & Finkler, A. E.: In vivo plasma vitamin B_{12}-binding in B_{12}-deficient and nondeficient subjects. J. Lab. clin. Med. *60:* 765, 1962.
9. Hall, C. A. & Finkler, A. E.: Measurement of the amounts of the individual vitamin B_{12}-binding proteins in plasma. I. Studies of normal plasma. II. Abnormalities in leukemia and pernicious anemia. Blood *27:* 611, 1966.
10. Hall, C. A. & Finkler, A. E.: A second vitamin B_{12}-binding substance in human plasma. Biochem. biophys. Acta (Amst.) *78:* 234, 1963.

1799

1850

11. KILLANDER, A.: B_{12} vitaminhalt i serum vid akut och kronisk leukemi. Nord. Med. *52:* 1513, 1954.

12. LAWRENCE, C.: B_{12}-binding protein deficiency in pernicious anemia. Blood *27:*389, 1966.

13. MENDELSOHN, R. S., WATKIN, D. M., HORNETT, W. P. & FAHEY, J.: Identification of the vitamin B_{12}-binding protein in the serum of normals and of patients with chronic myelocytic leukemia. Blood *13:* 740, 1958.

14. MILLER, A. & SULLIVAN, J. F.: Electrophoretic studies of the vitamin B_{12}-binding protein of normal and chronic meylogenous leukemia serum. J. clin. Invest. *38:* 2135, 1959.

15. MOLLIN, D. L. & ROSS, G. I. M.: Serum vitamin B_{12} concentrations in leukaemia and in some other haematological conditions. Brit J. Haemat. *1:* 155, 1955.

16. PITNEY, W. R., BEARD, M. D. & VANLOON, E. J.: Observations on the bound form of vitamin B_{12} in human serum. J. biol. chem. *207:* 143, 1954.

17. RACHMILEWITZ, M., IZAK, G., HOCHMAN, A., ARONOVITCH, J. & GROSSWICZ, N.: Serum vitamin B_{12} in leukemia and malignant lymphomas. Blood *12:* 804, 1957.

18. WEINSTEIN, L. B., WEISMAN, S. M. & WATKIN, D. M.: The plasma vitamin B_{12}-binding substance. J. clin. Invest. *38:* 1194, 1959.

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Tissue Distribution of Parenteral Co^{60} Vitamin B_{12} in Mouse, Hamster, Rat and Guinea Pig.* (22654)

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(Introduced by B. S. Walker.)

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Species differences in the tissue distribution of parenterally administered Co^{60} vit B_{12} ($Co^{60}B_{12}$) have been suggested by recent studies. In the rat, the kidneys were found to be the main site of $Co^{60}B_{12}$ localization even at a time when urinary excretion was absent(1,2,3). In man, the greatest percentage of a parenterally injected dose of $Co^{60}B_{12}$ was found in the liver(4). In the chick(5) minimal localization of $Co^{60}B_{12}$ was found in the liver and kidney. In this study parenteral $Co^{60}B_{12}$ was administered to hamsters, rats, mice and guinea pigs in order to elaborate on possible species differences in its tissue distribution.

Methods. Experimental plan: Eight adult male Swiss white mice weighing 25-40 g, 8 adult hamsters (4 male and 4 female) weighing 85-120 g, 7 adult male Sprague-Dawley rats weighing 200-230 g, and 4 adult male guinea pigs weighing 450-600 g, were subcutaneously injected under ether anesthesia with $Co^{60}B_{12}$ (secured from Merck and Co., Rahway, N.J.). The dose was contained in a volume of 1.0 ml, except for mice, where 0.5 ml was given. The dose injected ranged from 0.10 to 0.40 μc of Co^{60}. Mice received 0.15 μg of B_{12}, hamsters 0.70 μg, rats 0.30 μg and guinea pigs 0.26 μg. After injection, rats and guinea pigs were housed in metabolic cages so as to allow separate urine and stool collections. Hamsters were housed in wire cages under which 2 thicknesses of Whatman filter paper #3 were spread. All stools fell on the filter paper while the urine was absorbed into the paper. Mice were housed in 2,000 ml beakers. The bottoms of the beakers were also lined with this filter paper on which urine and stools were deposited. Four days after the injection of the Co^{60} B_{12}, animals were anesthetized, exsanguinated by cardiac puncture, and the appropriate organs removed.

Preparation of specimens for analysis: (a) *Organs.* The liver, kidneys, spleen, heart, testes and portions of the hind leg musculature were removed and weighed. Organs were placed into flasks containing 10 ml of 2N NaOH, except for the livers where 20 ml was used. The organs were digested in alkali at room temperature for 24 hours. Then, if necessary, gentle heating for 30 to 60 seconds brought the gel-like digest into a liquid state. Measurements of radioactivity were made on duplicate 4 ml aliquots of these tissue digests. The total volume of the tissue digest solution was assumed to be equal to 10 ml when organ weight was less than 0.75 g as the volume contributed by the dissolved organ was negligible. For heavier organs the total volume of the digest was measured in a 25 ml graduated cylinder. (b) *Urine, stools, and blood.* Independent total urine collections were secured for the first 24 hours and for the following 3 days for the rats and guinea pigs. The total urine volume was measured in a 50 ml graduated cylinder. Radioactivity was determined on 4 ml aliquots of urine. For hamsters and mice, the filter paper on which the urine had dried was cut into small pieces and placed into 1¾ by 3⅛ inch glass bottles in which radioactivity was measured. The radioactivity in 4-day stool collections was also determined in 1¾ by 3¼ inch glass bottles. One to 4 ml of oxalated blood (hamsters and rats were only done) was used for radioactivity assay. (c) *Carcasses.* Mice and hamster carcasses were placed into 1¾ by 3¼ inch glass bottles while the rat carcasses were placed into 2½ by 6 inch glass bottles. Car-

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TABLE I. Tissue Distribution and Excretion of $Co^{60}B_{12}$ in Mice, Rats, Hamsters and Guinea Pigs.

	Mice		Rats		Hamsters		Guinea pigs	
Organ	% of inj. dose*	% of dose* / g wet wt	% of inj. dose	% of dose / g wet wt	% of inj. dose	% of dose / g wet wt	% of inj. dose	% of dose / g wet wt
Kidney	6.2 ± .4	13.0 ± 1.4	16.2 ± 2.9	9.7 ± 1.9	7.2 ± .0	6.3 ± .1	2.4 ± .1	.3 ± 0
Liver	23.7 ± 2.5	11.2 ± 1.1	7.4 ± .4	.8 ± 0	18.0 ± 2.9	6.1 ± .1	29.7 ± 4.1	1.3 ± 0
Testes	1.5 ± .1	7.0 ± 1.1	1.0 ± 0	.6 ± 0	not done	—	.3 ± 0	.1 ± 0
Spleen	1.4 ± .2	1.7 ± .5	.8 ± 0	.0 ± 0	.3 ± 0	.8 ± 0	.2 ± 0	.2 ± 0
Heart	.4 ± .1	1.8 ± .1	.7 ± 0	.9 ± 0	.4 ± 0	.6 ± 0	.2 ± 0	.1 ± 0
Muscle	15.9 ± .0	1.3 ± 0	13.6 ± 1.1	.2 ± 0	7.7 ± 1.5	.3 ± 0	not done	—
Carcass	26.0 ± 2.9	—	34.5 ± 4.0	—	43.0 ± 8.8*	—	" "	—
Urine	5.9 ± 1.8	—	7.4 ± 1.1	—	14.1 ± 1.0	—	12.5 ± .9	—
Stool	15.5 ± 1.8	—	18.5 ± 6.2	—	3.1 ± .0	—	5.5 ± .1	—
Recovery	106.1		100.7		95.1		50.8	

* Mean and stand. error of mean.

cass radioactivity measurements were made in these bottles. Because of their large size, the guinea pig carcasses could not be counted.

Radioactivity determination: A 1:100 dilution of the injected $Co^{60}B_{12}$ was made and varying aliquots were then used as standards for the varying conditions employed in the counting of organ digests, excreta and carcasses. By adding water as needed, the height of the standard in the glass bottles was always the same as that of the specimen being counted *i.e.*, stools, urine or carcasses. Blood, tissue and liquid urine samples were counted in a scintillation well-type counter (thallium activated sodium iodide scintillation crystal). The radioactivity of the organ samples usually counted 3-12 times background, except for the muscle, heart, blood and spleen which usually counted 1-3 times background. Samples were counted long enough to give a counting error of less than 2%, except for the slow-counting samples where a 5-8% counting accuracy was obtained. Duplicate samples usually agreed within 1-5%. All samples contained in the 1¾ by 3¼ inch glass bottles were counted by placing them on top of the above-mentioned sodium iodide well crystal. The diameter of these was just about equal to that of the diameter of the well. Under similar geometry, a standard was also counted. The 6 inch bottles containing the rat carcasses were marked into 3 equal 2 inch segments. With the bottle in the horizontal position, and directly in contact with a solid 1 by 1 inch thallium activated sodium iodide crystal, each 2 inch segment of the bottle containing the carcass was counted. Under similar geometry the bottle containing the standard was counted.

Calculations. (a) The percent radioactivity in organ =

$$\frac{\text{cpm per ml organ digest} \times \text{total volume digest}}{\text{cpm injected}} \times 100$$

In calculating the total percent uptake in the muscle mass, 40% of the animal's weight was assumed to be muscle. For the total percent uptake in blood, a blood volume was estimated based on the figure of 69 ml/kg body weight.

Results. The organ distribution and excretion of a parenteral dose of $Co^{60}B_{12}$ in the rat, guinea pig, mouse and hamster are shown in Table I.

Discussion. In the rat the tissue distribution of a parenterally injected dose of $Co^{60}B_{12}$ was similar to that found by Rosenblum *et al.* (1). Similar studies on the mouse, hamster and guinea pig are not available in the literature. Differences in the major organ sites of $Co^{60}B_{12}$ localization were noted in the 4 species studied. In the hamster, guinea pig and mouse, the liver was the chief organ of localization whereas in the rat the kidney was the chief site. The high $Co^{60}B_{12}$ uptake of the rat kidney is probably unrelated to its known role in the excretion of large doses of parenterally injected vit B_{12}(3,6,7), since the dose given the rat was comparable to that given the other 3 species. Furthermore, the other 3 species studied had comparable amounts of

radioactivity excreted in the urine without an increase in kidney $Co^{60}B_{12}$ uptake. At the time the high rat renal $Co^{60}B_{12}$ uptake was found, urinary excretion of the radiovitamin was negligible making it highly unlikely that the $Co^{60}B_{12}$ in the kidneys was there to be excreted. This is further supported by the observations of Harte(2), who found that high renal $Co^{60}B_{12}$ levels were maintained for as long as 90 days after injection despite the absence of urinary excretion after the first day. It would thus appear that the kidney represents an important site of $Co^{60}B_{12}$ localization in the rat rather than a mere storage site for further urinary excretion. The high $Co^{60}B_{12}$ concentration in the liver of the mouse, hamster and guinea pig is comparable to that found in man after the injection of labelled vit B_{12}(4). The rat, with the chief localization of injected $Co^{60}B_{12}$ in the kidney, is not analogous to man. It would, therefore, appear hazardous to relate to man the results of the experiments in the rat.

Differences in the routes of $Co^{60}B_{12}$ excretion were noted in the 4 species studied despite the administration of comparable amounts of vit B_{12} in relation to body weight. The stools were the chief routes of excretion in the rat and mouse, whereas the urine was the chief excretory pathway in the hamster and guinea pig. In previous studies in the rat (1,8) the urine was the major excretory pathway containing 50-90% of a parenteral dose of $Co^{60}B_{12}$. These results differ markedly from those found in this study and are probably due to the large doses of vit B_{12} (4-20 μg) used in these studies. There is no available data on the excretion of parenteral $Co^{60}B_{12}$ in the other 3 species studied. The present investigation suggests that the stools may be an important route for the excretion of vit B_{12} in the rat and mouse. Radioactivity as measured in this study merely indicates the presence of the Co^{60} moiety of the vit B_{12} molecule. Thus, the radioactivity may be due to vit B_{12} or to an intermediate degradation product of the vitamin. After parenteral administration of $Co^{60}B_{12}$ to rats, the radioactivity excreted in the urine was found to be due to vit B_{12}, whereas that in the stool was unassociated with vit B_{12} activity(1). The radioactivity in the kidneys was found to be due to vit B_{12} in a similar study(8). It appears likely that the radioactivity in the kidneys in this study is due to vit B_{12}. However, concerning the other organs, this question cannot be answered.

Summary. The excretion and tissue distribution of parenterally administered $Co^{60}B_{12}$ has been studied in the rat, hamster, mouse and guinea pig. The liver contained more radioactivity than any other organ in the hamster, mouse and guinea pig, whereas the kidneys contained more radioactivity than any other organ in the rat. In the rat and mouse, more radioactivity was excreted in the stools than in the urine whereas in the guinea pig and hamster, the urine was the chief excretory pathway.

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1. Rosenblum, C., Chow, B. F., Condon, G. P., and Yamamoto, R., *J. Biol. Chem.*, 1952, v198, 915.

2. Harte, R. A., Chow, B. F., and Barrows, L., *J. Nutr.*, 1953, v49, 669.

3. Yamamoto, R., Barrows, C., Lang, C. A., and Chow, B. F., *ibid.*, 1951, v45, 507.

4. Glass, G. B. J., Boyd, L. J., and Gellin, G. A., *Blood*, 1955, v10, 95.

5. Monroe, R. A., Patrick, H., Comar, C. L., and Goff, O. E., *Poultry Sci.*, 1952, v31, 79.

6. Conley, C. L., Krevans, J. R., Chow, B. F., Barrows, C., and Lang, C. A., *J. Lab. Clin. Med.*, 1951, v38, 84.

7. Sokoloff, M. F., Sunneman, E. H., and Beard, M. F., *Blood*, 1952, v7, 243.

8. Barbee, K. W., and Johnson, B. C., Proc. Soc. Exp. Biol. and Med., 1951, v76, 720

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TREATMENT OF PERNICIOUS ANEMIA BY A SPECIAL DIET*

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This paper concerns the treatment in a series of forty-five cases of pernicious anemia in which the patients were given a special form of diet. While the problem of diet in the treatment of pernicious anemia is by no means new, in our opinion its possible importance has not heretofore been generally recognized. In 1863, seven years after the publication of Addison's second, but best known, description of the disease now called pernicious anemia, Habershon[1] wrote concerning this condition: "Many patients at an early stage completely recover under the influence of bracing air and a nutrient and stimulating diet." Other early investigators of the disease, as Biermer[2] in 1872, and Pepper,[3] in 1875, appreciated the desirability of prescribing easily digested foods as a form of medication, but no greater emphasis was placed on the value of diet. Osler,[4] however, in 1885, mentioned that "cases [of pernicious anemia] appear to have got well with change of air and a better diet after resisting all ordinary means."

During the last half century, many clinicians, following the suggestions of the pioneer writers on the subject of pernicious anemia, have advised various kinds of diet as an aid to induce a remission of the disease. More often than not, the recommendations have been of a general sort as might be given for many persons with an impaired condition of the gastrointestinal tract, which always is present in pernicious anemia. Thus, food for the pernicious anemia patient often has been selected because it appeared to be easily digested or because it seemed particularly nutritious and strength-giving. Rarely, diets have been chosen for some assumed direct effect on the blood.

The constant presence of achylia gastrica in pernicious anemia and the frequency of an abnormal bacterial activity within the intestines have been two main reasons for establishing certain forms of dietotherapy in the disease. On these accounts, Fenwick,[5] in 1880, and Naegeli,[6] among others, recommended diets relatively sparing in farinaceous foods and relatively rich in protein. For similar reasons, yet in contrast to the majority, Hunter,[7] in 1890, and others have advised quite the opposite type of diet. Grawitz[8] recommended a diet composed chiefly of fresh vegetables, followed by one with generous amounts of protein. The idea that forced feeding with any sort of food, but especially meats, is valuable to make weak and feeble individuals healthy and strong has caused the frequent use of this form of therapy in pernicious anemia, and Mosenthal[9] has shown that it can restore in these cases a positive nitrogen balance.

Meats and green vegetables, partly because of their iron content, have for a long time been thought to be useful to improve "an anemic state of the blood." Meat apparently has been chosen at times simply because it contained blood, which was supposed to be beneficial as food for persons who had an insufficient blood supply. The scientific foundations of the value of iron-containing foods to affect the blood-forming organs were laid by Menghini[10] in 1746, when he showed that iron could be increased in the blood by feeding such foods to animals. About 200 years later, Gibson and Howard[11] made important observations on the effect of a high iron content of the diet in anemia, and showed that in pernicious anemia it can have a most favorable influence on iron metabolism. They also showed that, in cases constantly losing nitrogen, a positive nitrogen balance could be obtained without forced feeding.

One thus finds that the diet usually advised for the pernicious anemia patient is one containing a relatively high nitrogen-content and often a relatively large number of calories. The recommendations of Smith[12] and of Barker and Sprunt[13] are of this sort and, like some others, the latter wisely recommend that the food be selected with a view to giving an ordinary, well balanced

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1. Habershon, S. O.: On Idiopathic Anemia, Lancet 1: 518, 551 (May 9) 1863.
2. Biermer: Hält Zunächst einen Vortrag über eine von ihm öfters beobachtete eigenthümliche Form von progressiver perniciöser Anämie, welche mit Capillaren Blutungen der Haut, Retina, des Gehirns &c., Corr.-Bl. f. schweiz. Aerzte 2: 15 (Jan. 15) 1872.
3. Pepper, W.: Progressive Pernicious Anaemia or Anhaematosis, Am. J. M. Sc. 70: 215 (Oct.) 1875.
4. Osler, William: Pernicious Anemia: A System of Practical Medicine, edited by Pepper, W., assisted by Starr, L., Philadelphia, Lea Brothers Company 5: 808, 1885.
5. Fenwick, S.: On Atrophy of the Stomach in Relation to Pernicious Anemia, Lancet 2: 77 (July 24) 1877.
6. Naegeli, O.: Blut Krankheiten und Blutdiagnostik, Leipzig, von Veit & Co., 1912.
7. Hunter, W.: Observations on Treatment of Pernicious Anemia Based on a Study of Its Causation, Brit. M. J. 2: 1, 81, 1890.
8. Grawitz, E.: Zur Frage der entwegen an Entstehung schwerer Anämien, Berl. klin. Wchnschr. 1: 641 (June 17) 1901.
9. Mosenthal, H.: The Effect of Forced Feeding on the Nitrogen Equilibrium and the Blood in Pernicious Anemia, Bull. Johns Hopkins Hosp. 29: 129 (June) 1918.
10. Menghini, quoted by Christian, H. A.: A Sketch of the History of the Treatment of Chlorosis with Iron, Medical Library and Historical Journal 1: 176 (July) 1903.
11. Gibson, R. B., and Howard, C. P.: Metabolic Studies in Pernicious Anemia, Arch. Int. Med. 31: 1 (July) 1923.
12. Smith, quoted by Fitch, W. E.: Dietotherapy, ed. 4, New York, D. Appleton & Co. 8: 237, 1922.
13. Barker, L. F., and Sprunt, T. P.: The Treatment of Some Cases of So-Called "Pernicious" Anemia, J. A. M. A. 60: 1919 (Dec. 6) 1913.

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diet to replace a quantitatively deficient and qualitatively ill balanced one, on which these patients are apt to have placed themselves during their illness.

In spite of attention to diet for the anemic patient, the influence of food on blood formation and destruction has received comparatively little consideration, and special sorts of food, because of some particular effect, have seldom been chosen for patients with pernicious anemia.

Complete starvation in man is not considered to cause anemia, but may do so in animals. However, it is known that improper food can cause, and suitable food alleviate, anemia; for example, the "iron starvation anemia" arising in infants who have partaken too long of only a milk diet, and who can be cured by food particularly containing complete proteins and iron. Incomplete diets, particularly those low in protein and relatively rich in concentrated carbohydrate food, can lead to anemia,[14] and even Shakespeare[15] recognized that improper food might impair the state of the blood. Likewise, patients with conditions due to, or associated with, vitamin deficiency experience anemia, and Jencks[16] has noticed that an abundance of vitamins favors blood regeneration. Certain foods, including liver, may benefit patients with sprue. This disease is considered by some partly dependent on a faulty diet, and resembles in numerous ways pernicious anemia, including the fact that the blood picture in the two diseases may be quite similar. Carnivorous animals and thin persons tend to have a greater percentage of hemoglobin in their blood than herbivorous animals and fat persons.[17] This further suggests, as do the observations of Morawitz and Kühl[18] on man, the favorable rôle that animal protein food may play in blood formation, although dehydration may account for the differences observed.

Some of the earlier experimental work concerning the effect of food on blood regeneration is reviewed by Pearce, Krumbhaar and Frazier.[19] Adequate proteins as well as iron are necessary for the formation of hemoglobin. Certain proteins will not suffice, such as gliadin.[20] However, the amino-acid tryptophane may have a special ability to enhance blood formation.[21] The most important recent work concerning the effect of food on blood regeneration has been done by Whipple and Robscheit-Robbins and their associates.[22] Their carefully controlled work on dogs has demonstrated clearly the value of certain foods, especially liver, on accelerating blood regeneration following acute hemorrhage and the value of iron added to the diet to decrease the anemia due to chronic blood loss.

McCollum[23] has pointed out that liver and kidneys give an exceptionally high quality protein for a low protein intake and can enhance remarkably the growth of animals. These foods are rich in nucleins, and Calkins, Bullock and Rohdenburg[24] have shown that the products of nuclein hydrolysis can stimulate growth. Whipple[25] has suggested that in pernicious anemia there may be a scarcity of the material from which the stroma of the red blood cells are formed, or that a disease of the stroma-forming cells of the marrow exists. Thus, theoretically perhaps liver and other foods rich in complete proteins may enhance the formation of red blood cells in this disease, especially by supplying material to build their stroma.

Fresh red marrow was first used as a means of treatment for pernicious anemia by Fraser[26] in 1894. He reported beneficial results when a patient ate for some time about 100 Gm. a day. It was then and has since been given apparently on the supposition of some hormone effect. Thus, numerous reports have appeared concerning the use of preparations of small amounts of concentrated bone marrow, but without definite evidence of advantage to the pernicious anemia patient. Reports regarding the effect of eating generously of fresh marrow are few and brief, but suggest that it may be beneficial. The nutritional composition of red bone marrow is similar to that of liver and kidneys. If generous amounts of red marrow and liver can improve the state of the blood in pernicious anemia, may their influence not be due to the same, but unknown, cause?

Various investigators have commented on the blood-destroying properties of certain substances derived from fats, and the rôle they may play in pernicious anemia. Stoeltzner[27] recently has reviewed the subject. Also, lipoids have been shown by Baker and Carrel[28] to be a factor in serum that can inhibit growth. Thus, founded on somewhat theoretical grounds, it seemed to us, as it did to Stoeltzner[27] and to Gibson and Howard,[11] that decreasing the amount of fat in the diet of the pernicious anemia patient might have a favorable effect on the state of his blood. Excess of fat in a diet is considered by some to favor putrefaction within the intestine, a condition frequent in pernicious anemia. Hence one might attribute any benefit derived from a low fat content of the diet to alterations in the bacterial flora rather than to some more direct effect on blood formation or destruction.

A further hypothetic reason for decreasing the fat in the diet is that we have noted it is not uncommon for these patients to have consumed throughout life unusually large amounts of food rich in fats. Patients with pernicious anemia also may give a history of partaking for years of some other type of one-sided diet. It is common for them to do so after the definite onset of their illness, when it is not unusual to find that they have a disgust for meat. Pernicious anemia is rare in certain parts of the world where diets are quite different (containing fewer dairy products, less free sugar and muscle meat) from those of the northern parts of Europe and

14. McCarrison, Robert: Faulty Food in Relation to Gastro-Intestinal Disorder, J. A. M. A. 78: 1 (Jan. 7) 1927. Benedict, F. G.; Miles, W. R.; Roth, P., and Smith, H. M.: Human Vitality and Efficiency Under Prolonged Restricted Diet, pub. 280, Carnegie Inst. of Washington, 1919, p. 364.

15. Shakespeare: Henry IV, act 2, scene 3.

16. Jencks, Z.: Studies on the Regeneration of Blood, Am. J. Physiol. 69: 240 (Feb.) 1924.

17. Hammarsten, O.: A Text Book of Physiological Chemistry, trans. by Mandel, J. A., ed. 5, New York, J. J. Wiley and Sons, 1908, p. 244.

18. Morawitz, C., and Kühl, G.: Der Blutumsatz des Normalen unter verschiedenen Bedingungen (Eisen, Essen, Fleisch), Klin. Wchnschr. 4: 7, 1925.

19. Pearce, R. M.; Krumbhaar, E. B., and Frazier, C. H.: The Spleen and Anemia, Philadelphia, J. B. Lippincott Company, 1918.

20. Smith, A. H., and Moise, T. S.: Diet and Tissue Growth: The Regeneration of Liver Tissue During Nutrition on Inadequate Diets and Fasting, J. Exper. Med. 40: 209 (Aug.) 1924.

21. Hirasawa, quoted by Wells, H. G.: Chemical Pathology, ed. 5, Philadelphia, W. B. Saunders Company, 1925, p. 334.

22. Whipple, G. H.; Hooper, C. W., and Robscheit, F. S.: Blood Regeneration Following Simple Anemia, Am. J. Physiol. 63: 151, 167 (Sept.) 1920. Whipple, G. H.; Robscheit, F. S., and Hooper, C. W.: Blood Regeneration Following Anemia, ibid. 63: 236 (Sept.) 1920. Whipple, G. H., and Robscheit Robbins, F. S.: Favorable Influence of Liver, Heart and Skeletal Muscle in Diet on Blood Regeneration in Anemia, ibid. 72: 404 (May) 1925 (cf. p. 431); Iron Reaction Favorable, Arsenic and Germanium Dioxide Almost Inert, in Severe Anemia, ibid. 72: 419 (May) 1925.

23. McCollum, E. V.: The Newer Knowledge of Nutrition, New York, the Macmillan Company, 1923.

24. Calkins, G. N., Bullock, F. D., and Rohdenburg, G.: The Effects of Chemicals on the Division Rate of Cells with Especial Reference to Possible Pre-Cancerous Conditions, J. Infect. Dis. 10: 421 (May) 1912.

25. Whipple, G. H.: Pigment Metabolism and Regeneration of Hemoglobin in the Body, Arch. Int. Med. 28: 711 (June) 1922.

26. Fraser, T. R.: Bone Marrow in the Treatment of Pernicious Anemia, Brit. M. J. 1: 1172 (June 2) 1894.

27. Stoeltzner, W.: Ein Versuch zur Behandlung der Biermerschen Anämie, München. med. Wchnschr. 68: 1538 (Dec. 2) 1921.

28. Baker, J. E., and Carrel, Alexis: Lipoids as the Growth Inhibiting Factor in Serum, J. Exper. Med. 42: 143 (July) 1925.

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America, in which areas the disease is relatively common. These different facts permit one to speculate on the possible partial rôle that some nutritional excess or deficiency may play in the etiology of the disease. Similar thoughts have occurred to others, including the idea that a vitamin deficiency might be a causative factor, as has been mentioned, for example, by Elders.[29]

Leafy vegetables and fruits usually are considered desirable for anemic patients, especially because of their iron content, and strawberries rich in iron appear beneficial for patients with sprue, a disease, as noted, resembling pernicious anemia. We prefer to add these foods to the pernicious anemia patient's diet not only because they are healthful ones for any person to eat, but also because, as Whipple and Robscheit-Robbins[30] have shown, certain ones have an especially favorable influence on hemoglobin production. It is quite probable, however, that their chief effect is not because of their iron content. It seems that such a factor as the character of the proteins or amino-acids in the diet is of much more importance than the iron content for pernicious anemia patients.

Numerous authorities hold the view that an intestinal bacterial toxemia plays an important etiologic rôle in this disease. One may choose to believe that any benefit these patients derive soon after beginning to take certain foods is to be attributed to changing rapidly the intestinal flora, thus decreasing a bacterial toxemia, rather than considering that the foods influence in some unknown, but more direct, manner the formation or destruction of red blood cells.

Gibson and Howard,[14] taking cognizance of Whipple and Robscheit-Robbins' work and the fact that certain lipoid substances could enhance hemolysis, fed pernicious anemia patients a relatively low caloric diet (from 1,500 to 1,900) "rich in iron [liver (daily), fruits, green vegetables, egg yolk] and low in fat" and adequate in vitamins. A somewhat similar diet but containing a less amount of food rich in purines was recommended by Fenton[31] in 1921. Gibson and Howard,[15] besides demonstrating the favorable influence of their diet on nitrogen and iron metabolism in pernicious anemia and some other anemias, suggested that it enhanced a remission in pernicious anemia and urged its use.

MATERIAL STUDIED AND OBSERVATIONS

Following the work of Whipple and Robscheit-Robbins, we made a few observations on patients concerning the influence of a diet containing an abundance of liver and muscle meat on blood regeneration. The effect appeared to be quite similar to that which they obtained in dogs. These observations, together with the information given above, led us to investigate the value of a diet with an abundance of food rich in complete proteins and iron—particularly liver—and relatively low in fat, as a means of treatment for pernicious anemia.

Observations set forth below have been made on forty-five patients with typical pernicious anemia first partaking of such a diet when in a relapse and continuing it to date (except temporarily omitted by three), or from six weeks to two and a half years.

29. Elders, C.: The Form, Course and Prognosis of the Anemia in Infants Sprue, and the Etiology of Pernicious Anemia, Nederlandsch. Tijdschr. v. Geneesk. 66: 2267, 1922.

30. Footnote 23, third reference.

31. Fenton, R. L.: A Diet for Pernicious Anemia, J. Iowa State M. Soc. 11: 50 (Feb.) 1921.

The special diet[32] used was made as palatable as possible and for each day was practically as follows:

1. From 120 to 240 Gm., and even sometimes more, of cooked calf's or beef liver. An equal quantity of lamb's kidneys was substituted occasionally.
2. One hundred and twenty grams or more of beef or mutton muscle meat.
3. Not less than 300 Gm. of vegetables containing from 1 to 10 per cent of carbohydrate, especially lettuce and spinach.
4. From 250 to 500 Gm. of fruit, especially peaches, apricots, strawberries, pineapple, oranges and grapefruit.
5. About 40 Gm. of fat derived from butter and cream, allowed in order to make the food attractive. However, animal fats and oils were excluded so far as possible.
6. If desired, an egg and 240 Gm. of milk.
7. In addition to the above mentioned foods, breads especially dry and crusty, potato, and cereals, in order to allow a total intake of between 2,000 and 3,000 calories composed usually of about 340 Gm. of carbohydrate, 135 Gm. of protein, and not more than 70 Gm. of fat. Grossly sweet foods were not given, but sugar was allowed very sparingly.

This diet is rich in iron and purine derivatives, containing about 0.03 Gm. of the former and about 1 Gm. of the latter.

At the time the diet was advised for many of the patients, they were able to take only a small amount of food of any sort. Under these circumstances they were encouraged to take as much as possible of liver and fruits, and at least some vegetables, while other sorts of food were not forced. During the first week of the diet, the intake was often less than a thousand calories. After about this period of time, the patients usually felt distinctly better and their appetite began to improve. Then the food was increased gradually until the complete diet was taken. The patients as a rule did so within two weeks after the diet was begun. In fact, frequently they soon became "ravenously hungry" and often anxious to eat more than the customary allowance of liver and meat.

Twenty-four of the forty-five patients carried out the regimen by weighing portions of liver and meat and estimating the amounts of the rest of their food for at least three weeks, and often for the first six after commencing the diet. The other patients, like those after leaving the hospital, have taken their diet at home, following out written directions but not weighing any of their food. Our data strongly suggest that the patients who commenced treatment in the hospital and those few able to have a trained nurse at home have improved on the average rather faster and to an even better degree than the others. When the patients had remained much better for many weeks, their diet was sometimes modified particularly by decreasing the amount of liver and fruit.

The therapeutic regimen for these forty-five patients, besides the special diet, included rest, usually at first in bed for twenty-four hours a day. All but three also took each day about 15 cc. of diluted hydrochloric acid (U. S. P.). These three, however, improved at least as much as the majority of the others. None of the patients received any especial treatment shortly before or after the diet was begun except as follows: A man, aged 69, with pronounced spinal cord lesions and advanced arteriosclerosis, was given five transfusions of blood within about six weeks while attempts were made to get him to eat. Now, three months later, he remains the least well of all forty-five, except for one woman who recently has omitted her diet. Blood was

32. Details concerning this diet with sample menus are given in a paper to be published soon in the Boston Medical and Surgical Journal.

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transfused to three others at about the time they first took the special diet. The red blood cell count of none was over 1,400,000 per cubic millimeter four days after transfusion.

The forty-five patients represent an essentially consecutive series seen in a relapse, and are all that have taken the special diet except one noted below. The series is not entirely consecutive, because during the time the forty-five cases were seen the following additional ones came under observation:

1. Four patients who had had their disease a long time were exceedingly sick, able to take little or no food, and died within a few weeks after they were seen. They ate no liver or kidneys.

2. Five patients consulting us but once and not taking the special diet. Letters indicate that three improved somewhat and two did not.

3. One patient that was in much better condition soon after taking the diet. This patient is not included in the series of forty-five because of several unusual complications.

Many of the forty-five patients had had definite symptoms due to pernicious anemia for more than two years, and two of them experienced such symptoms ten years before taking the special diet. A number of the cases were observed during a year or more before the diet was begun, others for several weeks, and some for only a few days. Many of the patients had remained in distinctly poor health and were unable to do their usual work for from a few months to more than a year before eating the food especially prescribed. During this time, many received various forms of therapy without distinct benefit, including transfusions of blood.

When the special diet was started, the forty-five patients that have continued to eat this kind of food fell naturally into the three following groups: (1) twelve in their first distinct relapse; (2) seventeen in their second relapse; (3) sixteen having had two or more relapses. It is thus evident that all sorts of variations of the disease occurred among the patients, and that the series was not composed chiefly of those in their first relapse, following which considerable "spontaneous" improvement is the rule.

The condition of all forty-five patients became much better rather rapidly soon after commencing the diet. All except one, who has recently omitted her diet, are now at the least in a very fair state of health, and if it were not for disorders in some due to spinal cord lesions, would have an appearance to a layman of being essentially well. However, there are only eleven patients who began the diet a year or more ago, two of whom have taken it for more than two years. Eighteen began taking the diet less than five months ago.

One of the earliest signs of improvement has been a change in the frequency of bowel movements believed to be due particularly to the diet and probably not to diluted hydrochloric acid. Within a few days, those who had had a tendency to diarrhea often began to have one formed stool a day, while, interestingly enough, those who had had normal movements or had been constipated frequently had for several days a few loose stools in each twenty-four hours. The latter patients, then, had a more natural regularity of their bowel movements and a more normal stool than they had had for some time before the diet was taken. The laxative effect of the diet has been observed also to occur in some normal persons.

Clinical improvement has been obvious usually within within two weeks. This has been heralded in the peripheral blood before the end of the first week by the beginning of a most definite rise of the reticulocytes (young red blood corpuscles) of from about 1.0 per cent to usually about 8.0 and even to 15.5 per cent of all the red blood cells. This rise occurred in all fifteen patients that have had such counts made every day or so for from one to three weeks before and some weeks after beginning the diet. By the end of the second week, these cells usually had returned close to their normal percentage. Later, when the red blood cell counts were distinctly high, it was frequent to find, as we have noted formerly, an abnormally small number of reticulocytes. Before they began to increase, the icterus index of the blood serum in these fifteen patients started to fall, and soon the yellow tint of the patient's skin disappeared. This index reached normal in from two to four weeks, and often has fallen to below normal even when the red blood cell count had increased to only 2,500,000 per cubic millimeter.[15]

The accompanying chart and table give in a synopsized manner the trends of the state of the blood in the forty-five patients, taking into consideration, on the one hand, the character of the case, and, on the other, the level of the red blood cells when the diet was begun. The data are given for all forty-five patients before

*Average Red Blood Corpuscle Count**

Before Diet Started		After Diet Started					
		About 1 Month		About 2 Months		4 to 6 Months	
Number of Cases	Average R. B. C. Count in Millions	Number of Cases	Average R. B. C. Count in Millions	Number of Cases†	Average R. B. C. Count in Millions	Number of Cases†	Average R. B. C. Count in Millions
19	0.00	19	3.28	19	4.08	12	4.50
15	1.00	15	3.25	13	4.09	10	4.51
11	2.30	11	3.35	9	4.41	5	4.47
43	1.47	45	3.40	37	4.16	27	4.50

* The figures represent the count per cubic millimeter before and after starting special diet in three groups of cases of pernicious anemia: (1) with less than 1.2 million; (2) having from 1.2 to 2 million, and (3) having from 2 to 2.75 million before diet was begun. Also, averages for all forty-five cases are shown.

† The differences in the number of cases after about one month is because some have not taken the diet for as long as two, and others as long as four months.

and about one month (from four to six weeks) after the diet was started. Although all the patients have been observed repeatedly, data can be given for only thirty-seven at the end of about two months (from eight to eleven weeks) of treatment, and for twenty-seven between four and six months after treatment began, because eight have taken the diet for less than two months, and eighteen for less than four months. As a measure of the patient's condition, we have chosen to give in the table and chart the red blood cell count rather than the hemoglobin percentage, partly because the latter in pernicious anemia may be at about the same high level (80 per cent) with red blood cell counts of from 2.5 to 4 million per cubic millimeter. It is recognized that figures for both may vary considerably within a few hours. The figure used in synopsizing the data often represents in each instance an average of several counts made within a few days of each other.

Inspection of the chart and table shows the rapidity with which the red blood corpuscles increased, the high level they attained at the end of about one month, two months and from four to six months after the diet was begun, and the rather slight differences that occurred

15. These changes in the blood and numerous others will be presented in a subsequent paper.

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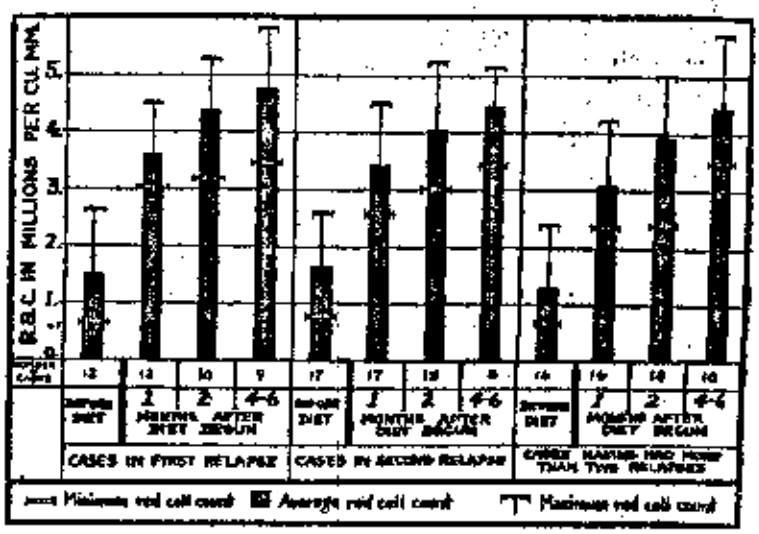
in the bloods in the cases falling into the three groups based on the number of relapses that had occurred. The percentage increase of cells (and the same is true of the hemoglobin) at the end of a month was usually very much greater in patients starting the diet when their red blood cell count was less than 1,200,000 per cubic millimeter than in those in whom it was distinctly higher. This occurs in other pernicious anemia patients rapidly restoring their blood. The blood of patients with rather high counts of their red blood corpuscles and prominent signs of injury to the spinal cord responded more slowly perhaps and less well than others, and, as is to be expected, no striking change occurred in very marked symptoms or signs due to spinal cord degeneration.

In pernicious anemia, remissions after two relapses are frequently less marked than previous ones, so that the red blood cell count is apt to be lower in a third or subsequent relapse than in a former one. In spite of the excellent remissions our patients had soon after beginning the diet, the data in the chart show what might be expected; namely, that not only did the third group of patients (those having had more than two relapses) have on the average a slightly lower red blood cell count before the diet was started, but also that afterward their counts were apt to increase more slowly and not become quite so high as in the other two groups. Only four of the patients had red blood cell counts as low as between 3 and 2.5 million per cubic millimeter after taking the diet for about a month. The cases of three belong to this third group. Even so, two had 4,000,000 or more red blood cells per cubic millimeter at the end of four months. The other, the patient transfused several times, has now, after three months of dieting, only 2,600,000 per cubic millimeter. However, his hemoglobin has risen from 25 to 70 per cent. The fourth case with a red blood cell count of less than 3,000,000 per cubic millimeter at the end of a month belongs to the second group, and now, two and a half months after the diet was started, shows a red blood cell count of 3,300,000 per cubic millimeter.

The data from which the table and chart were prepared have been analyzed in various ways, and the following statements indicate in a different manner than they do what satisfactory improvement was shown in the patients' blood: Seventy-six per cent of all the patients had 2,000,000 or less red blood corpuscles per cubic millimeter, with their hemoglobin usually 55 per cent or less before beginning the diet. In contrast to this, approximately a month (from four to six weeks) later, 91 per cent had over 3,000,000 and 42 per cent over 3,500,000 red blood cells per cubic millimeter, with corresponding rises in the hemoglobin percentage. After taking the diet for about two months (from eight to eleven weeks), 89 per cent (of the thirty-seven that had taken the diet this length of time) had 3,500,000 or more red blood corpuscles per cubic millimeter, while 73 per cent had 4,000,000 or more. All had a hemoglobin of approximately 80 per cent or over. None of the patients studied after they had eaten the food selected for them for between four and six months had less than 3,500,000 red blood cells per cubic millimeter; 81 per cent had 4,000,000 or more, and the counts of 30 per cent were over 5,000,000 per cubic millimeter. The hemoglobin was 80 per cent or above in all, often 90 per cent, and in several cases reached more than 100 per cent. However, it is to be noted that none of these cases observed between four and six months after the diet was started had appeared as advanced as several of those in patients that improved the least, but which have not yet had the diet for four months. The observations on the eighteen patients who have been on the diet for more than six months show that their count may fluctuate, though it has remained above 3,200,000 per cubic millimeter, and usually has been over 4,000,000, with the hemoglobin remaining at 80 per cent or more. There are three exceptions to this statement, for three patients had a relapse about eight weeks after changing their diet. One did so a year and another seven months after the special diet was begun. There was a count slightly below 3,000,000 per cubic millimeter in both for two or three weeks. Their red blood cells and hemoglobin then very rapidly increased under rest and on eating an increased amount of liver and fruit. The third patient's red cell count was 4,200,000 per cubic millimeter a month before she changed her diet. She has just resumed the special diet, and her red cell count is 1,900,000 per cubic millimeter and hemoglobin 50 per cent.



Red blood cell counts in forty-five cases of pernicious anemia before and after beginning special diet. Cases grouped according to the number of relapses the patients had had. One and two months after diet began indicates an approximate amount of time and for any given case is not less and often somewhat more than four or eight weeks. The differences in the total number of cases after about one month are caused by the fact that some patients have had the diet for less than two, and others for less than four, months.

COMMENT

Cases of pernicious anemia undergoing distinct remissions often show rapid and striking improvement, such as occurred in almost all our patients. A considerable number of them have made such remarks as, "I feel better than for several years," "better than for two years," and "stronger than after the two times my blood went low before." Such statements, to be sure, are made by pernicious anemia patients having remissions that have not taken this diet, and there is no case in this series of forty-five that cannot be paralleled by a similar one having a so-called spontaneous remission. However, the records of eleven cases show that the

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red blood cell count in the remission following the "liver diet" has remained distinctly higher, not only than in a former remission, but also in three cases higher, for at least two months, than in their three previous remissions. It is, thus, again pointed out here that it is rather unusual to find the red blood corpuscle count in a late remission distinctly above the level obtained in several earlier ones. A few of the patients observed for many months before they took the special diet ate, by our advice, relatively small amounts of liver two or three times a week, together with other food of the sort contained in the special diet. Under such a regimen a moderate degree of improvement occurred in some, to be followed later by a relapse of their case. It was rather striking that when the same patients were placed on a diet rich in liver they improved markedly. This suggests, as do similar observations we made some time ago in other cases, that if liver and food like it play a rôle in improving the blood of pernicious anemia, it is desirable for the patients to take such food daily and in large amounts.

The spontaneous remissions of pernicious anemia and the bizarre course it often runs make it notoriously difficult to determine accurately the effect of any procedure on the disease. All sorts of therapeutic procedures have been advised; many because a few cases improved promptly after their trial. Waves of enthusiasm for certain methods have vanished soon when it was shown that the earlier reports of benefit could be attributed readily to the natural course of the disease. There is, however, no doubt, as shown by some of the early and more recent investigators, that a well balanced, nutritious diet sometimes aids to enhance a remission. The patient may be helped by numerous other forms of treatment, such as those to change the intestinal flora, the injection of protein substances, the taking of arsenic, the transfusion of blood, and splenectomy.

At least one remission, as has been noted by Cabot,[34] takes place at some time, but at no regular time, in about 80 per cent of pernicious anemia cases. Precise data are sparse concerning the frequency, degree and rate of remissions in similar groups of cases treated in different ways. Splenectomy has caused quick and marked improvement in 64 per cent of the patients undergoing this operation, while about 15 per cent more have shown some benefit from the procedure.[35] However, the remissions that followed have been of no longer duration than those heretofore reported as of a spontaneous nature. Excluding desperately ill patients, Minot and Lee[36] noted in 1917 that about 35 per cent of forty patients treated in no especial manner had a moderate or better remission soon after they were seen. Following the transfusion of blood into forty-six similar patients, about 50 per cent continued to have definitely improved health for at least many weeks than for some time before the procedure. Not more than 20 per cent of the ninety-six patients of these two groups soon had rapid and marked increase in their red blood cells. An analysis of fifty other cases observed between 1916 and 1923 in sequence, except for several in a terminal condition, indicates that 45 per cent developed a definite remission soon after we saw them. These patients were treated in various ways by numerous physicians, but did not eat large amounts of liver or similar food. The remissions were seldom of marked degree, with the red blood corpuscles reaching 4,000,000 or more per cubic millimeter.

No entirely satisfactory data have been found concerning the frequency of remissions following the use of a nutritious high caloric diet and such a regimen as that prescribed by Barker and Sprunt. We have treated in this manner twenty-five partially selected cases, from which it appears that distinct remissions may follow such therapy in about 65 per cent of the instances. Even so, apparently the red blood cell counts of patients on such a diet and who were improved distinctly at the end of one or two months averaged less than for all forty-five who have eaten generously of liver for the same amount of time.

The evidence at hand suggests that the dietetic treatment of pernicious anemia is of considerable importance. It has been possible to demonstrate in forty-five cases seen essentially in sequence that following a diet rich in liver and low in fat a distinct remission of the anemia occurred rather promptly. The promptness and rapidity with which the red blood corpuscles and hemoglobin increased, coincident with at least rather marked subjective improvement in the sense of well being and clinical appearance of all the patients and the strikingly better health of many, is at least unusual in pernicious anemia. It is also not customary for the red blood cell counts during remissions of pernicious anemia to be so frequently of the height that occurred in these patients. We are inclined to believe that something contained in the foods rich in complete proteins is particularly responsible for the improvement in the state of the blood. The low fat content of the diet is assumed to have a less important effect than the character and amount of protein, although probably excess of nitrogen per se is unimportant. If liver and similar food is of value, every means must be taken, including the skill of the nurse and cook, to get patients to eat daily as much as possible, preferably 200 Gm. or more. Failure could be attributed to taking too little of such food.

There are no data to indicate whether the remissions in these forty-five cases will last longer than those of others.

It is possible that this series of cases eventually may be proved to be unusual in that there happened to be treated a group that would have taken a turn for the better under other circumstances. Also, time may show that the special diet used, or liver and similar food, is no more advantageous in the treatment of pernicious anemia than any ordinary nutritious diet. Let this be as it may, at the present time it seems to us, as it has to Gibson and Howard, that it is wise to urge pernicious anemia patients to take a diet of the sort described.

SUMMARY

The dietetic treatment of pernicious anemia is of more importance than hitherto generally recognized.

Forty-five patients with pernicious anemia observed essentially in sequence are continuing to take a special diet that they have now been living on for from about six weeks to two years but which was temporarily omitted by three. This diet is composed especially of foods rich in complete proteins and iron—particularly liver—and containing an abundance of fruits and fresh vegetables and relatively low in fat.

34. Cabot, R. C.: Pernicious Anemia, in Osler and McCrae's Modern Medicine, ed. 2, Philadelphia, Lea and Febiger 4, 1915.
35. Krumbhaar, E. B.: Late Results of Splenectomy in Pernicious Anemia, J. A. M. A. 67:738 (Sept. 2) 1916.
36. Minot, G. R., and Lee, R. I.: Treatment of Pernicious Anemia Especially by Transfusion and Splenectomy, Boston M. & S. J. 177:761 (Nov. 29) 1917.

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Following the diet, all the patients showed a prompt, rapid and distinct remission of their anemia, coincident with at least rather marked symptomatic improvement, except for pronounced disorders due to spinal cord degeneration. Improvement was often striking, so that where the red blood cell count averaged for all before starting the diet 1,470,000 per cubic millimeter, one month afterward it averaged 3,400,000; and for the twenty-seven cases observed from four to six months after the diet was begun, the average count was 4,500,000 per cubic millimeter.

Patients having had two or more relapses showed on the average slightly lower red blood corpuscle counts about one and two months after commencing the diet than did those who had started it in their first or second relapse.

Change in the frequency of bowel movements, temporary increase of reticulocytes in the peripheral blood, and decrease of the icterus index of the blood serum were among the earliest signs that heralded the patient's better health.

All the patients have remained to date in a good state of health except three, who discontinued the diet; two rapidly improved on resuming it and the other has just commenced it again. As the diet was advised for most of the patients less than eight months ago, enough time has not yet elapsed to determine whether or not the remissions will last any longer than in other cases.

SUBSEQUENT OBSERVATIONS

Since the data presented in this paper were compiled, the following additional information has been obtained: The eight patients who had taken the diet for only about one month had red blood cell counts at the end of about two months of between 3,500,000 and 6,000,000, with an average of 4,400,000 per cubic millimeter. One of these had but 2,500,000 at the end of one month and now at the end of three and a half months has 4,500,000 per cubic millimeter.

The ten patients recorded as having taken the diet for only about two months showed in four to six months after starting it as follows: Seven had an average red blood cell count of 5,100,000 per cubic millimeter. One who had had about 5,000,000 had but 3,500,000 per cubic millimeter. Another who had 2,500,000 per cubic millimeter at the end of the second month had the same number two months later, although symptomatically he seemed better. The tenth patient could not obtain the proper diet between the second and fourth month, and had at the latter time 3,000,000 per cubic millimeter.

The patients who had the diet from four to six months, or longer, when the data were compiled, continued in the next two and a half months to have on the average as satisfactory counts, except as noted below. The majority of these have shown higher counts than formerly. Two of the cases have had at three different times red blood cell counts of 6,000,000 or more per cubic millimeter. Two patients who have had the diet for more than six months have recently eaten very little liver, and their counts have fallen in two months from about 4,000,000 to about 3,000,000 per cubic millimeter. The red blood corpuscles of the patient referred to on page 474 as in a relapse increased 3,000,000 per cubic millimeter during the first eight weeks after the diet was resumed.

Information at hand suggests that some cases in which transfusion is done many times before the diet is started may respond but little to it.

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Ind. Jour. Med. Res., 55, 6, June, 1967.

STUDIES ON THE SERUM B_{12} AND FOLIC-ACID LEVELS IN INDIAN INFANTS UNDER OPTIMAL NUTRITIONAL CONDITIONS.

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THERE are scanty reports about serum B_{12} and folic-acid levels in normal Indian children, in contrast to the numerous reports in the literature on serum B_{12} and folic-acid concentration in various diseases. Satoskar *et al.* (1961, 1962) presented serum B_{12} values in normal children of 1 to 8 years. The range was 50 to 1280 μμg./ml. Baker *et al.* (1959, 1960) studied serum folic acid in 26 infants, the range was 3·5—250·0 mμg./ml. by *L. casei* method. Satoskar *et al.* (*loc. cit.*) found a range of 3·0 to 37·0 mμg./ml. by *Streptococcus faecalis* method in 17 children of 1 to 8 years of age. It has also been reported that the serum B_{12} and folic-acid levels are higher in non-vegetarian and better socio-economic groups (Satoskar *et al.*, *loc. cit.*). A review of normal (average) serum B_{12} and folic-acid levels available at various ages by Indian and Western authors is presented in Tables I and II.

MATERIALS AND METHOD.

Thirty-six infants who were fed on a patent milk preparation† from birth onwards were selected for this study.

Table III gives the distribution of age and sex of these infants.

1. *Haemoglobin estimation.*—The cyanmethaemoglobin method (Crosby *et al.*, 1964).

2. Lobe average (neutrophilic segmentation) peripheral smear was stained by May Grünwald Geimsa technique. One hundred neutrophilic leucocyte counts

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†COMPOSITION OF MILK

Each 100 g. contains :—

Protein	26 g.	Vitamin B_1	0·6 mg.
Fat	19 g.	Vitamin B_2	1·0 mg.
Carbohydrate	46 g.	Vitamin B_6	0·3 mg.
Calcium	1·33 g.	Vitamin B_{12}	1·0 mg.
Phosphorus	0·80 g.	Folic acid	0·1 mg.
Iron	4·0 mg.	Niacinamide	6·0 mg.
Sodium iodide	118 mcg.	Vitamin C	30·0 mg.
Vitamin A	1500 IU	Sodium citrate	0·5 g.
Vitamin D	600 IU		

TABLE I.

Serum vitamin B_{12} levels μμg./ml. of normal subjects.

Authors.	Test organism.	Number of cases.	Mean.	Range.	S.D.
Ross (1950)	*Eu. gracilis*	12	490	350—750	..
Mollin and Ross (1952)	*Eu. gracilis*	223	356	100—900	183
Heinrich and Lahann (1953)	*Eu. gracilis*	..	238	62—460	..
Killander (1957)	*Eu. gracilis*	56	360	100—720	..
Lear *et al.* (1954)	*Eu. gracilis*	20	532	292—856	161
Pitney *et al.* (1954)	*Eu. gracilis*	13	195	122—460	..
Pitney and Beard (1954)	*Eu. gracilis*	56	212	86—460	..
*Dixit *et al.* (1956)	*Eu. gracilis*	37	259	92—800	..
*Dixit (*loc. cit.*)	*Eu. gracilis*	78	592	72—2160	..
Killander (*loc. cit.*)	*Eu. gracilis*	156	501	130—1200	200
*Parekh *et al.* (1958)	*Eu. gracilis*	62	593	80—1520	..
Tasker *et al.* (1958)	*Eu. gracilis*	223	356	100—900	..
Chatterjea (1958)	*Eu. gracilis*	37	282·7	98—615	..
Herbert *et al.* (1960)	*Eu. gracilis*	..	..	200—660	..
*Satoskar *et al.*	*Eu. gracilis*	100	..	..	..
		L. Veg.	78·9	20—200	..
		N. Veg.	515·0	190—800	..
*Satoskar *et al.*	*Eu. gracilis*	17	..	50—1280	..
Adams and Scragg (1962)	*Eu. gracilis*	59	681	207—1701	..
Adams and Scragg (1962) Newborn infants	*Eu. gracilis*	17	760	172—1573	..
*Gupta (*loc. cit.*)	*Eu. gracilis*	..	..	..	..
	Low income	30	341	150—575	..
	Good income	30	580·5	180—625	..
	Veg.	48	327	150—450	..
	Non.-Veg.	12	434	215—612	..
Gird Wood (1954)	*L. leichmannii*	50	320	50—870	..
Nieweg *et al.* (1954)	*L. leichmannii*	36	528	310—1050	167
Unglaub *et al.* (1954)	*L. leichmannii*	31	210	70—420	81
Spray (1955)	*L. leichmannii*	111	437	150—830	..
Kristensen *et al.* (1957)	*L. leichmannii*	84	375	150—770	..
Matthews (1962)	*L. leichmannii*	..	..	120—1150	..
*Satyanarayana (1963)	*L. leichmannii*	17	124	50—204	..
Grosswiez *et al.*	*E. coli* 113-3	30	..	200—1000	..

* Indian workers.

were calculated according to the following formula for the lobe average.—

$$\text{Lobe average} = \frac{\text{Number of lobes in 100 successive neutrophils}}{100}$$

3. Estimations of serum B_{12} and folic acid.

(*a*) *Collection of samples.*—7 to 10 ml. of venous blood was withdrawn with a acid-treated dry-sterile all-glass syringe, atleast three hours after the last feed. The serum was separated and stored at —20°C. until assayed. No vitamin C was added as samples were analysed within a fortnight after collection (Waters and Mollin, 1961).

(*b*) Extraction of serum vitamin B_{12}.

(Dacie and Lewis, 1963)

(*c*) Extraction of serum folic acid.

(Waters and Mollin, *loc. cit.*)

TABLE II.

Serum folic-acid levels mμg./ml. of normal subjects.

Authors.	Test organism.	Number of cases.	Mean.	Range.	S.D.
Baker *et al.*	*L. casei*	10	..	7·5—24·0	..
Cooperman *et al.* (1960)	*L. casei*	38	15·9	4·0—45·6	..
Cooperman *et al.* (Infants)	*L. casei*	40	27·5	2·7—201·6	..
Baker *et al.* (Infants) *loc. cit.*	*L. casei*	26	..	3·5—250·0	..
Herbert *et al.* (1960)	*L. casei*	..	..	7·5—24·0	..
Cooper and Lowenstein (1961)	*L. casei*	71	..	3·0—76·0	..
Waters and Mollin (*loc. cit.*)	*L. casei*	100	9·9	5·9—21·0	..
Grossowicz *et al.*	*L. casei*	43	8·3	3·2—15·0	..
Stevens *et al.* (1912)	*L. casei*	20	..	5·0—28·0	..
Solomons *et al.* (1962)	*L. casei*	36	7·8	..	..
Hansen and Weinfeld (1962)	*L. casei*	..	5·4	3·7—9·3	..
Hansen and Van Klewesahl Palm (1963)	*L. casei*	..	5·4	3·2—13·6	..
Maoth *et al.* (1964)	*L. casei*	..	6·3	1·5—30·0	..
Toennies and Usdin (1964)	*L. casei*	9	4·4	2·7—10·6	..
Magnus (1965)	*L. casei*	..	..	3·0—11·0	..
Jeejeebhoy *et al.* (1965)	*L. casei*	7	9·1	4·0—16·1	..
*Gupta *loc. cit.*	*L. casei*	Low Socio Eco. 30	6·05	4·0—8·5	..
		Good Socio Eco. 50	7·68	3·5—11·0	..
		Veg. 48	6·9	4·0—11·0	..
		Non. Veg. 12	7·86	5·9—10·0	..
Vanier and Tyas. Infants (1966)	*L. casei*	6—8 months	7·70	3·5—16·0	..
		1 year	9·30	3·0—35·0	..
*Satoskar *et al.*	*St. faecalis*	100	26·6	16·0—40·0	..
		Lac. Veg.	..	..	..
		Non. Veg.	39·5	16·0—59·0	..
*Satosker *et al.*	*St. faecalis*	7	..	3·6—37·0	..
Grossowicz *et al.*	*St. faecalis*	43	0·75	0·25—1·7	..
*Banerji and Chatterji (1965)	*P. cerevisiae*	..	..	3·0—22·0	..
Grossowicz *et al.*	*St. faecalis*	29	0·4	0·1—1·4	..

* Indian workers.

TABLE III.

Age and sex distribution of the material.

Age groups (months).	Male.	Female.
2— 5	5	7
6—12	10	10
13—24	3	1
Total ..	18	18

The accuracy of the methods (*b* and *c*) was investigated as follows :—

(*i*) *Recovery experiments.*—Cyanocobalamine or folic-acid solutions of known concentrations were subjected for extraction and estimation to find out the recovery rate of these vitamins by the methods used.

(*ii*) *Purity of chemicals.*—During the process of extraction, distilled water was used in place of serum and the whole process repeated.

(*d*) Estimations of serum B_{12} (spray) and of folic acid (Waters and Mollin, *loc. cit*). Aqueous B_{12} and vials of dried folic acid were specially prepared by Glaxo Laboratories (India) Pvt. Ltd., Bombay, for the authors as Macrabin batch No. 605, containing cyanocobalamine 105 mcg./ml. and folic acid ($C_{19}H_{19}O_6N_8$, 91 per cent) respectively, and were stored at 4°C. in dark. The organisms used for B_{12} and folic acid were *Lactobacillus leichmannii* ATCC 7830 Code No. 3215 and *Lactobacillus casei* ATCC 7469 Code No. 3210, respectively. The media used for B_{12} were Bacto-B_{12} culture agar U.S.P. Code No. 0541, B_{12} inoculum broth U.S.P. Code No. 0542, B_{12} assay medium U.S.P. Code No. 0457, and for folic acid were *Lactobacillus* agar AOAC Code No. 0900, *Lactobacilli* broth AOAC, Code No. 0901 and folic acid *casei* medium Code No. 0822. The organisms and the media were supplied by Difco Laboratories. The makers instructions for rehydration of the media maintenance of cultures, and for preparation of the inoculum (Difco Manual*) were followed exactly. The density of bacterial growth for B_{12} and folic acid was measured spectrophotometrically after thorough mixing against uninoculated control at 700 mμ and 520 mμ, respectively.

Observations.

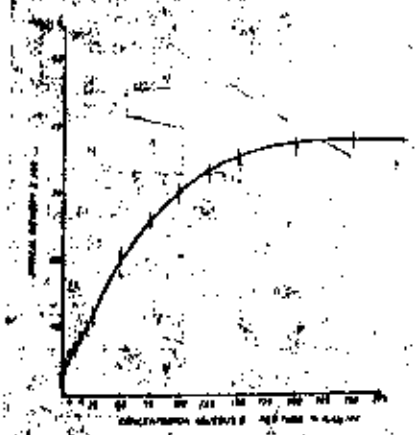
I. Experimental data on serum B_{12} and folic-acid assays :—

(A) Standard curves.

(*a*) Vitamin B_{12} (Graph 1) (*b*) Folic acid (Graph 2)

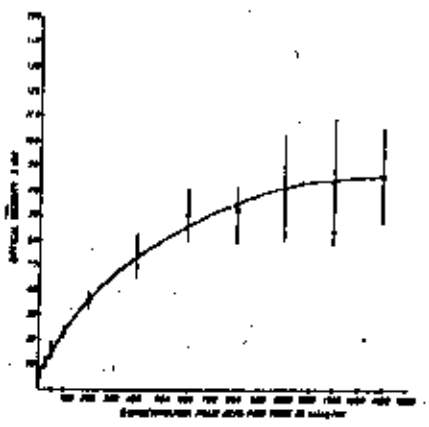
Graph 1.

Standard curve for vitamin B_{12} with standard deviation.



Graph 2.

Standard curve for folic acid with standard deviation.



(B) Microbiological assay for serum B_{12} and folic acid.

Estimation of serum B_{12} and folic acid are difficult processes and results show considerable degree of variation. Thus, the authors investigated recovery, variability of the methods and purity of chemicals.

*Microbiological Assay of Vitamins and Amino Acids (1964) Difco Laboratories, Detroit 1, Michigan, U.S.A.

(*a*) The serum B_{12} was estimated after some modifications in the method described by Spray (1955). The accuracy of the method was studied in the following ways :—

(*i*) Recovery for B_{12} ranged from 83 to 118 per cent.

(*ii*) The variation in three consecutive assays for the serum showed good agreement, i.e. mean 983 SD ± 130 μμg./ml.

(*iii*) The triplicate determinations in 2 different dilutions gave mean 975 SD ± 31·2 μμg./ml.

(*iv*) In test tubes containing distilled water no growth was found.

(*b*) The serum folic acid was estimated according to Waters and Mollin (*loc. cit.*) method.

(*i*) Recovery of folic acid was 81 to 117 per cent.

(*ii*) The variation between different folic acid assays gave a mean 16·0 SD ± 1·54 mμg./ml.

(*iii*) Sera determination (as in B_{12}), mean 14·48 SD ± 0·43 mμg./ml.

(*iv*) Distilled water, very minimal growth was observed and the test values were corrected accordingly.

II. *Clinical material :*

Mean values for haemoglobin, lobe average, serum B_{12} and folic acid (Table IV).—The mean haemoglobin was 10·26 ± 0·42 g. per cent (range 8·00—1300 g. per cent). The mean lobe average determined in 16 children was 3·00 ± 0·184 (Range 2·70—3·26). The serum B_{12} in 36 cases showed a mean value of 713·66—3322·50 μμg./ml. (Range 240·00—1400·00 μμg./ml.) and serum folic acid determined on 28 subjects showed a mean value of 17·95—9·39 mg./ml. (Range 4·70—32·00 mμg./ml.). The values are comparable to those of other authors using similar techniques (Tables I and II). Observations on serum folic acid and B_{12} estimations in the present study show that though the methods are sensitive enough, still there is a considerable degree of variation.

TABLE IV.

Haemoglobin, lobe average, serum folic acid and vitamin B_{12} values of the control material.

Investigations.	Number of cases.	Range.	Median.	Mean.	S.D. ±
I. Haemoglobin, g. per cent	36	8·00— 13·00	10·00	10·26	0·42
II. Lobe average	16	2·70— 3·26	3·10	3·00	0·184
III. Vitamin B_{12}, μμg./ml.	36	240·00—1400·00	670·00	713·66	322·50
IV. Folic acid, mμg./ml.	28	4·70— 32·00	16·38	17·95	9·39

DISCUSSION.

The information in the literature on the serum B_{12} and folic-acid concentrations in normal subjects and in patients with various diseases differs widely. These differences

cannot be attributed solely to the use of various test organisms, since the results obtained by investigators using the same organism vary as greatly as those found when different test organisms have been employed (Tables I and II). It is not known to what extent the differences are due to the use of various techniques for assay or to dissimilarity in the composition of the series investigated. Thus, rigidly controlled and standardized assay conditions are needed and each investigator must define normal and pathological serum B_{12} values under his own conditions of assay.

The daily requirements of folic acid and vitamin B_{12} at various ages are essentially unknown. It is probably that 0·5-1·0 mg./day and 1 to 2 mcg./day of folic acid and vitamin B_{12}, respectively, will maintain good health in adults under most conditions (Davidson and Girdwood, 1947; Burton, 1959; Prondfit and Robinson, 1960; Sheehy *et al.*, 1960). The maximum capacity of the intestinal wall to absorb vitamin B_{12} even when there is an excess of the intrinsic factor does not exceed 0·7—1·3 μg./daily (Glass *et al.*, 1954; Callender and Evans, 1955). The need for these vitamins in growing infants is difficult to assess. In the present study, infants were receiving about 0·1-0·2 mg./day and 1-1·5 mcg./day of folic acid and vitamin B_{12}, respectively. The infants, on breast or other commercial milk preparations, generally receive supplemented vitamin and iron tonics which, in turn, supply approximately similar quantities of these vitamins.

These infants showed a mean haemoglobin value of 10·26 ± 0·42 g. per cent which is comparable to the normal values reported by Guest and Brown (1957). The mean lobe average determined in 16 children was 3·00 ± 0·184 and peripheral smears in all the 36 cases showed normocytic normochromic picture, and a sufficient number of platelets. The normal lobe average reported by Herbert (1965) is 3·17 ± 0·25. The difference in the lobe average in present study compared to that Herbert (*loc. cit.*) is not clearly understood. The normal haemoglobin, peripheral smear report and lobe average have been used to substantiate that none of the infant was suffering from any haematological disorders. The growth and development data of these infants approached western standards (Khanduja *et al.*, in press).

The serum B_{12} estimation in 36 subjects gave a mean value of 714 ± 322·5 μμg./ml. (Range 240-1400 μμg./ml.) and serum folic-acid estimation in 28 infants gave a mean value of 17·95 ± 9·39 mμg./ml. (Range 4·70—32·00 mμg./ml.). These values are comparable with those in adults (non-vegetarian subjects) by Satoskar *et al.* (*loc. cit.*), and Gupta (1965). Somewhat higher serum B_{12} and folic-acid levels observed in the present material in comparison to data of other Indian workers may possibly be due to optimal nutritional conditions.

Summary.

A group of 36 normal infants on optimal diet showed a mean haemoglobin of 10·26 ± 0·42 g. per cent, mean lobe average 3·00 ± 0·184, mean serum B_{12} 713·66 ± 322·5 μμg./ml., and mean serum folic acid 17·95 ± 9·39 mμg./ml.

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REFERENCES.

ADAMS, E.C., and SCRAGO, J.N. (1962) .. Serum vitamin B_{12} concentrations in megaloblastic anemia associated with kwashiorkor and marasmus. *Jour. Pediat.*, 60, 580.

BAKER, H., HERBERT, V., FRANK, O., PASHER, I., HUTNER, S.H., WASSERMAN, L.R., and SABOTKA, H. (1959) A microbiologic method for detecting folic acid deficiency in man. *Clin. Chem.*, 5, 275.

BAKER, H., FRANK, O., PASHER, I., ZIFFER, H., and SABOTKA, H. (1960) Pantothenic acid, thiamine and folic acid levels at parturition. *Proc. Soc. Exper. Biol. N.Y.*, 103, 321.

BANERJEE, D.K., and CHATTERJEE, J.B. (1965) Observations on the folate activity of human serum. *Bull. Cal. School Trop. Med.*, 13, 45.

BURTON, B.T. (1959) .. .. .. 'The Heinz Book of Nutrition', McGRAW-HILL, N.Y. 99.

CALLENDER, S.T., and EVANS, J.R. (1965) .. The urinary excretion of labelled vitamin B_{12}. *Clin. Sci.*, 14, 295.

CHATTERJEA, J.B. (1958) .. .. Nutritional macrocytic anaemia in India. *Jour. Ind. Med. Ass.*, 31, 4.

COOPER, B.A., and LOWENSTEIN, L. (1961) .. Evaluation of assessment of folic acid deficiency by serum folic acid activity measured with *L. casei*. *Canad. Med. Ass. Jour.*, 85, 987.

COOPERMAN, J.M., LUHBY, A.L., and AVERY, C.M. (1960) Serum folic acid activity by improved *L. casei* assay. *Proc. Soc. Exper. Biol. N.Y.*, 104, 536.

CROSBY, M.H., MUNN, J.I., and FURTH, F.W. (1964) Standardizing method for clinical haemoglobinometry. *U.S. Armed Forces Med. Jour.*, 5, 693-703.

DACIE, J.V., and LEWIS, S.M. (1963) .. 'Practical Haematology', 3rd ed., J. & A. Churchill Ltd., 104, Gloucester Place, W.1., London.

DAVIDSON, L.S.P., and GIRDWOOD, R.H. (1947) Folic acid as a therapeutic agent. *Brit. Med. Jour.*, 1, 587.

DIXIT, C.H., MODY, B.M., JHALA, H.I., PAREKH, J.G., and RAMASARMA, G.B. (1956) Serum vitamin B_{12} concentrations in normal subjects. *Ind. Jour. Med. Sci.*, 10, 419.

GLASS, G.B., BOYD, L.J., and STEPHANSON, L. (1954) Intestinal absorption of vitamin B_{12} in humans as studied by isotope technic. *Proc. Soc. Exper. Biol. N.Y.*, 86, 522.

GIRDWOOD, R.H. (1954) .. .. Rapid estimation of serum vitamin B_{12} levels by a microbiological method. *Brit. Med. Jour.*, 2, 954-956.

GROSSOWICZ, N., ARONOVITCH, J., and RACHMILEWITZ, M. (1954) Determination of vitamin B_{12} in human serum by mutant of *E. coli* (21428). *Proc. Soc. Exper. Biol., N.Y.*, 87, 513.

GROSSOWICZ, N., RACHMILEWITZ, M., IZAK, G., and ZAN, S. (1962) Determination of folic acid metabolites in normal subjects and in patients with nutritional megaloblastic anaemia. *Proc. Soc. Exper. Biol. Med.*, 109, 770.

GUEST, G.M., and BROWN, E.W. (1957) .. Erythrocytes and haemoglobin of the blood in infancy and childhood. *Amer. Jour. Dis. Child.*, 93, 486.

GUPTA, O.P. (1965) .. .. .. Folic acid estimation inenormal subjects and in megaloblastic anaemia cases. *Jour. Ind. Med. Ass.*, **45**, 302.

HANSEN, H.A., and VAN KLEWESAHLPALM, H. (1963) Blood folic acid levels and clearance rate of injected folic acid in normal pregnancy and puerperium. *Scand. Jour. Clin. Lab. Invest.*, **15**, 78.

HANSEN, H.A., and WEINFELD, A. (1962) .. Metabolic effects and diagnostic values of small doses of folic acid and B_{12} in megaloblastic anaemias. *Acta Med. Scand.*, **172**, 427.

HEINRICH, H.C., and LABANN, H. (1953) .. *Z. Naturforsch*, **86**, 589.

Idem (1957) .. .. .. Cf. Killander, A. *Acta Med. Scand.*, **159**, 307.

HERBERT, V. (1965) .. .. .. Megaloblastic anaemias : Mechanism and Management. *Disease a month*, Aug. Year Book Medical Publishers. Inc. Chicago.

HERBERT, V., BAKER, H., FRANK, O., PARKER, J., SABOTKA, H., and WASSERMAN, L.R. (1960) The measurement of folic acid activity in serum : A diagnostic aid in the differentiation of the megaloblastic anaemias. *Blood*, **51**, 228.

JEJEEBHOY, K.N., PATHARE, S.M., and NORONHA, J.M. (1965) Observations on conjugated and unconjugated blood folate levels in megaloblastic anaemia and effects of vitamin B_{12}. *Ibid.*, **26**, 354-391.

KHANDUJA, P.C., AGARWAL, K.N., and TANEJA, P.N. Long term growth study in first year of life on optimal nutritional conditions. *Ind. Ped.*, (In press).

KILLANDER, A. (1953) .. .. *Svensk Lakartidn*, **50**, 2260.

Idem (1957) .. .. The use of the serum vitamin B_{12} assay in the diagnosis of vitamin B_{12} deficiency. *Acta Med. Scand.*, **159**, 307.

KRISTENSEN, H.P.O., LUND, J., OHLSEN, A.S., and PEDERSEN, J. (1957) Maintenance therapy in pernicious anaemia, (controlled by determining vitamin B_{12} level in plasma). *Lancet*, **i**, 1266.

LEAR, A.A., HARRIS, J.W., CASTLE, W.B., and FLEMING, E.M. (1954) The serum vitamin B_{12} concentration in pernicious anaemia. *Jour. Lab. Clin. Med.*, **44**, 715.

MAGNUS, E.M. (1965) .. .. Folic acid activity in serum and red cells in patients with vitamin B_{12} deficiency, Relation to treatment. *Brit. Jour. Haemat.*, **11**, 188.

MATOTH, Y., PINKAS, A., ZAMIR, R., MOOALLEM, F., and GROSSOWICZ, N. (1964) Studies on folic acid in infancy. 1. Blood levels of folic and folinic acid in healthy infants. *Pediat.*, **33**, 507.

MATTHEWS, D.M. (1962) .. .. Observations on the estimation of serum vitamin B_{12} using *Lactobacillus leichmannii*. *Clin. Sci.*, **22**, 101.

MOLLIN, D.L., and ROSS, G.I.M. (1952) .. The vitamin B_{12} concentrations of serum and urine of normals and of patients with megaloblastic anaemia and other diseases. *Jour. Clin. Path.*, **5**, 129.

NIEWEG, H.O., FABER, J.G., DEVRIES, J.A., and KROOSE, W.E.S. (1954) The relationship of vitamin B_{12} and folic acid in megaloblastic anaemias. *Jour. Lab. Clin. Med.*, **44**, 118.

PAREKH, J.G., DIXIT, C.H., JHALA, H.I., MODY, B.M., and RAMA SARMA, G.B. (1958) Serum vitamin B_{12} concentrations in patients with anaemias and other diseases. *Jour. Ass. Phys. Ind.*, **6**, 173.

PITNEY, W.R., and BEARD, M.F. (1954) .. Serum and urine concentrations of vitamin B_{12} following oral administration of the vitamin. *Amer. Jour. Clin. Nutr.*, 2, 89.

PITNEY, W.R., BEARD, M.F., and VANLOON, B.J. (1954) Observation on the bound form of vitamin B_{12} in human serum. *Jour. Biol. Chem.*, 207, 143.

PRONDFIT, F.T., and ROBINSON, C.H. (1960) 'Nutrition and Diet Therapy', 135. The McMILLAN Co. N.Y.

ROSS, G.I.M. (1950) .... .. Vitamin B_{12} assay in body fluids. *Nature* (London), 166, 270.

SATOSKAR, R.S., KULKARNI, B.S., MEHTA, B.M., SANZGIRI, R.R., BAMJI, M.S. (1962) Serum B_{12} and folic acid (P.G.A.) levels in hypoproteinaemia and marasmus in Indian children. *Arch. Dis. Child.*, 37, 9.

SATOSKAR, R.S., KULKARNI, B.S., and REGE, D.V. (1961) Serum proteins, cholesterol, vitamin B_{12} and folic-acid levels in lactovegetarian and non-vegetarians. *Ind. Jour. Med. Res.*, 49, 887.

SATYANARAYANA, N.S. (1963) .. .. Plasma vitamin B_{12} levels in some nutritional deficiency states. *Ibid.*, 51, 103-112.

SHERBY, T.W., RUBIN, M.E., SIGLER, M.H., PEREZ-SANTIAGO, E., BACODAMENA, R., and SANTINI, R. (1960) Folic acid requirements in man. *Fed. Proc.*, 19, 56.

SOLOMONS, E., LEE, S.L., WASSERMAN, M., and MALKIN, J. (1962) Association of anaemia in pregnancy and folic acid deficiency. *Jour. Obstet. Gynaec. Brit. Commonwealth.*, 69, 724.

SPRAY, G.H. (1955) .. .. .. An improved method for the rapid estimation of vitamin B_{12} in serum. *Clin. Sci.*, 14, 661.

STEVENS, K., METZ, J., and BRANDT, V., and VON BROEKHUIZEN, L. (1962) Serum vitamin B_{12}, folic acid and urinary formiminoglutamic acid in megaloblastic anaemias in South African Bantu adults. *E. Afr. Med. Jour.*, 39, 222.

TASKER, P.W.F., MOLLIN, D.L., and BERRIMAN, H. (1958) Vitamin B_{12} deficiency in the megaloblastic anaemias of Malaya. *Brit. Jour. Haemat.*, 4, 167.

TOENNIES, G., and USDIN, E. (1964) .. (Cf. LUHY, A.L., and COOPERMAN, J.M. 'Advances in Metabolic Disorders', I, 316-17, Table III).

UNGLAUB, W.G., ROSENTHAL, H.L., and GOLDSMITH, G.A. (1954) Studies of vitamin B_{12} in serum and urine following parenteral administration. *Jour. Lab. Clin. Med.*, 43, 143.

VANIER, T.M., and TYAS, J.F. (1966) .. Folic acid status in normal infants during the first year of life. *Arch. Dis. Child.*, 41, 658.

WATERS, A.H., and MOLLIN, D.L. (1961) .. Studies on the folic acid activity of human serum. *Jour. Clin. Path.*, 14, 335.

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Deranged B_{12} Metabolism: Studies of Fibroblasts Grown in Tissue Culture

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We have recently described the case of an infant who had a decreased concentration of plasma methionine and increased concentrations of plasma and urinary homocystine and cystathionine (1, 2). It was shown (3) that these abnormalities may be ascribed to a specific deficiency in the activity of N^5-methyltetrahydrofolate-homocysteine methyltransferase (N^5-methyltetrahydrofolate methyltransferase).[1] In addition, the child excreted elevated amounts of methylmalonic acid (2), a finding which in mammals, including humans, has been related to decreased activity of methylmalonyl-CoA isomerase (methylmalonyl-CoA CoA-carbonylmutase, EC 5.4.99.2).[2] N^5-Methyltetrahydrofolate methyltransferase and methylmalonyl-CoA isomerase are the two enzymes in mammals known to depend for activity upon B_{12}-containing cofactors. Deficient activity of both these enzymes could have a common basis if there were a derangement in B_{12} metabolism so that coenzymatically active derivatives of B_{12} failed to accumulate normally. This hypothesis received some support when it was demonstrated that tissues of the patient had an

[1] The abbreviations used are: N^5-methyltetrahydrofolate methyltransferase, N^5-methyltetrahydrofolate-homocysteine methyltransferase; B_{12} is used as a generic abbreviation for the cobalamin group of compounds. The ligand of the cobalt atom above the plane of the corrin ring is specified in each case as a prefix. For example, when the ligand is the cyanide moiety, as it is in vitamin B_{12} itself, we will use the term "cyano-B_{12}."

[2] Enzyme numbers are those designated in "Enzyme Nomenclature," Commission on Enzymes (Elsevier, New York, 1965).

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abnormally low content of deoxyadenosyl-B_{12}, the coenzyme for methylmalonyl-CoA isomerase (1). We now report the results of studies of fibroblasts grown in tissue culture from a skin biopsy of our patient. These results confirm and extent previous observations and indicate that the abnormalities of both the sulfur amino acids and methylmalonic acid are primarily due to a derangement in B_{12} metabolism.

CLINICAL

Clinical and biochemical studies of the patient, E. M., suspected of having deranged B_{12} metabolism, have been published (1–3). Another patient, L. A., whose fibroblasts we studied, died with ketoacidosis and methylmalonic aciduria. His liver contained a normal concentration of deoxyadenosyl-B_{12}. Extracts supplemented with deoxyadenosyl-B_{12}, failed to catalyze the conversion of methylmalonyl-CoA to succinyl-CoA, probably due to a defective methylmalonyl-CoA isomerase apoenzyme (4). In agreement, fibroblasts cultured from the skin of this patient accumulated methylmalonate and failed to convert propionate-^{14}C to CO_2, aspartate, or glutamate as readily as control cells (5). Control cell lines were obtained from normal volunteers or patients without known abnormalities of amino acid or propionate metabolism.

METHODS AND MATERIALS

Skin biopsies were obtained, and tissue cultures were initiated as described (6). Tissue culture reagents were prepared by the NIH media production unit or obtained from Grand Island Biological Company, Grand Island, New York. Cells were grown in a medium consisting of 90% Eagle's minimal essential medium (MEM) containing the nonessential amino acids (7), 0.16% sodium bicarbonate and neomycin sulfate (50 μg/ml), and 10% fetal bovine serum. The B_{12} concentration of this medium was 50–100 pg/ml due to the presence of B_{12} in the fetal bovine serum. For some experiments, as specified, commercial dialyzed serum was used. The dialysis removed little, if any, of the B_{12}. Cells were grown as monolayer cultures in commercially available 32 oz prescription bottles. At the time of harvesting they were rinsed with Ca^{2+}, Mg^{2+}-free Dulbecco's saline (8) and removed from the glass by treatment with this solution containing 0.025% trypsin, 0.0025% EDTA, and 0.2% methylcellulose. The cells were suspended in 40 ml of Dulbecco's saline containing 0.1% methylcellulose. After a 0.5-ml aliquot had been removed for cell enumeration in the Coulter counter, the cells were sedimented by centrifugation at 600g and washed by resuspension in Dulbecco's saline–methylcellulose. The washed pellet was stored at −85° until processed further. For extraction, the cells were suspended in potassium phosphate buffer, pH 6.9, 0.03 M, and subjected to two

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bursts of sonication using the microtip of the Branson sonifier (Model S-75) set at a meter reading of 0.7–0.8 amp. Each burst lasted 30 sec, with 30-sec periods of cooling between. After centrifugation at 2000g for 8 min the supernatant fluid was used for all enzyme assays except that of N^5-methyltetrahydrofolate methyltransferase. For the latter, cells were sonicated in potassium phosphate buffer, pH 7.5, 0.1 M containing reduced glutathionine, 10^{-3} M. The sonicate was centrifuged at 48,000g for 90–120 min and the supernatant fluid was either dialyzed or gel filtered against the same buffer. All sonications and manipulations of cell-free extracts were carried out at 0–3°.

Enzyme assays, determination of protein concentration, and other methods have been described (3, 9). N^5-Methyltetrahydrofolate methyltransferase assays were carried out under N_2. If alkyl-B_{12} compounds were used, the experiments were performed in the dark. All enzyme assays, unless otherwise noted, were performed within 2–3 hr of preparation of the extracts. Reaction mixtures for assay of N^5-methyltetrahydrofolate methyltransferase were modified from that described (3) as follows: (a) The amount of S-adenosylmethionine added was varied as specified in each experiment. The S-adenosylmethionine routinely used was a preparation of the iodide salt (Calbiochem). For the experiment in which homocysteine dependence of the reaction was examined, it was desirable to decrease possible contamination with S-adenosylhomocysteine (which might give rise to homocysteine). The S-adenosylmethionine was purified by preparative paper chromatography with ethanol, 160, HCl, 0.105 N, 76 (v/v). (b) Cyano-B_{12} was not added routinely. For each experiment, any supplementation with a B_{12} derivative will be specified. Paper chromatography was performed by the descending method. Radioactivity on paper chromatograms was determined as described (9). Total B_{12} was measured by a radioisotope dilution method (10) at Leary Lab., Boston.

The ability of cells to convert propionate-1-^{14}C to $^{14}CO_2$ was measured by a modification of the technique of Blass *et al.* (11). Cells were harvested in the standard manner except that after trypsinization they were suspended in a medium consisting of: Ca^{2+} and Mg^{2+}-free Dulbecco's saline, containing 0.4% methylcellulose (viscosity, 4000 cps), 25 ml; a 50-fold concentrate of the amino acids of Eagle's MEM, 2 ml; neomycin sulfate, 10 mg/ml, 0.5 ml; Hanks' balanced salt solution (12) to bring to 100 ml. After centrifugation the pellet was resuspended in this medium at a concentration of approximately 8×10^6 cells/ml. Nonwetting glassware and pipettes, were used. The reaction mixture was composed of the following, in milliliters: 0.05 ml potassium phosphate buffer, pH 7.4, 0.2 M; 0.01 ml Hanks' balanced salt solution (10-fold concentrate); 0.06 ml Eagle's MEM (6.7-fold concentrate); 0.10 ml

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of a solution of propionate-1-^{14}C, 0.27 M, containing about 1.8×10^{9} count/min/ml; 0.15 ml water. To start the assay, up to 0.8 ml of cell suspension was added. If less than 0.8 ml was used, the difference was made up by addition of the medium in which the cells were suspended. To the control vessel was added 0.8 ml of this medium only. The cell suspensions were incubated in 20 ml polyethylene vials which were closed with 14-mm sleeve-type rubber stoppers (Arthur H. Thomas Co.), from which were suspended disposable center wells (Kontes Glass Co.) containing filter paper wicks. After incubation at 37° for 90 min with slow shaking, the reaction was stopped by successive injection of 0.2 ml of 8% NaOH into the center well, and 1.0 ml of 0.6 N H_2SO_4 into the cell suspension. After collection of CO_2 for 30 min at 37° with slow shaking, the center wells were transferred into counting vials containing 1 ml p-dioxane and 15 ml of a mixture of the following (in milliliters): toluene, 667; methanol, 333; p-dioxane, 105; Liquifluor (New England Nuclear Corp.), 45. Radioactivity was determined in a Packard Tri-Carb liquid scintillation counter. The value from the control vessel without cells was subtracted and the results were expressed as count/min/10^{8} cells/90 min. In almost all experiments, each cell preparation was assayed in duplicate or at two cell concentrations. The results usually agreed closely and were averaged. Mean values are reported.

Oxidation of glutamate to CO_2 was assayed in the same way with substitution of glutamate-U-^{14}C, 0.22 M, containing approximately 1.7×10^{9} count/min/ml, 0.10 ml, for propionate-1-^{14}C in the reaction mixture.

Several experiments were carried out to determine accumulation of intermediates during propionate-1-^{14}C oxidation. For those experiments, the standard incubation was modified as follows: (a) As many as 12.7 × 10^{8} cells were incubated in a single vial. (b) The propionate-1-^{14}C concentration was raised 4.3-fold. (c) Sodium succinate, 7.9 μmole, was added to each vial. (Control experiments showed that this addition caused little alteration in $^{14}CO_2$ evolution from propionate-1-^{14}C.) (d) The time of incubation was 165 min. For each of the four cell lines studied, several aliquots of cells were incubated in separate vials. The reaction mixtures were combined after acidification and CO_2 collection at the end of the experiment. The combined acidified suspensions were centrifuged. The supernatant fluids were extracted continuously with ether overnight to yield ether-soluble and aqueous fractions.

The etheral solutions were evaporated to dryness, and the residues were dissolved in ethanol. The ethanolic solutions were subjected to preparative paper chromatography with authentic succinic and methylmalonic acids, using isoamyl alcohol saturated with 4 N formic acid (solvent 1) (18) as developing solvent. The chromatograms were dried

steamed for a few minutes to remove solvent, and sprayed with bromcresol green (14) to detect acidic compounds. Methylmalonic acid separated from an area to which succinate and lactate moved together. The "methylmalonate" and "succinate" areas were each eluted. The solutions were taken to dryness, and the residues were dissolved in ethanol. Aliquots of these solutions were further analyzed by paper chromatography with ethanol, concd. NH_3, water (80:5:15 v/v) (solvent 2) (15) as developing solvent. Radioactivity migrating with authentic methylmalonate, succinate, or lactate in these chromatograms was taken as a measure of accumulation of radioactivity in these compounds after correction for recovery in the preparative steps. As a further check on the identity of the methylmalonate-^{14}C accumulated by cells from E. M. and L. A., portions of the eluates from the "methylmalonate" areas of the chromatograms developed with solvent 1 were repeatedly recrystallized from acetic acid: toluene (10:90 v/v) (16) with authentic carrier. The specific radioactivity of the methylmalonic acid was constant during the second and third crystallizations and was in excellent agreement with the value calculated from the methylmalonate-^{14}C content of the eluates measured by the amount of radioactivity migrating with methylmalonate in solvent 2.

The aqueous solutions after ether extraction were diluted and sulfate was removed with barium hydroxide. The supernatant fluids were concentrated and aliquots were chromatographed with isopropyl alcohol, formic acid, and water (70:10:20, v/v). The chief radioactive products formed by control cells migrated with glutamate and aspartate. The identity of these products was confirmed by chromatography with tertbutyl alcohol, formic acid, water (70:15:15 v/v). Accumulation was calculated from the amounts of radioactivity present in these areas.

RESULTS

The cells grown from the skin biopsy of E. M. have been evaluated with respect to the two metabolic reactions in which B_{12} derivatives are known to play a role in mammals.

Homocysteine Metabolism

One of the mammalian enzymes which utilizes a B_{12} derivative as coenzyme is N^5-methyltetrahydrofolate methyltransferase, the enzyme catalyzing the terminal step in de novo formation of the methyl group of methionine by transfer of a methyl group from N^5-methyltetrahydrofolic acid to homocysteine. To investigate homocysteine metabolism by cells from E. M., several types of experiments were performed.

Nutritional Experiments. Mangum and North (17) have briefly re-

ported that established lines of heteroploid mammalian cells fail to survive or grow on a medium in which homocysteine replaces methionine unless hydroxy-B_{12} is added to the medium. We have preformed similar experiments on four of our control diploid human fibroblast cell lines. Each grew satisfactorily in a medium in which methionine had been replaced by homocysteine. In contrast, cells from E. M. did not grow in the homocystine-containing medium although upon a methionine-containing medium these cells grew at a rate which was not unusual compared to our general experience with human fibroblasts (Fig. 1). When the homocystine-containing medium was supplemented with graded concentrations of hydroxy-B_{12} the ability of the cells from E. M. to grow was partially restored (Table 1). Even in the presence of the highest concentration of hydroxy-B_{12} tested (10 μg/ml), the cells from E. M. did not grow as well upon homocystine as upon methionine (Table 1).

N^5-Methyltetrahydrofolate Methyltransferase Activity in Cell-Free Extracts. Extracts were prepared from cells from control subjects and from E. M., subjected to gel filtration, and assayed for N^5-methyltetrahydrofolate methyltransferase activity. Both control extracts and extracts of the patient's cells contained methyltransferase activity when assayed under our usual conditions (3). Omission of S-adenosylmethionine led to a complete loss of activity. Omission of homocysteine decreased activity by about 95%. These are the dependencies expected by analogy with the B_{12}-dependent methyltransferase of animal (18, 19), *E. coli* (19), and human (3) tissues. In the experiments reported in this paper, S-adenosylmethionine has been added at various concentrations between 0.05 and 0.45 mM. An increase in S-adenosylmethionine from 0.05 to 0.30 mM increased the methyltransferase of control extracts by an average of 11%. The low rate of methionine formation in the absence of added homocysteine is tentatively attributed to homocysteine present in, or arising from, one of the other components of the reaction mixture (3). Methionine formation was linearly proportional to enzyme concentration and to time of incubation. For example, the reaction catalyzed by extracts of E. M. cells supplemented with methyl-B_{12} was linear from the shortest to the longest time tested (8–90 min).[5]

A striking difference between control extracts and extracts from E. M. was noted when the B_{12} dependencies were compared (Table 2). If no B_{12} supplement was added to the assay medium, the methyltransferase activity of control extracts decreased about 50%. With extracts of

[5] For data illustrating the dependencies and linearity of product formation with enzyme concentration, order document NAPS 01172, from National Auxiliary Publications Service, c/o CCM Information Corporation, 909 Third Avenue, New York, New York, 10022.

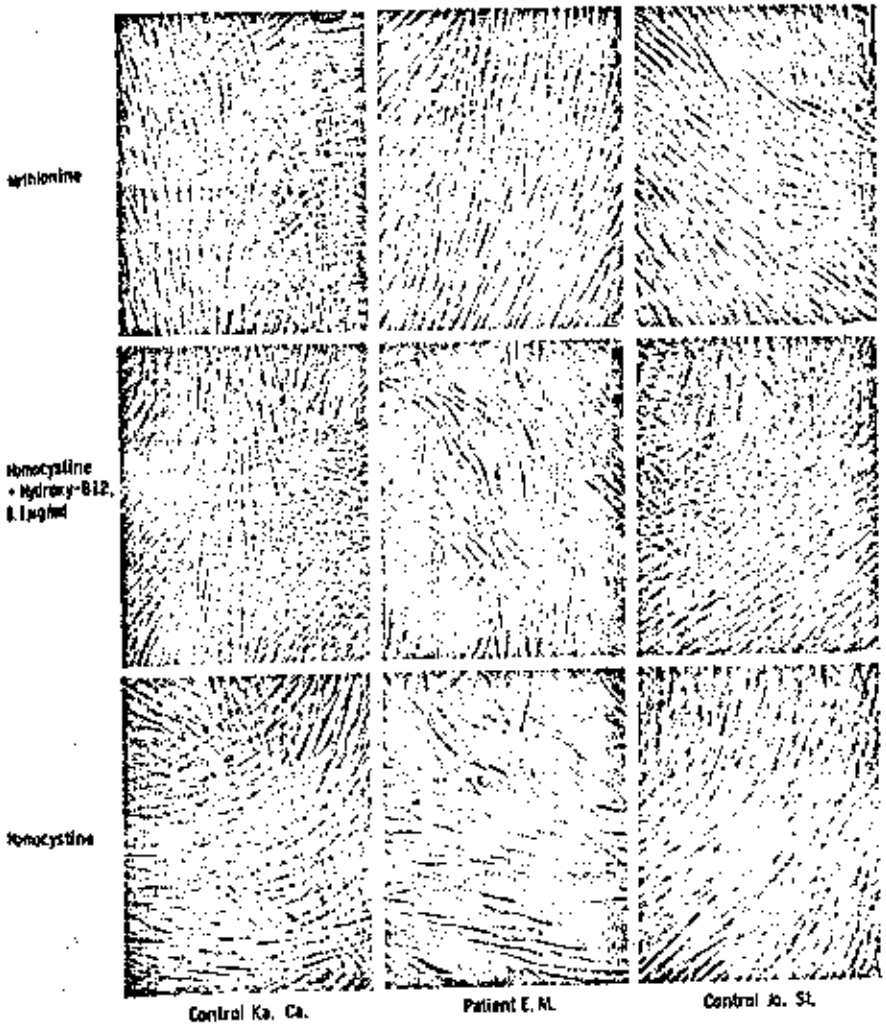

FIG. 1. Growth of fibroblasts from E. M. and from control subjects in media containing methionine, or homocystine and hydroxy-B_{12}, 0.1 µg/ml, or homocystine. Cells were grown in the dark on basal medium made with dialyzed serum. Methionine and choline were omitted and replaced as follows: upper row, methionine; middle row, homocystine + hydroxy-B_{12}, 0.1 µg/ml; bottom row, homocystine. The amino acids were added at a concentration of 15 µg/ml. The cells were grown for 12 days with two changes of medium. Photographs were taken on day 12. Cells were from the following subjects: left column, control Ka. Ca.; middle column, patient E. M.; right column, control Jo. St.

TABLE 1

GROWTH OF FIBROBLASTS FROM E. M. AND CONTROL SUBJECTS IN METHIONINE- OR HOMOCYSTINE-CONTAINING MEDIA[a]

Supplement to medium	No. of cells, millions			
	Experiment A		Experiment B	
	Control Jo. Me.	Patient E. M.	Control Ro. Te.	Patient E. M.
Methionine	2.6	1.3	18.6	11.4
Homocystine	2.9	0.48	16.6	4.4
Homocystine + hydroxy-B_{12}, 0.1 μg/ml	2.8	0.54	—	5.3
Homocystine + hydroxy-B_{12}, 1.0 μg/ml	2.7	0.88	—	6.8
Homocystine + hydroxy-B_{12}, 10 μg/ml	—	—	—	7.6

[a] Cells were grown in the dark in basal medium made with dialyzed serum. Methionine and choline were omitted and replaced by the supplements shown. Methionine or homocystine were added at a concentration of 15 μg/ml. For Expt. A, the cells were grown for 14 days in small plastic tissue culture flasks. For Expt. B, large glass bottles were inoculated with 5.2×10^6 cells. The cells were grown for 8 days with two changes of medium. At the termination of each experiment the cells were harvested and counted as usual.

TABLE 2

B_{12} EFFECTS ON N^5-METHYLTETRAHYDROFOLATE METHYLTRANSFERASE ACTIVITY IN EXTRACTS FROM CONTROL CELLS AND CELLS FROM E. M.[a]

B_{12} Supplement	Activity, % of maximum			
	Ro. Pa.[b]	Combined[c]	E. M.[b]	E. M.[c]
None	51	47	2	0
Hydroxy-B_{12}	77	71	15	52
Cyano-B_{12}	91	87	50	71
Deoxyadenosyl-B_{12}	59	86	1	0
Methyl-B_{12}	100	100	100	100

[a] The results of two experiments are shown. The standard reaction mixture was used with S-adenosylmethionine, 90 nmole, and B_{12} derivatives, as noted, 13.6 μg.

[b] In this experiment, extracts of cells from control subjects Ro. Pa. and E. M. were compared. The specific activities in the presence of methyl-B_{12} were 7.68 and 3.78 nmole methionine/mg protein/60 min, respectively.

[c] In this experiment, a preparation made by combination of extracts from several control cell lines was compared to a preparation made by combination of several extracts from cells of E. M. These extracts had been stored at −60° after gel filtration and before assay. The specific activities in the presence of methyl-B_{12} were 9.41 and 5.04.

cells from E. M., essentially no activity was detected in the unsupplemented condition. Methyltransferase activity in extracts from E. M.'s cells was thus completely dependent upon addition of a B_{12} supplement. Deoxyadenosyl-B_{12} was completely ineffective in restoration of activity in the extracts from cells of E. M. and relatively ineffective in stimulation of activity in control extracts. Hydroxy-B_{12} and cyano-B_{12} were each partially effective (Table 2). In other experiments, not shown, it was demonstrated that (a) methyl-B_{12}, 13.6 μg, led to approximately maximal activity with E. M. extracts. In one experiment, decrease in methyl-B_{12} to 3.4 μg decreased methyltransferase activity to 81%; increase of methyl-B_{12} to 54.4 μg was without significant effect. (b) Deoxyadenosyl-B_{12}, 13.6 μg, or hydroxy-B_{12}, 13.6 μg, each inhibited the stimulatory effect of a simliar amount of methyl-B_{12}. (c) Activities in control extracts assayed without B_{12} supplementation or with cyano-B_{12}, and in E. M. extracts supplemented with cyano-B_{12}, were each dependent upon S-adenosylmethionine and homocysteine and linear with enzyme concentration. (d) The restoration of activity to extracts from E. M. cells and the stimulation of activity in control extracts were quantitatively similar when measured as functions of the concentractions of added cyano-B_{12}.

Increase in Methylfolate-H_4 Methyltransferase Activity Due to Hydroxy-B_{12} Added to the Cell Growth Medium. While the present work was in progress, Mangum and North (17) reported that when HEp-2 cells were grown in a medium in which methionine had been replaced by homocysteine and hydroxy-B_{12}, the N^5-methyltetrahydrofolate methyltransferase activity of the cell extracts was elevated. We have carried out similar experiments and can extend the observations of these authors to diploid human cells. Further, in agreement with the recent full-length report from the same laboratory (20), we find it is the hydroxy-B_{12} which brings about this stimulation either in the presence of methionine, of homocystine, or a combination of the two. Homocystine, by itself, or in combination with methionine, does not stimulate. The pattern of response was the same whether the cell-free extracts were assayed either unsupplemented or supplemented *in vitro* with methyl-B_{12} or cyano-B_{12}.[4]

As hydroxy-B_{12} was raised in the growth medium, the methyltransferase activity of control cells increased and dependence upon *in vitro* addition of methyl-B_{12} decreased (Fig. 2). Thus, control cells grown in the basal medium yielded enzyme which was stimulated 100% by methyl-B_{12} supplementation whereas cells grown with 10 μg/ml hydroxy-B_{12} yielded

[4] For data illustrating the effect of hydroxy-B_{12} in the growth medium upon the activity of N^5-methyltetrahydrofolate methyltransferase order document NAPS (01172) (see footnote 3).

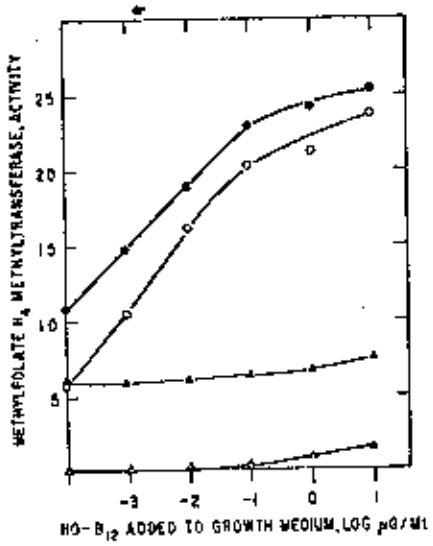

FIG. 2. Effect of hydroxy-B_{12} added to growth medium upon N^5-methyltetrahydrofolate methyltransferase activity of extracts prepared from control cells and cells from E. M. Cells were grown for 5 days in either basal medium containing 0.5–1.0 × 10^{-4} μg/ml B_{12} (points on ordinate) or basal medium to which hydroxy-B_{12} had been added as indicated. Extracts were prepared and assayed as usual either in the absence of added B_{12} (open symbols) or in the presence of methyl-B_{12}, 13.6 μg (closed symbols). All assay mixtures contained S-adenosylmethionine, 90 nmoles. Extracts of cells from control subject Jo. St., ○ and ●; extracts of cells from E. M., ▲ and △.

enzyme which was stimulated only 7%. Cells from E. M. were different in that (a) there was little stimulation of maximal enzyme activity by hydroxy-B_{12} in the growth medium, and (b) even with 10 μg/ml hydroxy-B_{12} in the growth medium, the methyltransferase activity of the cell-free extracts was largely dependent upon *in vitro* supplementation with methyl-B_{12} (although there was a small measurable activity without such supplementation).

The possibility that the decreased dependence of methyltransferase activity upon *in vitro* B_{12} supplementation as hydroxy-B_{12} was raised in the growth medium was due to carry-over of B_{12} to the gel-filtered extracts used for assay was shown to be unlikely because: (a) The total B_{12} content of the gel-filtered extracts prepared from cell growth with hydroxy-B_{12}, 1 μg/ml, was such that the amount added to the reaction mixtures would have been less than 0.005 μg; (b) twice this amount of cyano-B_{12} caused no measurable stimulation of methyltransferase activity in extracts from control cells or cells from E. M.

Cells from a number of control subjects and from cystathionine

synthase-deficient subjects were grown in basal medium and in the same medium with hydroxy-B_{12}, 1 μg/ml, added. The methyltransferase of all such cell lines tested had properties similar to those already described (Table 3). Extracts from cells grown in basal medium were stimulated 2-fold by *in vitro* addition of methyl-B_{12}. Growth in medium containing 1 μg/ml hydroxy-B_{12} caused a several-fold increase in enzyme activity, but there was far less dependence upon B_{12} supplementation in the reaction mixture. The methyltransferase activities of extracts of cells from cystathionine synthase-deficient individuals did not differ significantly from those of control cells. Shown also in Table 3 are the cumulative results of methyltransferase assays on extracts of cells from E. M. The cells from E. M. were reproducibly different in the ways described above.

TABLE 3

N^5-METHYLTETRAHYDROFOLATE METHYLTRANSFERASE ACTIVITY IN CELL-FREE EXTRACTS OF CELLS FROM CYSTATHIONINE SYNTHASE-DEFICIENT SUBJECTS, CONTROL CELLS, AND CELLS FROM E. M.

		nmole methionine/mg protein/60 min		
Growth media[a]	Methyl-B_{12} added to assay	Control subjects	Cystathionine synthase-deficient subjects	Patient E. M.
Basal	−	4.0 ± 0.7 (4)	5.5 ± 1.6 (3)	0.02 ± 0.04 (6)
Basal	+	8.2 ± 1.0	9.3 ± 1.8	4.7 ± 0.9
Basal + 1.0 μg/ml hydroxy-B_{12}	−	15.7 ± 2.0 (9)	17.8 ± 0.4 (3)	0.9 ± 0.6 (3)
Basal + 1.0 μg/ml hydroxy-B_{12}	+	17.3 ± 6.0	21.7 ± 1.9	6.4 ± 0.7

[a] Cells were grown in the media indicated. Cell-free extracts were prepared and subjected to gel filtration. In each case, the extract was assayed with either no added B_{12} or 13.6 μg methyl-B_{12} added to the assay reaction mixture. Each value is a mean ± SD. The numbers of control subjects and cystathionine synthase-deficient subjects are indicated in parentheses. The number of cell preparations from E. M. are in parentheses. The control subjects included five normal volunteers, two individuals with metabolic diseases not known to involve amino acid metabolism, and two relatives of such individuals.

N^5-Methyltetrahydrofolate Methyltransferase Activity in Extracts of Control Cells and Cells from E. M. Supplemented with Cyano-B_{12}. In an early series of experiments, N^5-methyltetrahydrofolate methyltransferase was assayed in gel-filtered extracts prepared from cells of 14 control subjects and from cells of E. M. The extracts were supplemented with cyano-B_{12}, 13.6 μg, and S-adenosylmethionine, 10 nmole. The values for the control extracts ranged from 3.2 to 6.2 nmole methionine/mg protein/60 min (mean ± 1 SD = 4.45 ± 0.80). An extract of cells from L. A.

had a specific activity of 6.5. The mean of several determinations with E. M. extracts was 3.1. Thus, when assayed in this manner, the extracts from cells of E. M. had a specific activity lower than the lowest control value and significantly lower than the control mean ($p < .05$ by the one-tailed probability test (21)). These results confirm under slightly different conditions and in a large series, the evidence in Table 3 that extracts of cells of E. M., grown in basal medium, contain a specific activity of N^5-methyltetrahydrofolate methyltransferase somewhat below normal when the extract is assayed with B_{12} supplementation.

Stability of N^5-Methyltetrahydrofolate Methyltransferase Activity in Cell-Free Extracts. The methyltransferase activity in extracts of fibroblasts from control subjects or from E. M. was stable for at least several months during storage at —55° in a medium containing potassium phosphate, pH 7.5, 0.1 M, and reduced glutathionine, 10^{-3} M.

Cystathionine Synthase and Methionine-Activating Enzyme Activities in Extracts of Fibroblasts from E. M. Extracts of cells from E. M. had a specific activity of cystathionine synthase of 23.6 nmole/mg protein/135 min, a value within our control range and far greater than values found in cells of patients with deficiency of this enzyme (22). This result confirms the studies of tissues of E. M. which showed that lack of activity of cystathionine synthase could not explain the homocystinuria found in this patient. Methionine-activating enzyme activity (ATP: L-methionine S-adenosyltransferase, EC 2.5.1.6) in extracts of E. M. cells was 2.5 nmole S-adenosylmethionine/mg protein/30 min. This value is also within the normal control range (22) and further indicates the specificity of the abnormalities in E. M.

Propionate Metabolism

Methylmalonyl-CoA isomerase is the other mammalian enzyme known to require a B_{12} derivative as coenzyme. In this instance, deoxyadenosyl-B_{12} is the active compound. Methylmalonyl-CoA isomerase catalyzes a step in the pathway by which the carbon of propionate enters the Krebs cycle and is metabolized to CO_2. As one means of evaluating propionate metabolism, cells from E. M. and control subjects were incubated with propionate-1-^{14}C. Evolution of $^{14}CO_2$ was measured (Table 4). Under our standard conditions $^{14}CO_2$ production from propionate-1-^{14}C was directly proportional to the number of cells added and to the time of incubation (Fig. 3). As shown in Table 4, cells from E. M., grown on the basal medium (containing 50–100 pg/ml B_{12}), produced $^{14}CO_2$ on the average only 11% as rapidly as control cells. To evaluate the activity of the Krebs cycle, $^{14}CO_2$ production from glutamate-U-^{14}C was also studied. The cells from E. M. were normal for this activity (Table 4).

TABLE 4

$^{14}CO_2$ PRODUCTION FROM PROPIONATE-1-^{14}C OR GLUTAMATE-U-^{14}C

	$^{14}CO_2$, count/min/10^6 cells/90 min		
	Hydroxy-B_{12} added to basal medium[a]		
	None	0.1 μg/ml	1.0 μg/ml
Propionate-1-^{14}C → $^{14}CO_2$			
Control subjects	2000 ± 890 (8)	2570 ± 1110 (4)	3740 ± 1770 (6)
Patient L. A.	210 ± 230 (2)	220 ± 270 (2)	—
Patient E. M.	210 ± 100 (7)	1610 ± 620 (6)	2170 ± 320 (6)
Glutamate-U-^{14}C → $^{14}CO_2$			
Control subjects	1110 ± 260 (4)	1260 ± 280 (4)	—
Patient L. A.	1960 (1)	1950 (1)	—
Patient E. M.	1290 (1)	1580 (1)	—

[a] Cells were grown for 5 days with hydroxy-B_{12} added to the basal medium as indicated. All assays were carried out under the standard conditions. Each value is a mean ± 1 SD. The number of control subjects are indicated in parentheses. One of the control subjects was cystathionine synthase-deficient. The number of cell preparations for L. A. and E. M. are in parentheses.

When relatively high concentrations of hydroxy-B_{12} were added to the medium in which the cells were grown, there was a moderate stimulation of $^{14}CO_2$ production from propionate-1-^{14}C by control cells (mean increases of 1.3- and 1.9-fold with hydroxy-B_{12} at 0.1 and 1.0 μg/ml, respectively). The cells from E. M. were stimulated much more by these concen-

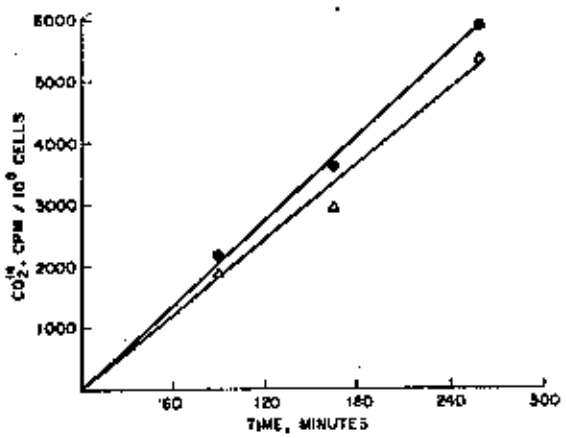

FIG. 3. Time course of $^{14}CO_2$ production from propionate-1-^{14}C. Incubations were carried out for various times under standard conditions with 6.3 × 10^6 cells from control subject Jo. Me. No succinate added, ●; sodium succinate, 7.9 μmoles, added, △.

trations of hydroxy-B_{12} (7.7- and 10.3-fold stimulations) and, under these conditions, produced $^{14}CO_2$ at rates within the control ranges. Glutamate-U-^{14}C conversion $^{14}CO_2$ was not markedly affected by the presence of hydroxy-B_{12} in the growth medium for either control cells or cells from E. M.

Cells from L. A., a patient with a probable defective methylmalonyl-CoA isomerase apoenzyme (4, 5) were similar to those from E. M. in their slow rate of propionate-1-^{14}C oxidation when grown upon basal medium. The oxidation rate for these cells, however was not restored to normal when hydroxy-B_{12} was added to the growth medium.

Intermediates Formed from Propionate-1-^{14}C. To define more precisely the reason the cells from E. M. were abnormally slow in converting propionate-1-^{14}C to $^{14}CO_2$, these cells were incubated with propionate-1-^{14}C, and the major radioactive compounds accumulating during the incubation were identified. For comparison, cells from L. A. and from two control patients (Ja. Pe. and St. Wi.) were studied similarly (Table 5). The control cells accumulate glutamate, aspartate, and lactate as major radioactive metabolites. Smaller amounts of radioactivity were found in succinate and only traces in methylmalonate. These results agree with those reported by Morrow *et al.* (5) in their studies of control cell lines. In addition, we noted an unidentified metabolite (A in Table 5) which moved with methylmalonate in solvent 1, and with an $R_{succinate}$= 2.85 in solvent 2.

TABLE 5

RADIOACTIVITY IN CO_2 AND INTERMEDIATES UPON INCUBATION OF CELLS WITH PROPIONATE-1-^{14}C[a]

Compound	Radioactivity, count/min/10^6 cells			
	Ja. Pe.	St. Wi.	L. A.	E. M.
CO_2	12,000	2,700	650	1,100
Glutamate	1,420	339	31	44
Aspartate	448	296	0	48
Lactate	1,545	338	23	47
Succinate	94	18	0	0
Compound A	337	51	83	82
Methylmalonate	28	5	214	110
Compound B	18	0	221	410
Compound C	0	0	141	180

[a] The following numbers of cells were used (in millions): Ja. Pe., 30.2; St. Wi., 38.2; L. A., 37.9; E. M., 33.3. For each cell line, three separate incubations were performed, and the acidified suspensions were combined as described in Methods. For details of the means used to identify each compound, see the description in the text.

Cells from E. M. and L. A. each accumulated intermediates different from those found in the control cells, but similar to one another. Only traces of radioactivity were present in glutamate, aspartate, and lactate. No radioactivity was found in succinate, but substantial amounts were present in methylmalonic acid. For both E. M. and L. A., this radioactive material was unequivocally identified as methylmalonate by recrystallization to constant specific radioactivity. These results for L. A. are also in agreement with those of Morrow *et al.* (5). Cells from E. M. and L. A. each accumulated three further major radioactive metabolites. The radioactivity in Compound A was not very different from the amount of radioactivity found in this compound in the control incubations. Two metabolites, (Compounds B and C, Table 5), were found only in the incubations with cells from E. M. and L. A. Compound B, present in the aqueous phase after ether extraction, moved with an $R_{glutamate}$= 1.59 during paper chromatography with isopropyl alcohol, formic acid, water (70:10:20). Compound C was extracted into ether from acidic solution and moved with succinate in solvent 1 and $R_{lactate}$= 1.10 in solvent 2. No further attempts have been made to identify compounds A, B, or C.

Specificity of Hydroxy-B_{12} in Stimulating $^{14}CO_2$ Production from Propionate-1-^{14}C by Cells of E. M. Cells from E. M. were grown in the

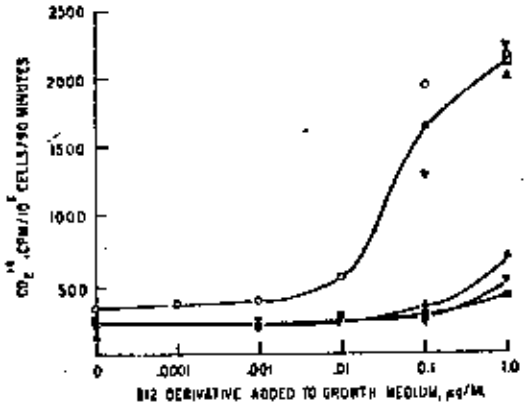

FIG. 4. Effect of B_{12} derivatives added to the growth medium upon $^{14}CO_2$ production from propionate-1-^{14}C catalyzed by cells from E. M. Cells were grown for 5 days in the dark with B_{12} derivatives added to the growth medium as indicated. Assays of $^{14}CO_2$ evolution from propionate-1-^{14}C were carried out under the standard conditions. The results of four separate experiments are plotted. Open symbols represent addition of hydroxy-B_{12}; closed symbols represent other B_{12} analogs. Expt. A: hydroxy-B_{12}, ○; Expt. B: deoxyadenosyl-B_{12}, ▼, and hydroxy-B_{12}, ▽; Expt. C: methyl-B_{12}, ■ and hydroxy-B_{12}, □; Expt. D: cyano-B_{12}, ▲, and hydroxy-B_{12}, △.

presence of several B_{12} derivatives to test the effectiveness of these compounds in restoration of propionate oxidation (Fig. 4). In each experiment the cells were responsive to hydroxy-B_{12}, but neither deoxyadenosyl-B_{12}, methyl-B_{12}, nor cyano-B_{12} caused more than a slight stimulation. No attempt was made to rule out the possibility that these slight effects were due to traces of hydroxy-B_{12} present in, or arising as a breakdown product of, the other analogs.

Effect of Time and Concentration on Hydroxy-B_{12} Stimulation. The effect of a given concentration of hydroxy-B_{12} was dependent upon the time during which the cells were grown in its presence (Fig. 5). In one experiment (not shown), cells from E. M. were grown for 5 days in the presence of media containing concentrations of hydroxy-B_{12} from 0.001–0.1 μg/ml. The growth media were changed daily, instead of after 4 days, as in the standard experiment. The response was as usual. The more frequent changes of media did not lead to any enhancement of the stimulation due to hydroxy-B_{12}. In another experiment hydroxy-B_{12}, added directly to the medium for assay of $^{14}CO_2$ production, at a final concentration of 0.01 μg/ml, was without effect.

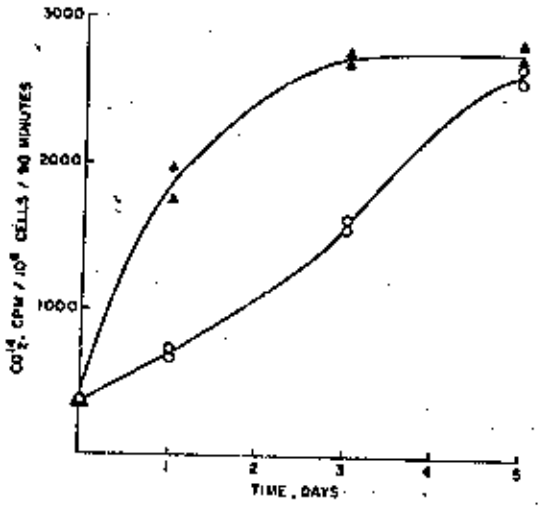

FIG. 5. Effect of time and concentration of hydroxy-B_{12} added to growth medium upon $^{14}CO_2$ production from propionate-1-^{14}C catalyzed by cells from E. M. A large sample of cells was divided into aliquots, each of which was grown for 5 days until harvested for assay. Hydroxy-B_{12} was added either 5 days, 3 days, or 1 day before harvest. The time of exposure to hydroxy-B_{12} is plotted. The "zero time" cells were grown for 5 days in basal medium without added B_{12}. Hydroxy-B_{12}: 0.1 μg/ml, ○; 1.0 μg/ml, ▲. Duplicate determinations for each cell preparation are shown.

DISCUSSION

The studies reported here establish that cells cultured from the skin of patient E. M. differ from control cells in two areas:

Homocysteine Metabolism. Cell-free extracts of cells from E. M. were able to metabolize homocysteine normally to cystathionine. However, the E. M. cells were not able to convert homocysteine to methionine at a normal rate. If methionine in the growth medium was replaced by homocystine, cells from E. M. did not grow. Control human diploid fibroblasts lines grew normally under these conditions.[5] Cells from E. M. grew slowly, however, if the B_{12} in the medium was raised by about four orders of magnitude.

Normal mammalian liver can methylate homocysteine to form methionine by the action of at least two enzymes: (a) betaine-homocysteine methyltransferase (EC 2.1.1.5), and (b) N^5-methyltetrahydrofolate methyltransferase. The physiologic contributions of these two enzymes in liver remain to be finally evaluted. Two lines of evidence make it clear that diploid human fibroblasts utilize only N^5-methyltetrahydrofolate methyltransferase: (a) choline (the source of betaine) could be removed from the homocystine medium without affecting the growth of control cells, and (b) no betaine–homocysteine methyltransferase activity was detected in extracts of control cells. The failure of cells from E. M. to grow on a medium in which they were required to form methionine from homocystine is explained by the observation that gel-filtered extracts from these cells grown on basal medium have little or no N^5-methyltetrahydrofolate methyltransferase activity unless B_{12} is added *in vitro*. In contrast, extracts from control cell lines assayed in the absence of added B_{12} have substantial activities of this enzyme. Thus control cells possess appreciable holoenzyme which contains a B_{12} coenzyme not removed by gel-filtration. Cells from E. M. possess no similar holoenzyme, although apoenzyme is present as shown by the activity when extracts were supplemented with B_{12}. The ability of E. M. cells to grow slowly on a homocystine medium supplemented with hydroxy-B_{12} correlates with the

[5] The control cells did not require added B_{12} to grow upon the homocystine-containing medium. This result differs from that obtained by Mangum and North (17) with established mammalian cell lines. Growth of these lines was dependent upon addition of hydroxy-B_{12} to a medium in which methionine had been replaced by homocysteine. This discrepancy may result from differences in the B_{12} content of the unsupplemented basal media or from the fact that established lines grow more rapidly than diploid lines and might be expected to have a greater methionine requirement which could not be satisfied by the relatively low concentration of N^5-methyltetrahydrofolate methyltransferase in the established lines grown without added B_{12}.

appearance of small amounts of methyltransferase holoenzyme when the cells were grown in the presence of hydroxy-B_{12} (Fig. 2 and Tables 1 and 3).

More detailed interpretation of the present results is difficult because the N^5-methyltetrahydrofolate methyltransferase of mammalian cells has not been studied as extensively as the *E. coli* enzyme. Available evidence suggests that the role of B_{12} in the two systems is similar, possibly identical, so we may examine our results in the light of what is known about the bacterial enzyme. As usually purified, enzyme from *E. coli* grown on cyano-B_{12} contains a bound B_{12}-derivative which, after separation from the protein by alcohol extraction, appears to be sulfito-B_{12} (23). To activate the enzyme, this bound B_{12} is methylated by S-adenosylmethionine. Thereafter, enzyme-bound methyl-B_{12} functions as a transient intermediate with the B_{12} cycling between methylated and unmethylated forms. The latter receives methyl groups predominantly from N^5-methyltetrahydrofolate (24, 25). By cautious isolation procedures, the enzyme may be isolated with its B_{12} largely methylated (26). Deoxyadenosyl-B_{12} is not itself a coenzyme for the *E. coli* methyltransferase (19, 27, 28).

The *in vitro* effect of B_{12} derivatives upon N^5-methyltetrahydrofolate methyltransferase of *E. coli* depends upon (a) whether the enzyme is initially in the apoenzyme or holoenzyme form, (b) whether the assay is performed aerobically or anaerobically, and (c) the structure of the B_{12} compound. Holoenzyme, obtained from bacteria grown on media containing cyano-B_{12}, has no need for B_{12} if assayed under strictly anaerobic conditions. If assayed aerobically, holoenzyme requires a B_{12} derivative for maximum activity, but only to satisfy the need for anaerobiosis. Many B_{12} compounds fulfill this function (28) which resembles that first described by Peel (29). The methyltransferase of pig, chicken, and rat liver extracts also showed a dependence upon B_{12} compounds (cyano-, methyl-, or deoxyadenosyl-) if assayed aerobically, but no dependence if assayed anaerobically (19). The bacterial apoenzyme, obtained after growth on media not supplemented with B_{12}, has a B_{12} requirement even when assayed anaerobically. The B_{12} requirement is then relatively specific. Methyl-B_{12} is most effective. Cyano-B_{12} is partially effective under conditions where reduction of the vitamin may occur (19, 27). Deoxyadenosyl-B_{12} itself is inactive (19, 27).

The ability of B_{12} derivatives to promote conversion of apoenzyme to holoenzyme reflects these effects on activity. Methyl-B_{12} is most effective and is active in the absence of a reducing system (28, 30–32). Cyano-B_{12} or hydroxy-B_{12} requires a reducing system for activity (19, 30, 31, 33). Deoxyadenosyl-B_{12} is active only in the presence of another enzyme, "holoenzyme synthetase," and a reducing system (31, 34).

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By analogy with the *E. coli* system, the N^5-methyltetrahydrofolate methyltransferase of control human fibroblasts grown on our basal medium behaves as though it were about 60–65% in the holoenzyme form.[6] The stimulation caused by addition of methyl-B_{12} to the control extracts indicates the presence of apoenzyme which is converted by methyl-B_{12} to holoenzyme. The relatively small stimulation caused by deoxyadenosyl-B_{12} suggests this is not a nonspecific effect on preformed holoenzyme due to attainment of strict anaerobiosis. If control cells are grown in the presence of high concentrations of hydroxy-B_{12}, the extracts are less stimulated by the *in vitro* methyl-B_{12}, as though a higher portion of the total enzyme were present in holoenzyme form.

The extracts from cells of E. M. were completely dependent upon addition of methyl-B_{12} for activity, as are extracts of *E. coli* containing only apoenzyme. Similarly, for both E. M. extracts and bacterial apoenzyme, cyano-B_{12} restores partial activity, and deoxyadenosyl-B_{12} is not only inactive, but actually inhibits methyl-B_{12}-promoted activity (27). Growth of the fibroblasts from E. M. in the presence of high concentrations of hydroxy-B_{12} led to formation of a minimal amount of holoenzyme. The major portion of the methyltransferase was still present as apoenzyme.

The cells from E. M. differed from control cells not only by having a greater portion of their methyltransferase as apoenzyme, but also by containing less total methyltransferase (i.e., activity measured in the presence of methyl-B_{12}). Whether grown on basal or B_{12}-supplemented media, the cells from E. M. had only one-third to one-half the mean control specific activity of total methyltransferase.[7]

The combined results from the studies of N^5-methyltetrahydrofolate methyltransferase suggest that cells from E. M. are unable to efficiently

[6] The results obtained under our assay conditions are most validly compared to the anaerobic experiments described in the literature. Absolute anaerobiosis was probably not achieved in our experiments, but the high concentrations of 2-mercaptoethanol used would have led to some degree of anaerobiosis, even if the assays had been carried out under air (28). In fact, the assays were performed after thorough gassing with N_2.

[7] There is a possible alternative explanation of these observations. If, under our assay conditions, holoenzyme formation from apoenzyme and methyl-B_{12} (or cyano-B_{12}) were rate-limiting, then the low total activity in extracts from E. M. cells compared to control cells could be due to incomplete conversion of the greater amount of apoenzyme in the former extracts. This possibility is unlikely because formation of holoenzyme from bacterial apoenzyme in the presence of methyl-B_{12} is not stimulated by "holoenzyme synthetase" (34) and is complete within a few minutes (31). The linear time course of methionine formation by extracts of E. M. cells supplemented with methyl-B_{12} also suggests that holoenzyme formation is not rate-limiting under our conditions.

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utilize B_{12} in the growth medium to promote accumulation of the B_{12}-containing coenzyme required for methyltransferase activity. Thus, they do not form a normal portion of holoenzyme. The deficient methyltransferase activity in extracts from cells of E. M. is due to a lack of a factor other than deoxyadenosyl-B_{12} since this compound itself is completely ineffective in stimulating activity under our assay conditions. The increase in total methyltransferase which occurs in control cells grown with high concentrations of hydroxy-B_{12} in the medium, but to a much lesser extent with cells from E. M., suggests that methyltransferase accumulates due to a B_{12} derivative present in control cells, but not in cells from E. M. The methyltransferase-B_{12}-coenzyme would be a reasonable agent to promote such accumulation, perhaps by stabilization of enzyme protein as holoenzyme,[8] or perhaps by other mechanisms. The accumulation of methyltransferase under these conditions in many respects resembles the pyridoxine-promoted rise in hepatic tyrosine transaminase activity (35) which is probably due to increased synthesis of enzyme protein (36).

Propionate Metabolism. Cells from E. M. are unable to convert propionate-^{14}C to $^{14}CO_2$ at a normal rate. The reason for this abnormally slow conversion was pinpointed by the findings that, whereas control cells accumulate substantial amounts of radioactivity originating in propionate in the forms of aspartate, glutamate, succinate, and lactate, the cells from E. M. do not do so. Rather than such compounds, the cells from E. M. accumulate unusually large amounts of radioactivity in methylmalonic acid.[9] The cells from E. M. thus form methylmalonate, but are unable to further metabolize this compound to succinate. Normal mammalian cells metabolize propionate sequentially to propionyl-CoA, D-methylmalonyl-CoA, L-methylmalonyl-CoA, and then to succinyl-CoA. These experimental results with cells from E. M. are compatible with a block in either methylmalonyl-CoA racemase (EC 5.1.99.1), the enzyme which catalyzes the conversion of the D to the L form of methylmalonyl-CoA, or in methylmalonyl-CoA isomerase, the deoxyadenosyl-B_{12}-dependent enzyme which completes the conversion to succinyl-CoA. The latter step is implicated as the site of the block in cells from E. M. by the experiments in which cells were grown in the presence of unusually high concentrations of hydroxy-B_{12} in the medium. Propionate metabolism was stimulated to rates within the control range. The cells from E. M. behave as though they are unable to utilize the low concentration of B_{12}

[8] The *E. coli* apoenzyme is strikingly unstable compared to its holoenzyme (33).

[9] The accumulated compound may have been methylmalonyl-CoA rather than methylmalonate itself. Coenzyme A derivatives would have been hydrolyzed by the prolonged exposure to strong acid used in processing the cell extracts in the experiments described.

in the basal medium to accumulate deoxyadenosyl-B_{12} sufficient for normal methylmalonyl-CoA isomerase activity. Only when the B_{12} in the medium is raised three to four orders of magnitude do the cells from E. M. accumulate enough deoxyadenosyl-B_{12} so that methylmalonyl-CoA isomerase activity is no longer severely rate-limiting in the conversion of propionate-1-^{14}C to $^{14}CO_2$.

In addition to accumulating abnormal amounts of radioactivity in methylmalonate, the cells from E. M. accumulated excessive radioactivity in two unidentified compounds, (called here B and C). Cells from L. A., which are similar to those from E. M. in being unable to convert methylmalonyl-CoA to succinyl-CoA at a normal rate, also accumulated compounds B and C. We suggest that these compounds are formed directly or indirectly from methylmalonyl-CoA and accumulate when the normal pathway for disposal of this compound is inoperative.

Together, the results of studies of both propionate metabolism and homocysteine metabolism in cells of E. M. indicate that these cells are unable to utilize with normal efficiency the B_{12} in the medium to foster accumulation of coenzyme derivatives of B_{12}. Accumulation of both deoxyadenosyl-B_{12} (the coenzyme for methylmalonyl-CoA isomerase) and of the coenzyme for N^5-methyltetrahydrofolate methyltransferase (methyl-B_{12} or a closely related reduced form of B_{12}) appear to be defective. Growth of the cells from E. M. in the presence of hydroxy-B_{12} restores the ability of these cells to convert propionate-1-^{14}C to $^{14}CO_2$ at a rate within the control range. Yet the methyltransferase activity of extracts of such cells is still far below control levels. Does this indicate that under such conditions the cells accumulate normal amounts of deoxyadenosyl-B_{12}, but not of the methyltransferase-B_{12}-coenzyme? Not necessarily. Conversion of propionate-1-^{14}C to $^{14}CO_2$ requires many separate enzymic steps, and it is unlikely that methylmalonyl-CoA isomerase is severely rate-limiting in control cells, since these cells do not accumulate appreciable radioactivity in methylmalonate. Thus, restoration of methylmalonyl-CoA isomerase activity to only a few percent of normal may restore normal rates of the overall conversion of propionate-1-^{14}C to $^{14}CO_2$. This may be what occurs when cells from E. M. are grown with hydroxy-B_{12}.

Experiments are now in progress to test directly the hypothesis that cells of E. M. do not accumulate normal concentrations of the coenzyme derivatives of B_{12}. If this hypothesis is correct, there are several possible explanations: (a) The cells of E. M. could have a failure in cellular uptake of B_{12}. If so, the failure of uptake must be due to an abnormality of the cells themselves rather than extracellular factors since in tissue culture the cells from E. M. and from control subjects respond differently

during growth in identical media. (b) There may be one or more metabolic steps which are shared in common in the conversion of vitamin forms of B_{12} to the two known coenzyme forms. For example, the recent work of Walker *et al.* (37) indicates that deoxyadenosyl-B_{12} is formed from hydroxy-B_{12} by two successive reductive steps which are followed by an adenosyl transfer from ATP to B_{12s} (the doubly-reduced B_{12} derivative). These steps are each catalyzed (in *Clostridium tetanomorphum*) by separate enzymes (37). It is generally agreed that, to convert cyano-B_{12} or hydroxy-B_{12} to the coenzyme for the methyltransferase, reduction of the cobamide moiety is required (30, 31, 33). It may be in such a step that cells from E. M. are blocked. (c) Finally, there may be excessive degradation of alkyl-B_{12} derivatives in cells of E. M. Fibroblasts provide an excellent experimental system in which to distinguish between these possible abnormalities. Such work is now in progress.

The specificity of hydroxy-B_{12} in restoring propionate oxidation by cells of E. M. may have therapeutic implications. The failure of deoxyadenosyl-B_{12} to restore activity strongly suggests that this B_{12} derivative fails to reach its intracellular site of action. Although other cells may behave differently, this finding offers no positive indication that deoxyadenosyl-B_{12} might be more efficacious therapeutically than is hydroxy-B_{12}.[10] Similarly, cyano-B_{12} was far less effective in restoring propionate oxidation by the fibroblasts of E. M. than was hydroxy-B_{12}. Before his death, E. M. was treated briefly with cyano-B_{12} without striking beneficial effect. The present evidence suggests that hydroxy-B_{12} might have been more helpful. As patients similar to E. M. are discovered, it will be of interest to ascertain whether a correlation can be established between clinical benefit from various B_{12} analogs and the effectiveness of these analogs in restoration of missing activities to fibroblasts cultured from these patients.

SUMMARY

Studies are reported of fibroblasts cultured from the skin of a patient with abnormalities of sulfur amino acid and of methylmalonic acid metabolism. These cells are unable to grow as do normal cells if methio-

[10] Lindblad *et al.* (38) recently demonstrated that large doses of deoxyadenosyl-B_{12} administered to two patients with methylmalonic acidemia decreased the amounts of methylmalonate in serum and urine after valine loads. These authors neither explored experimentally, nor speculated upon, the comparative efficiences of deoxyadenosyl-B_{12}, hydroxy-B_{12}, or other B_{12} derivatives in bringing about such effects. Rosenberg has stated (39) that his methylmalonic acidemic patient responded to deoxyadenosyl-B_{12} exactly as he did to vitamin B_{12}.

nine in the medium is replaced by homocystine. Addition of hydroxy-B_{12}, 1 μg/ml, to the medium allowed the cells of the patient to grow slowly. Extracts of the cells contained a normal concentration of cystathionine synthase, but an abnormally low concentration of holoenzyme for N^5-methyltetrahydrofolate methyltransferase activity. N^5-Methyltetrahydrofolate methyltransferase apoenzyme was present, as indicated by assay in the presence of appropriate B_{12}-containing compounds. Growth of control cells in media containing hydroxy-B_{12}, 1 μg/ml, led to a several-fold increase in total methyltransferase activity and a decrease of dependence of activity upon *in vitro* supplementation with B_{12}-containing compounds. Growth of the patient's cells under these conditions led to formation of a small, but detectable, amount of holoenzyme. However, the major part of the methyltransferase activity in extracts of his cells grown on hydroxy-B_{12} remained dependent upon *in vitro* addition of a B_{12}-containing compound.

The patient's cells converted propionate-1-^{14}C to $^{14}CO_2$ at a slow rate compared to control cells. During incubation with propionate-1-^{14}C his cells accumulated abnormally great amounts of methylmalonate-^{14}C but failed to form the usual amounts of radioactive succinate, lactate, glutamate, or aspartate. Growth of the patient's cells with supplemental hydroxy-B_{12} in the medium partially restored the rate at which these cells converted propionate-1-^{14}C to $^{14}CO_2$. The specificity of this restorative effect as well as the effects of variation in concentration and time of exposure to hydroxy-B_{12} are reported.

All the available data are consistent with the hypothesis that cells from the patient are deficient in their ability to accumulate coenzymatically active derivatives of B_{12} with resultant deficits in the activities of N^5-methyltetrahydrofolate methyltransferase and methylmalonyl-CoA isomerase, the two enzymes known in mammals to depend upon B_{12} derivatives.

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REFERENCES

1. Mudd, S. H., Levy, H. L., and Abeles, R. H., *Biochem. Biophys. Res. Commun.* 35, 121 (1969).
2. Levy, H. L., Mudd, S. H., Schulman, J. D., Dreyfus, P. M., and Abeles, R. H., *Amer. J. Med.* 48, 390 (1970).
3. Mudd, S. H., Levy, H. L., and Morrow, G., III., *Biochem. Med.* 241,

4. Morrow, C., III., Barness, L. A., Cardinale, G. J., Abeles, R. H., and Flaks, J. G., *Proc. Nat. Acad. Sci. U. S. A.* 63, 191 (1969).
5. Morrow, G., III., Meldman, W. J., Barness, L. A., and Dimitrov, N. V., *Pediat. Res.* 3, 217 (1969).
6. Uhlendorf, B. W., Holtz, A. J., Mock, M. B., and Fredrickson, D. S., *in* "Inborn Disorders of Sphingolipid Metabolism," (S. M. Aronson and B. W. Volk, eds.), p. 443. Macmillan (Pergamon) New York, 1967.
7. Eagle, H., *Science* 130, 432 (1959).
8. Dulbecco, R., and Vogt, M., *J. Exp. Med.* 99, 167 (1954).
9. Mudd, S. H., Finkelstein, J. D., Irreverre, F., and Laster, L., *J. Biol. Chem.* 240, 4382 (1965).
10. Lau, K. S., Gottlieb, C., Wasserman, L. R., and Herbert, V., *Blood* 26, 202 (1965).
11. Blass, J. P., Avigan, J., and Uhlendorf, B. W., *J. Clin. Invest.* 48, 423 (1970).
12. Hanks, J. H., and Wallace, R. E., *Proc. Soc. Exp. Biol. Med.* 71, 196 (1949).
13. Flavin, M., and Ochoa, S., *J. Biol. Chem.* 229, 965 (1957).
14. Smith, I., "Chromatographic and Electrophoretic Techniques," Vol. I, p. 279 Wiley (Interscience), New York, 1960.
15. Giovanelli, J., and Stumpf, P. K., *J. Biol. Chem.* 231, 411 (1958).
16. Gibbholzer, V. C., Levin, B., Burgess, E. A., and Young, W. F., *Arch. Dis. Childhood* 42, 492 (1967).
17. Mangum, J. H., and North, J. A., *Biochem. Biophys. Res. Commun.* 32, 105 (1968).
18. Mangum, J. H., and Schimgeour, K. G., *Fed. Proc.* 21, 242 (1962).
19. Weissbach, H., Peterkofsky, A., Redfield, B. G., and Dickerman, H., *J. Biol. Chem.* 283, 3318 (1963).
20. Mangum, J. H., Murray, B. K., and North, J. A., *Biochemistry* 8, 3496 (1969).
21. Fisher, R. A., "Statistical Methods for Research Workers," Oliver & Boyd, Edinburg, 1948.
22. Uhlendorf, B. W., and Mudd, S. H., *Science* 160, 1007 (1968).
23. Ertel, R., Brot, N., Taylor, R., and Weissbach, H., *Arch. Biochem. Biophys.* 126, 353 (1968).
24. Taylor, R. T., and Weissbach, H., *Arch. Biochem. Biophys.* 129, 728 (1968).
25. Taylor, R. T., and Weissbach, H., *Arch. Biochem. Biophys.* 129, 745 (1968).
26. Rüdiger, H., and Jaenicke, L., *Eur. J. Biochem.* 10, 557 (1969).
27. Weissbach, H., Redfield, B. G., and Dickerman, H., *J. Biol. Chem.* 239, 146 (1964).
28. Weissbach, H., Redfield, B., and Dickerman, H., *Biochem. Biophys. Res. Commun.* 17, 17 (1964).
29. Peel, J. L., *J. Biol. Chem.* 237, PC263 (1962).
30. Guest, J. R., Friedman, S., Woods, D. D., and Smith, E. L., *Nature London* 195, 340 (1962).
31. Weissbach, H., Redfield, B. G., Dickerman, H., and Brot, N., *J. Biol. Chem.* 240, 856 (1965).
32. Taylor, R. T., and Weissbach, H., *J. Biol. Chem.* 242, 1502 (1967).
33. Takeyama, S., Hatch, F. T., and Buchanan, J. M., *J. Biol. Chem.* 236, 1102 (1961).
34. Brot, N., and Weissbach, H., *J. Biol. Chem.* 241, 2024 (1966).
35. Greengard, O., and Gordon, M., *J. Biol. Chem.* 238, 3708 (1963).

1915

36. Holten, D., Wicks, W. D., and Kenney, F. T., *J. Biol. Chem.* **242**, 1053 (1967).
37. Walker, G. A., Murray, S., and Huennekens, F. M., *Arch. Biochem. Biophys.* **134**, 95 (1969).
38. Lindblad, B., Lindstrand, K., Svanberg, S., and Zetterström, R., *Acta Paediat. Scand.* **58**, 178 (1969).
39. Rosenberg, L. E., *N. Engl. J. Med.* **281**, 145 (1969).

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/ - Proc. Soc. Exptl. Bio. + Med. 108 (2) 337-340 (1961)

Effect of Vitamin B_{12}, Unsaturated Fat and Hydrolyzed Glucosecyclo-acetate on Bile Acid Excretion in Experimental Hyperlipemia.* (26931)

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Many workers have reported that cholesterol administered to animals is metabolized and eliminated mostly as bile acids(1-3). The effect of different food fats on elimination of the bile acids has also been studied. Haust and Beveridge(4) demonstrated an increased output of bile acids in the feces of human subjects receiving corn oil in the diet as compared to those receiving butter fat. Srikantia and Gopalan(5) have recently shown that fecal output of bile acids in human subjects receiving unsaturated fat is higher than in those receiving saturated fat. Recent work of Gordon *et al.*(6) supports the view that hypocholesterolemic effect of sunflower oil is associated with an increase in fecal output of bile acids. As the unsaturated fats are known to lower the higher cholesterol levels of the serum and tissues, it is reasonable to assume that unsaturated fat may lower serum

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cholesterol levels by promoting the fecal output of bile acids.

Nath and Saikia(7) have reported that high fat (saturated) given to rabbits for a long period, induces hypercholesterolemia as indicated by elevated cholesterol levels and C/P ratio whereas unsaturated fat (linseed oil) and the same level of mixed fat (saturated and unsaturated) were found to maintain lower ester cholesterol values and C/P ratio. Vit. B_{12}, essential fatty acids, inositol and aqueous extract of hydrolyzed glucose cycloacetoacetate (GCA) also checked the rise of cholesterol levels of plasma and tissues in experimental hyperlipemia(8).

The present work was, therefore, undertaken to study the effect of hydrolyzed GCA as well as Vit. B_{12} and unsaturated fat (groundnut oil) on excretion of fecal bile acids in animals with hyperlipemia induced by feeding high saturated fat diet.

Materials and methods. Thirty male albino rats weighing an average 125 g were divided into 5 equal groups. Animals of Group I were given basal diet consisting of sucrose 16%, casein 20%, groundnut oil 20%, whole meal wheat flour 39%, salt mixture(9) 5%. Group II received saturated fat diet containing same percentage of sucrose, casein, whole meal wheat flour and salt mixture as in basal diet, but groundnut oil (20%) was replaced by Dalda† (20%). Group III animals were given the same diet containing mixed fat 20% (10% Dalda + 10% groundnut oil) in place of 20% Dalda. Vitamin mixture consisting of Vit. B_1 .4 mg, Vit. B_2 .4 mg, folic acid .2 mg, pyridoxine .3 mg, niacin 3 mg, calcium pantothenate 2 mg, inositol 20 mg, and codliver oil (Vit. A & D) 10-15 drops were added/100 g of the diet. The animals were supplied with food and water *ad libitum*. Animals of Groups IV and V which received high saturated fat diet as did Group II, were injected subcutaneously with hydrolyzed product of GCA (120 mg) and 5 μg Vit. B_{12}‡ respectively/100 g body wt/day.

† Hydrogenated groundnut oil, Hindusthan Lever Product Private, Ltd.

‡ Macrabin, Glaxo Laboratories, India Private Ltd.

GCA was prepared according to the method of West(10) as modified by Nath *et al.* (11). Hydrolyzed GCA was prepared by hydrolyzing 1 g of GCA with 5 cc of 2N HCl for 20 minutes in a boiling water bath. The pH was then adjusted to 7.2 with 2N NaOH. The solution was filtered and filtrate repeatedly washed with ether. Aqueous layer was separated and traces of ether were removed. The experiment was continued for 8 weeks, after which the rats were caged separately for fecal collection. Feces were collected for 4 days and stored in cold.

Bile acids estimation. Since bile acids in feces are not in the conjugated form, the measurements here were made on free bile acids, *i.e.*, cholic, and dihydroxycholanic acids. The method for determination of these 2 bile acids was similar to that adopted by Haust and Beveridge(4) for measurement of cholic and dihydroxycholanic acids in feces.

Extraction of feces and column chromatography. Extraction procedure followed was similar to that of Carey and Watson(12) and final resulting mixtures were subjected to column partition chromatography as suggested by Mosbach *et al.*(13), using celite column which utilized 70% aq. acetic acid as the stationary phase and petroleum ether-isopropyl ether (IPE) mixture as moving phase. Elution was started with a mixture containing 40% IPE in petroleum ether and followed by gradual increase in IPE up to 60% in petroleum ether. IPE 40 and IPE 60 fractions were collected separately and evaporated to dryness and dissolved in 65% sulphuric acid (AR Grade) and heated at 60°C for 15 minutes. The 2 bile acids were determined spectrophotometrically according to the method of Mosbach *et al.*(14) by differential absorption at 320 mμ and 385 mμ for cholic and dihydroxycholanic (deoxy cholic and chenodeoxycholic acids, the 2 isomers) acids respectively with the Beckmann DU Quartz Spectrophotometer. Since IPE 40 and IPE 60 fractions behaved spectrophotometrically like dihydroxycholanic acid and cholic acid respectively, they were reported as dihydroxycholanic and cholic acids.

At the end of the feeding period, the ani-

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TABLE I. Values of Bile Acid Excretion in Feces and Total Plasma Cholesterol of Rats from Different Groups, with Standard Deviation of Mean Values (6 Rats/Group).

Exp. groups		Cholic acid	Dihydroxycholanic acid	Total plasma cholesterol, mg %
		mg/rat/24 hr		
I	Basal diet	.530 ± .023	.553 ± .014	121 ± 12
II	Saturated fat (hydrogenated) diet	.372 ± .028	.414 ± .034	205 ± 11
III	Mixed fat (hydrogenated fat + groundnut oil) diet	.536 ± .052	.543 ± .036	174 ± 9
IV	Hydrogenated fat diet and GCA(h) inj. (120 mg/100 g body wt)	.801 ± .036	.726 ± .057	138 ± 15
V	Hydrogenated fat diet and Vit. B_{12} inj. (5 μg/100 g body wt)	.760 ± .033	.648 ± .06	152 ± 14

mals were sacrificed and total cholesterol of plasma was estimated according to the method of Schoenheimer and Sperry(15) as modified by Mookerjee and Sadhu(16).

Results. Table I shows bile acid values (cholic and dihydroxycholanic acids) of the feces of the rats of different groups. Since total bile acids were not estimated, values of 2 bile acids obtained here do not account for the total fecal bile acids. Bergstrom and Norman(17) demonstrated the modification of cholic acid to compounds containing ketonic groups in the feces. But we obtained values for cholic and dihydroxycholanic acids in feces of rats, as Haust and Beveridge reported in human feces.

The animals receiving 20% hydrogenated fat excreted less bile acids than those receiving basal diet containing unsaturated fat and mixed fat containing 10% hydrogenated fat + 10% groundnut oil. Plasma cholesterol levels of saturated fat ingested animals are higher than those of animals receiving unsaturated fat and mixed fat. Hydrolyzed GCA and Vit. B_{12} when injected to the saturated fat ingested animals show pronounced effect on excretion of fecal bile acids. Plasma cholesterol levels of the animals of Groups IV and V injected with hydrolyzed GCA and Vit. B_{12} respectively, are also much lower than those receiving hydrogenated fat.

Discussion. From the above observations, it is noted that elimination of bile acids in feces is affected by the nature of the fat. The hydrogenated fat (20%) in absence of unsaturated fat appears to have increased plasma cholesterol levels through its diminishing effect on fecal elimination of bile acids. When equal quantities of saturated and unsaturated fats are given maintaining total dietary fat at 20%, bile acid excretion is increased, accompanied by fall in total plasma cholesterol level. This effect can be attributed to the unsaturated fat.

Gordon *et al.*(6) have reported an increase of fecal bile acid elimination with a concomitant fall in serum cholesterol levels when sunflower seed oil was substituted for coconut oil in the diet. Byers and Friedman(18) have also observed that bile obtained from the common bile duct was richer in cholic acid following the feeding of unsaturated fats than following saturated fats.

The observations of other workers, and our findings support the possibility that increased excretion of the end products of cholesterol as bile acids may be one of the possible mechanisms by which substances like Vit. B_{12}, hydrolyzed GCA and unsaturated fat bring about reduction in plasma cholesterol levels. The mechanism of inhibition of bile acid excretion in animals fed hydrogenated fat can be supported by the observation of Alfin-Slater *et al.*(19) that in the absence of essential fatty acids, there is marked increase in the deposition of cholesterol esters of fatty acid which are not available for normal metabolism.

Vit. B_{12} and hydrolyzed GCA which was found to contain dienol glucose by Nath and Bhattathiry(20) may, by stimulating the cholesterol metabolism, restore the normal metabolism of cholesterol which is hampered by saturated fat. Nath and Saikia(21) have suggested that hydrolyzed GCA and Vit. B_{12} through their property of increasing the con-

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tent of methionine in the liver, aid the biosynthesis of choline which results in the increase of phospholipid, and thus may prevent the rise of C/P ratio and atherosclerosis. Besides this, hydrolyzed GCA and Vit. B_{12} may be helpful in cholesterol metabolism.

Summary. The effect of Vit. B_{12}, unsaturated fat and hydrolyzed glucose cycloacetoacetate (GCA) on total plasma cholesterol levels and on excretion of fecal bile acids in rats with hyperlipemia induced by feeding saturated fat has been studied. These substances have been found to increase the output of fecal bile acids with a concomitant fall in total plasma cholesterol levels, thus indicating their important role in increasing the metabolism of cholesterol in the living system through its conversion to bile acids. Hydrolyzed GCA was found to be most effective in this process.

1. Byers, S. O., Biggs, M. W., *Arch. Biochem. Biophys.*, 1952, v39, 301.

2. Siperstein, M. D., Chaikoff, I. L., *Fed. Proc.*, 1955, v14, 767.

3. Siperstein, M. D., Jyako, M. E., Chaikoff, I. L., Dauben, W. G., PROC. SOC. EXP. BIOL. AND MED., 1952, v81, 720.

4. Haust, H. L., Beveridge, J. M. R., *Arch. Biochem. Biophys.*, 1958, v78, 367.

5. Srikantia, S. G., Gopalan, C., *Ind. J. Med. Res.*, 1960, v48, 503.

6. Gordon, H., Lewis, B., Eales, L., Brock, J. F., *Nature*, 1957, v180, 923.

7. Nath, M. C., Saikia, A., *Ind. J. Med. Res.*, 1958, v46, 579.

8. ———, *Arch. Biochem. Biophys.*, 1959, v84, 162.

9. Hawk, B. P., Oser, B. L., Summerson, W. H., *Practical Physiological Chemistry*, 13th ed., 1954, p1375. Blakiston.

10. West, E. S., *J. Biol. Chem.*, 1927, v74, 561.

11. Nath, M. C., Chitale, R. P., Belavady, B., *Nature*, 1952, v170, 545.

12. Carey, J. B., Jr., Watson, C. J., *J. Biol. Chem.*, 1955, v216, 847.

13. Mosbach, E. H., Zomely, C., Kendall, F. E., *Arch. Biochem. Biophys.*, 1954, v48, 95.

14. Mosbach, E. H., Kalinsky, H. J., Halpern, E., Kendall, F. E., *ibid.*, 1954, v51, 401.

15. Schoenheimer, R., Sperry, W. M., *J. Biol. Chem.*, 1934, v106, 745.

16. Mookerjea, S., Sadhu, D. P., *Indian J. Physiol. Allied Sci.*, 1955, v9, 55.

17. Bergstrom, S., Norman, A., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 71.

18. Byers, S. O., Friedman, M., *ibid.*, 1958, v98, 523.

19. Altin-Slater, R. B., Aftergood, L., Deuel, H. J., Jr., *ibid.*, 1954, v86, 872.

20. Nath, M. C., Bhattathiry, E. P. M., *Nature*, 1956, v178, 1233.

21. Nath, M. C., Saikia, A., *Arch. Biochem. Biophys.*, 1959, v79, 216.

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EFFECT OF DIFFERENT FATS AND ESSENTIAL FATTY ACIDS ON VITAMIN B_{12} LEVEL IN PLASMA AND LIVER

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The role of vitamin B_{12} in lipid metabolism has been assessed by many workers (1, 2). Drill and McCormick (3), Nath *et al.* (4) and Bennett *et al.* (5) showed that vitamin B_{12} is effective in preventing fatty livers. Reports of check in the rise of cholesterol in serum and tissues afforded by vitamin B_{12} is well established (6, 7). Deficiency of vitamin B_{12} in hypercholesterolemia was reported earlier (8). Fox *et al.* reported that the requirement for vitamin B_{12} increased when the diet contained high fat (9). Severity of vitamin B_{12} deficiency has also been shown to increase by increasing the fat content of the diets (10). Fat intermediate metabolites, ketone bodies, have recently been shown to cause depletion of vitamin B_{12} in blood and liver of rats (11). Further, saturated fats have been found to be more ketogenic than the unsaturated ones (12). In view of the above observations, it was thought worthwhile to study the effect of different kinds of fats, both saturated and unsaturated, on the vitamin B_{12} level of plasma and liver in rats.

Vitamin B_{12} seems to be associated with the metabolism of polyunsaturated fatty acids (13). Insufficiency of polyunsaturated fatty acids in the ration can be compensated by adding thiamine or vitamin B_{12} to a degree (14). It was therefore also contemplated to study the additional effect of essential fatty acids such as linoleic acid on the vitamin B_{12} levels when kept on saturated fat diet.

EXPERIMENTAL

Male albino rats were divided into five groups and were put in individual cages and given different fats at 35% level as indicated in Table 1. Group I, II, III, IV and V animals were fed hydrogenated groundnut oil, hydrogenated groundnut oil+linoleic acid, sesame oil, butter fat and coconut oil respectively. Each group of animals received the respective diet and water *ad libitum*. Care was taken to prevent coprophagy during the experiment. The experiment was carried out for 120 days and the growth was recorded every fortnight. At the end of the experimental period, the animals were sacrificed and vitamin B_{12} activity in liver and plasma was determined microbiologically with *Euglena gracilis var. bacillaris* as the test organism (15). The estimation of ketone bodies of liver was done by the method of Greenberg and Lester (16). All the estimations were done in the fed state.

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TABLE 1
Composition of Rations (g per cent)

Ingredients	Group I Hydrogenated fat	Group II Hydrogenated fat + linoleic acid	Group III Sesame oil	Group IV Butter fat	Group V Coconut oil
Sucrose	40	40	40	40	40
Casein (vitamin-free)	20	20	20	20	20
Salt mixture [a]	5	5	5	5	5
Hydrogenated groundnut oil [b]	35	33	—	—	—
Sesame oil	—	—	35	—	—
Butter	—	—	—	35	—
Coconut oil	—	—	—	—	35
Linoleic acid	—	2	—	—	—
Vitamins [c]					

[a] Hubbel, Mendel and Wakeman, 1937.
[b] Dalda, Hindustan Lever Private Ltd.
[c] Vitamins added per 100 g of diet: thiamine hydrochloride, 0.4 mg; riboflavin, 0.5 mg; pyridoxine hydrochloride, 0.4 mg; calcium pantothenate, 4 mg; niacin, 4 mg; inositol, 20 mg; biotin, 0.02 mg; folic acid, 0.2 mg; choline, 400 mg; vitamin B_{12}, 1 μg; vitamin A, 0.31 mg; vitamin D, 0.0045 mg; α-tocopherol, 5 mg.

RESULTS AND DISCUSSION

The gain in body weight in all groups is indicated in Fig. 1. The highest growth was recorded in the animals fed sesame oil, butter fat and hydrogenated fat supplemented with linoleic acid. However, hydrogenated fat and coconut oil-fed animals exhibited increase in growth rate till 45 days, after which the increase in

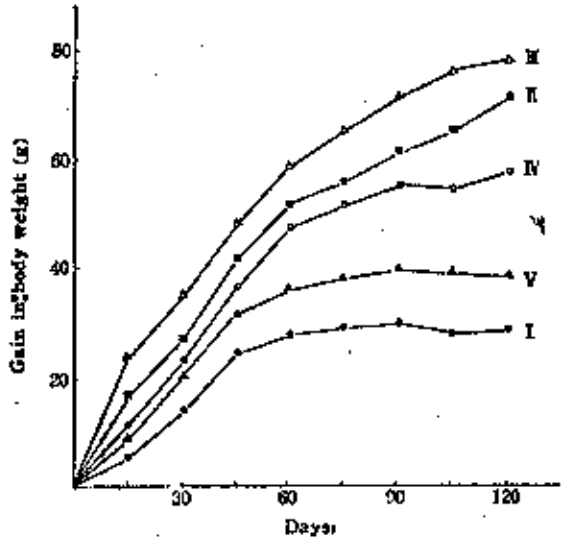

FIG. 1 *Growth Curve of Rats Fed Different Food Fats*
I, hydrogenated groundnut oil group; II, hydrogenated groundnut oil + linoleic acid group; III, sesame oil group; IV, butter fat group; V, coconut oil group.

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body weight was more or less negligible.

It is evident from Table 2, that at the end of four months liver ketone body level increased in all groups, fed high fat diet, except in group II where linoleic acid was supplemented. The saturated fats seemed to be more ketogenic than the unsaturated ones. It is also evident from Table 2 that plasma and liver vitamin B_{12} level of all high fat-fed groups show a significant decrease (P less than 0.01) as compared to group II, at the end of 120 days. This is in confirmation with the earlier report (13), which indicated steatosis of the liver and increased requirement of vitamin B_{12} of rats fed high fat diet (40 %). But our results have further shown that the effect of feeding saturated fats such as hydrogenated fat and coconut oil on the vitamin B_{12} status in liver and plasma were more pronounced than that of feeding the unsaturated fat such as sesame oil.

Butter fat, which is a saturated fat containing some unsaturated fatty acids (17), had comparatively shown a lesser decrease in vitamin B_{12} level of liver and plasma when compared to the other saturated fats. Also when linoleic acid was supplemented to the hydrogenated fat diet the animals had significantly higher levels of vitamin B_{12} in liver and plasma than those kept on the hydrogenated fat diet alone (P less than 0.01). These results indicate that the vitamin B_{12} level of liver and plasma is influenced by the amount of unsaturated fatty acids present in the fat.

The accumulation of ketone bodies in liver by high fat feeding for a long period, because of increased ketogenesis and decreased utilisation, has already been reported from this laboratory (18). Saturated fats have also been shown to be more ketogenic than the unsaturated ones (12). Recently ketone bodies have been found to increase the vitamin B_{12} requirement in rats (11). In the present investigation also the same relationship between vitamin B_{12} and ketone bodies is observed. The more ketogenic fat such as the saturated fat showed increased requirement of vitamin B_{12} than the lesser ketogenic fats, the unsaturated ones. The essential fatty acids seem to decrease the ketogenic potency of the fat and thus afford a protection to vitamin B_{12} level of liver and plasma.

TABLE 2

Effect of Different Fat Diets and Linoleic Acid on the Liver and Plasma Vitamin B_{12} Levels in Rats

Group	Liver		Plasma
	Ketone bodies	Vitamin B_{12}	Vitamin B_{12}
	mg/100 g	*mμg/g*	*mμg/ml*
I. Hydrogenated groundnut oil (10) [a]	7.0±0.09 [b]	79±14	0.31±0.18
II. Hydrogenated groundnut oil + linoleic acid (8)	2.9±0.37	139±11 [c]	0.53±0.09 [c]
III. Sesame oil (10)	3.4±0.21	110±11 [c]	0.39±0.07 [c]
IV. Butter fat (8)	4.6±0.33	100±12 [c]	0.37±0.10 [c]
V. Coconut oil (8)	6.8±0.24 [d]	88±8	0.30±0.12

[a] Figures in parenthesis shows the number of rats sacrificed. [b] Mean±S.E.
[c] Significantly increased from Group I at $P<0.01$.
[d] Significantly increased from Group III at $P<0.01$.

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SUMMARY

Effect of different high fat diets on vitamin B_{12} levels in plasma and liver in rats was studied, and the following findings were obtained.

1. High fat diet (35 %) produced vitamin B_{12} deficiency in rats after 120 day-feeding.

2. The effect of saturated fats on vitamin B_{12} levels in liver and plasma was more pronounced than that of the unsaturated ones.

3. Supplementation of linoleic acid in hydrogenated fat diet could improve the B_{12} status.

REFERENCES

1. Ling, C. T., and Chow, B. F., *J. Biol. Chem.* **206**, 797 (1954).
2. Yesair, D. W., Mooskar, D. E., and Daniel, L. J., *Arch. Biochem. Biophys.* **78**, 168 (1959).
3. Drill, V. A., and McCormick, H. M., *Proc. Soc. Exp. Biol. Med.* **72**, 388 (1949).
4. Nath, N., Yawalkar, V. D., and Nath, M. C., *Nature*, **208**, 1001 (1965).
5. Bennett, M. A., Joralemon, K., and Halpern, P. L., *J. Biol. Chem.* **193**, 285 (1951).
6. Chakrabarti, R. N., De, U. N., and Mukerji, B., *Ind. J. Med. Res.* **45**, 315 (1957).
7. Nath, M. C., and Saikia, A., *Arch. Biochem. Biophys.* **79**, 216 (1959).
8. Hsu, J. M., and Chow, B. F., *Federation Proc.* **16**, 63 (1957).
9. Fox, M. R. S., Ortiz, L. O., and Briggs, G. M., *Proc. Soc. Exp. Biol. Med.* **93**, 501 (1956).
10. Fox, M. R. S., Ortiz, L. O., and Briggs, G. M., *J. Nutrition* **68**, 371 (1959).
11. Nath, N., and Nath, M. C., *Proc. Soc. Exp. Biol. Med.* **124**, 210 (1967).
12. Brahmankar, D.M., and Nath, M. C., *Proc. Soc. Exp. Biol. Med.* **112**, 670 (1963).
13. Marcello, Martinelli, Lipidi, Aliment. Altivita 'Epathche, Atti, Conv. 2, Rimini, Italy, p. 39 (1961).
14. Pogorelko, M. A., Trudy-Oi (Pervoi). Nauch. Sessii Po Voprsam: Problemy Zhira V Pitani, Leningrad., p. 214 (1959).
15. Hoff-Jorgensen, E., In methods of Biochemical Analysis, Interscience Publisher Inc., N. Y., **1**, 81 (1954).
16. Greenberg, L. A., and Lester, D., *J. Biol. Chem.* **154**, 177 (1944).
17. Amberger, C., *Z. Untersuch. Nahr. Genussm.*, **35**, 313 (1918).
18. Khanade, J. M., and Nath, M. C., *Proc. Soc. Exp. Biol. Med.* **105**, 566 (1960).

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C. NAVA:

ALLERGIC REACTIONS TO VITAMINS B_{12}

The purpose of the study is: to describe some cases of allergy to vitamins B_{12}, observed in out-patient treated for allergies in our Clinic during the last five years (1966 to 1970). The investigation involved either subjects who had used vitamins B_{12} and/or streptomycins, or workers who came into contact with these substances for occupational reasons.

The research is justified by the fact that workers engaged in the extraction of streptomycin nutrient cultures (griseus, olivaceus, venezuelae,etc.) were exposed to both substance groups, and by the fact that in streptomycin preparations of fermentative origin, quantities of cyanobalamin were occasionally found to be present.

It was therefore proposed to determine how far the use of drugs, the presence of vitamins B_{12} as an impurity, and finally, exposure to these for reasons of work can be significant relatively to the occurrence of allergies.

Material and Methods.

On the following Table,I show (in synopsis) a description of the cases to which present investigation refers (see Table 1.).

For this purpose, 110 persons who had come into contact with vitamins B_{12} and stretomycin were subjected to control; from the above total, 42 subjects had reported disorders.

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The investigations consisted in intradermic tests with streptomycin and dihydrostreptomycyn sulfate (in physiologic solution: injected dose 0,02 ml, gamma 100), and with cobalamin and hydroxycobalamin: dose of 10 gamma, always to the amount of 0,02 ml. The test reading was performed 20', 24 and 48 hours after the inoculation.

The tolerance test, performed in subjects who had never come into contact with the above-mentioned products, consisted in the inoculation of 500 gamma streptomycin and 100 gamma vitamins B_{12} (without intolerance).

In cases with evidence of cutaneous hyperergy to vitamins B_{12}, subsequent control tests were performed, suitable for revealing eventual group sensitizations to parts of the molecule, to other components of the used products. In addition to the drugs cited earlier, intradermic tests were performed with the following substances, for which I include specifications and data on the injected volume:

1) commercial hepatic extract sol. 1 : 10, in physiologic : 0,02 ml;
2) extractive hepatic bovine extract sol. 1 : 10 in physiologic: 0,02 ml;
3) sieroalbumin of hogs, sol. 1 : 10 in phsyiologic: 0,02 ml;
4) bovine sieroalbumin sol. 1 : 10 in physiologic: 0,02 ml;
5) thiamine: gamma 100;
6) riboflavin: gamma 100;
7) nicotinamide: gamma 100.

TABLE 1. - Control tests performed in subjects exposed to contact with vit. B_{12} and streptomycin for occupational or therapeutic reasons.

Description	Number of cases	Positive reaction to B_{12} after 20'	Idem, to streptomycin
Group 1.: exposed for occupational reasons to vit.B_{12} and streptomycin			
a) no disorders	40	0	0
b) with disorders	10	1	7
Group 2.: exposed for therapeutic reasons to vit.B_{12} and/or streptomycins			
a) no disorders	28	0	1
b) with disorders	32	2	12
Totals	110	3	20

8) hyper-purified cobalamin: gamma 10;
9) hydroxycobalamin, hyper-purified : gamma 10;
10) coenzyme B_{12} (as benzilcobamid coenzyme): gamma 10;
11) pyridoxine: gamma 100;
12) cobalt chloride: 5.10^{-7} grams;
13) benzilic alcohol: gamma 75;
14) cytidine: gamma 75;
15) uridine: gamma 100.

In all cases the cutaneous reactivity was additionally checked with intradermics in a tamponed physiologic solution with pH 7,4, and (a physiologic solution) of bihydrochloric histamine.

RESULTS

Group 1.: workers engaged in the production of vitamins B_{12} and of streptomycins.

50 workers are involved, from some industries in Lombardia where the production is implemented with fermentation processes, using cultures of selected germs; the investigation was performed in particular in workers who had been exposed to strains of streptomices, used for the double production of vitamins and antibiotics. The workers came from various production sectors (fermentation, extraction, refining, packing) as well as from the chemical laboratories where the contents of the cultures are labelled as vitamin and streptomycin (for more details on processing reference is made to the respective literature) (BORENSZTAJN, 1954; FABIANI and ROUBERT, 1961).

The heterogeneous origin of the workers excluded the determination of the exposure to the substances in question; furthermore, in most industries, work in the production departments is alternated in cyles with the preparation of other antibiotics, and -- in chemical laboratories--with the performance of analyses involving other products.

As the Table shows (see above), 20 % of the tested workers reported disorders which included: disosmus bad taste in the mouth, pruritus on exposed skin locations, and widespread pyroses at the mucous orifices, as well as a sensation of excitement and insomnia.

Finally, the occurrence of blepharospasm, rhinorrhea and dyspnea was a possibility for those who were exposed to said substances in pulverized form.

Objectively, the presence of dermatitis and dermatosis of the dry type and conjunctival phlogoses was verified in 8 cases; bronchospasm was found in one case; clinical findings were negative for the rest of the workers who reported, or failed to report, disorders.

The allergy tests indicate that the major part of the verified allergic afflictions are attributable to streptomycins; in fact seven of the ten workers who reported disorders showed cutaneous sensitivity to these antibiotics ; besides sensitivity to streptomycins, allergic reaction to vitamins B_{12} was present in a single case only.

The following description refers to the last-mentioned case:

Case 1. female pharmaceutic worker, 42 years old. Close association *) through diathesis negative non-significant pathologic symptoms in the past. For approximately five years, engaged in the preparation, refining and labelling of the B_{12} and streptomycin content in germ cultures , in particular of the streptomices (types: griseus,venezuelae, olivaceus). Four years after starting said occupation, the worker began to complain of pruritus in exposed areas and endonasal areas, sternutation crises, blepharospasm and dyspnea after exposure to vitamins B_{12} and streptomycin in dissolved and pulverized form.

The results of the performed allergy tests are as follows:

a) Intradermic tests with inhalants, foods, mycelia, pollen and bacteria; positive reactions were obtained for the following antigens: environmental dusts, mites (dermatophagoides pteronissimus), aspergellus, moniliae, penicillins, streptomices.

b) The epicutaneous tests performed with the specified drugs yielded negative test readings. The same tests, when performed intradermically,showed positive reaction to streptomycins, vitamins B_{12} in all formulations 20' after the inoculation; after approximately 1 hour, the cutis, site of the test, had reverted to normal.

*) "familiarità"

Group 2: subjects who used streptomycins and/or cyanocobalamin for therapeutic purposes.

60 individuals were involved; approximately 50% of these had reported disorders attributed to the use of the drugs; the rest of the cases was tested so as to evaluate the eventual presence of cutaneous sensitivity to the above-mentioned drugs.

The following disorders predominated: pruritus, more or less widespread; insomnia; pyrosis at the mucous orifices. Anaphylactic shock and asthma occurred in a single case (see Case 2).

In this group as well, the allergenic capacity of the streptomycins proved to be superior to the allergenic capacity of the cyanocobalamins (12 cases of allergy to streptomycins as compared to 2 cases).

The drugs were administered enterally or parenterally to all subjects.

As for the group of subjects without complaints: it should be pointed out that positive reaction to streptomycins was obtained in one of the cases. In that case, as of course in all other allergies as well, the patients were advised against the therapeutic use of, or occupational contact with the drugs mentioned above. A description of two cases with hypersensitivity to vitamins B_{12} follows (from Group 2):

Case 2: female elementary school teacher, 60 years old. Close association through diathesis negative. Afflicted by chronic bronchitis for 10 years (asthmatic form), with crises mainly in winter. In 1967 underwent allergy tests by means of scarification, without formulation of a well-defined etiologic diagnosis.

In September 1968, a few minutes after intramuscular administration of a polyvitamin preparation containing raw liver extract as well, she suffered a serious asthmatic crisis and collapse, which required hospitalization. Allergy tests performed following this serious anaphylactic crisis yielded the following results: a) intradermic tests with inhalants, pollen, bacteria, mycelia: positive test readings were obtained 20' after the injection, for aspergellus, papularia, moniliae, chaetonium, botrytis, micrococci, pneumococci, bacilli aerogenes, staphilococci;
b) tests with specific drugs: as a precaution, the tests were performed epicutaneously (with test readings after 20', 2, 24 and 48 hours). Since the tests proved to be negative, intradermic tests were subsequently performed . Positive reactions were obtained to hepatic extracts and vitamins B_{12}. The results are shown in detail on Table 2.
c) Other antibiotics (penicillin, tetracylline ,lincomycin, gentamycin, chloramphenicol) were additionally tested, with negative results. After the tests with vitamin B_{12} the patient complained of an intensive pruritus at the site of the injection, which spread ; she also experienced a moderate sensation of retrosternal oppression. Apart from evident foci *) with a diameter in excess of 15 mm on the average with pseudopodia surrounded by intensive hyperemia, the objective evaluation showed no clinically relevant facts; furthermore, no variations of arterial pressure and temperature were determined within 30 minutes after the injections. After approximately 1 hour, the cutaneous reactions showed a pronounced regression; they disappeared within 24 hours.

*) "ponfi"

Case 3. Male teacher and geographer , 36 years old. Close (family) associations through diathesis negative. In 1966, following the oral ingestion of antibiotics, occurence of aphthous stomatitis; the use of polyvitamin, likewise orally ingested, caused pruitus at the mucous orifices and mucous edema of the mouth and prolabium. For an undefined period of time, afflicted by herpes labialis as well. The allergy tests supplied the following results: a) intradermic tests with inhalants, mycelia, food, bacteria: negative test reading after 20', 24 and 48 hours; b) tests for antibiotics and chemotherapy substances (penicillin, tetracylline, oleandromycin, streptomycin, chloramphenicol, erythromycin, lincomycin, sulfametoperazin) were initially performed epicutaneously with negative results; subsequently, they were carried out intradermically. The test with tetracycline HCl yielded positive reaction; c) tests with vitamins showed strong positive cutaneous reaction of the immediate type for cyanocobalamin (on the Table at the end of the description of the cases , results are displayed separately). In this case as well, the foci were conspicuously reduced an hour after the injection; they disappeared during the next 24 hours.

In conclusion, the following Table shows the reactions to the tested products.

TABLE 2. - Reactions to intradermic tests (test reading after 20', 24 and 48 hours)

Substance:	Case 1.	Case 2.	Case 3.
Streptomycin sulfate	++	-	-
dihydrostreptomycin sulfate	++	-	-
commercial hepatic extract	+	++	+
bovine hepatic extract	+	+	+
sieroalbumin of the hog	-	-	-
bovine sieroalbumin	-	-	-
thiamine	-	-	-
riboflavin	-	-	-
pyridoxine	-	-	-
nicotinamide	-	-	-
commercial cobalamin	++	++++	+++
hyper-purified cobalamin	++	++++	+++
commercial hydroxycobalamin	++	+++	+++
coenzyme B_{12}	++	+++	+++
cobalt chloride	-	-	-
benzilic alcohol	-	-	-
cytidine	-	-	
uridine	-	-	-

As usual, the interpretation of the intradermic reactions is as follows: focus*)with an average diameter of 5 mm = +; 10 mm = ++; 15 mm = +++; over 15 mm = +++.

*) "ponfo"

Discussion and Conclusions.

Medical literature reports adequately documented cases of allergy to vitamins B_{12}. Thus YOUNG (1950), BORODULIN (1961), ROY (1961), MARINKOVIC (1962) and RONDING (1968) report anaphylactic reactions of the cutaneous or generalized type; the case mentioned by ROY was lethal. Some reactions of minor significance, of the reaginic type (urticaria, dermatitis, asthma) are mentioned by BEDFORD (1952), GILLESPY (1955), UNGLEY (1955). On the other hand, reactions of the delayed type appeared after the use a cyanocobalamin in subjects sensitized previously to cobalt salts and with a history of dermatoses (ROSTEMBERG and PERKINS, 1951). According to the authors' hypothesis , the possibility of an allergic reaction to the cobalt in the molecule of B_{12} exists.

Finally, other authors attempted to induce sensitizing to B_{12} experimentally: TRAINA (1950), using guinea pigs, failed to obtain a shock reaction after parenteral application of vitamin doses. On the other hand, LIPTON and STEIGMANN (1963) obtained reactions of the anaphylactic type in guinea pigs when using cyanobalamide of the fermentative type (from germ cultures) and (liver) extract emulsified with FREUD adjuvant.

In the three reported cases, the clinical history and the allergy control tests seem to indicate that immediate reactions are attributable to hyperergy relatively to vitamins B_{12}.

These investigations made it possible to exclude the eventual sensitizing to additives or impurities when using hyper-purified products, and to control certain components of marketed products such as benzilic alcohol, cited as cause of urticaria (LEGERHOLM,1958).

In Case 1., sensitizing of the immediate type to streptomyces culture extracts, streptomycin and vitamins B_{12} seems significant: said poly-sensitivity apparently indicates that the operations performed by the female worker exposed her to contact and subsequently unleashed the allergic syndrome.

In Case 2., which involved anaphylactic shock following the injection of a product containing B_{12}, a well-defined cutaneous reaction of the immediate type was obtained; the regression of the cutaneous reaction within less than 1 hour leads to the supposition that said pathologic conditions is attributable to reaginic antobodies only, or at least to their prevalent form. Additional clarification would require the testing of other possibly present antibodies (using technical methods suitable to demonstrate the presence of precipitating or agglutinating antibodies, and also of other fractions, by means of immuno-electrophoresis).

It could not be definitely ruled out in the tested cases that exposure to a single group of substances occurred (streptomycins and vitamins B_{12}). It was therefore not possible to clarify the hypothesis which contends that allergologic control can determine the presence of a cobamide volume sufficient for producing sensitization, in streptomycin preparations of fermentative origin. It should furthermore be pointed out that among subjects exposed for occupational reasons,, a single case of hyperergy to B_{12} was found, besides a quite numerous group (reacting) to streptomycins. This seems to show, parallely with the efficient penetration of the streptomycin antigen, that the allergizing capacity of B_{12} is minor. It must therefore be ruled out--while the occurence of allergy cases is rather difficult during work when exposure is considerable--that the incidence of allergy cases results from the therapeutic use of a drug containing B_{12} as an impurity, and certainly in minor quantities.*)

*) Text garbled. (Translator).

Finally, it should be noted in the same context that double sensitization occurred in none of the cases with therapeutic exposure to these products.

It should be stated as well that the various application methods (inhalation, oral, parenteral) can trigger an allergy. It should finally be taken into consideration that the cutaneous reactions of our patients to all B_{12} formulations were nearly superimposable.

In conclusion: it remains to be clarified how the sensitization to vitamins B_{12}, introduced into our organism, occurs : it is hypothetically possible that our subjects had to create a B_{12}-protein compound, reacting to the same with the production of antibodies (a synthesis which in some way differs from the synthesis present in normal subjects). Said compounds, which are indispensable for the use of cyanocobalamide in erythropoiesis, were recently demonstrated with Co^{57} by MELWIN (1968), in case of therapeutic application as well as alimentary administration of B_{12}. The used technique could not discriminate any compounds differing relatively to origin. The hypothesis requires confirmation; it could explain possible allergic sensitization to other vitamins (of groups B,K,D) which have been reliably demonstrated, while their genesis has not been determined.

BIBLIOGRAPHY.

V. L. MELWYN (1968) Records of the International Symposium on erythropoiesis factors. Italseber ,publ. p. 165.

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Clinica del Lavoro « L. Devoto » della Università di Milano
Centro di studi e ricerche sulle Malattie professionali sotto il Patronato dell'INAIL
Direttore: Prof. E. C. Vigliani

MANIFESTAZIONI ALLERGICHE DA VITAMINE B_{12}

C. NAVA

Scopo del lavoro è quello di descrivere alcuni casi di allergia a vitamine B_{12}, osservati presso gli ambulatori di allergologia della nostra Clinica negli ultimi cinque anni (1966-1970). L'indagine si è rivolta sia verso soggetti che avevano usato vitamine B_{12} e/o streptomicine, sia verso operai che erano venuti a contatto con tali sostanze per ragioni di lavoro.

La ricerca ha la sua giustificazione nel fatto che gli operai addetti alla estrazione da brodi di cultura di streptomices (griseus, olivaceus, venezuelae ecc.) erano esposti ad ambedue i gruppi di sostanze e che in preparati di streptomicine di origine fermentativa, si è riscontrata, talora, la presenza di quantità di cianocobalamine.

Si è voluto pertanto accertare quanto possa avere importanza nella comparsa di allergie, l'uso dei farmaci, la presenza di vitamine B_{12} come impurità, e infine la esposizione a queste per ragioni di lavoro.

Materiale e metodi

Nella tabella seguente do, in sunto, la descrizione dei casi a cui si riferisce la presente indagine (v. tabella I).

Sono state controllate pertanto, complessivamente 110 persone che erano venute a contatto con vitamine B_{12} e streptomicine: di queste 42 avevano lamentato disturbi.

Le prove sono consistite in tests intradermici con streptomicina e diidrostreptomicina solfato fin soluzione fisiologica: dose iniettata in ml. 0,02 gamma 100) e con cobalamina e idrossicobalamina alla dose di 10 gamma sempre in ml 0,02. La lettura è avvenuta dopo 20', 24 e 48 ore dalla inoculazione.

Il test di tolleranza cutaneo praticato in soggetti che non avevano mai avuto contatti con i sopracitati prodotti, è consistito nella inoculazione di 500 gamma di streptomicine e in 100 gamma di vitamine B_{12} (senza intolleranza).

Nei casi in cui si è mostrata ipersensibilità cutanea a vitamine B_{12} si sono praticati ulteriori controlli atti a mostrare eventuali sensibilizzazioni di gruppo, verso parti della molecola, verso altre componenti dei prodotti usati. Oltre ai farmaci già citati, sono state praticate prove intradermiche con le seguenti sostanze di cui dò la descrizione e la quantità iniettata, qui di seguito:

1) estratto epatico in commercio sol. 1:10 in fisiologica: ml 0,02;
2) estratto epatico estrattivo di bue sol. 1:10 in fisiologica: ml 0,02;
3) sieroalbumina di maiale sol. 1:10 in fisiologica: ml. 0,02;
4) sieroalbumina di bue sol. 1:10 in fisiologica: ml 0,02;
5) tiamina: gamma 100;
6) riboflavina: gamma 100;
7) nicotinamide: gamma 100;

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TABELLA 1 - *Controlli praticati a soggetti esposti al contatto con vitamina B_{12} e streptomicine per ragioni di lavoro o per terapia.*

Descrizione	N. casi	Reaz. positive a B_{12} dopo 20'	idem a streptomicina
1° Gruppo: esposti per lavoro a vit. B_{12} e streptomicine			
a) senza disturbi	40	0	0
b) con disturbi	10	1	7
2° Gruppo: esposti per terapia a vit. B_{12} e/o streptomicina			
a) senza disturbi	28	0	1
b) con disturbi	32	2	12
Totali	110	3	28

8) cobalamina iperdepurata: gamma 10;
9) idrossicobalamina iperdepurata: gamma 10;
10) coenzima B_{12} (come benzilcobamide coenzima): gamma 10;
11) piridossina: gamma 100;
12) cobalto cloruro: gammi 5.10^{-7};
13) alcool benzilico: gamma 75;
14) citidina: gamma 75;
15) uridina: gamma 100.

In tutti i casi è stata inoltre controllata la reattività cutanea con intradermiche di sol. fisiologica tamponata a pH 7.4 e di istamina bicloridrato.

RISULTATI

1° Gruppo: *operai addetti alla produzione di vitamine B_{12} e di streptomicine.*

Trattasi di 50 operai, provenienti da alcune industrie della Lombardia, ove la produzione avviene attraverso processi di fermentazione di culture di germi selezionati; in particolare si sono controllati quegli operatori che avevano avuto esposizione a ceppi di streptomices, utilizzati per la doppia produzione di vitamine e antibiotici. Gli operai provenivano dai vari settori di produzione (fermentazione, estrazione, raffinazione, confezionamento) e inoltre dai laboratori chimici adibiti alla titolazione del contenuto in vitamine e streptomicine nei brodi di cultura. (Per maggiori dettagli sulle lavorazioni si rimanda alla letteratura in proposito) (BORENSZTAJN, 1953, 1954; FABIANI e ROUBERT, 1961).

L'eterogeneità della provenienza degli operai non ha permesso di accertare il grado di esposizione alle sostanze in oggetto; inoltre, nella maggior parte delle industrie, nei reparti di produzione, il lavoro è ciclicamente alternato con l'allestimento di altri antibiotici e, nei laboratori chimici, con la esecuzione di analisi su altri prodotti.

Come appare dalla tabella (v. sopra), il 20 % degli operai controllati lamentavano disturbi: si trattava di disosmie, senso di cattivo sapore in bocca, prurito alle sedi cutanee esposte e diffusamente, pirosi agli orifizi mucosi e, ancora, senso di eccitazione ed insonnia.

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Per coloro che erano esposti a queste sostanze in forma pulverulenta era infine possibile la comparsa di blefarospasmo, rinorrea, dispnea.

L'obbiettività ha rivelato la presenza di dermatiti o di dermatosi di tipo secco e di flogosi congiuntivale in otto casi, di broncospasmo in un caso; negativo il reperto clinico nei restanti operai che avevano o non avevano denunciato disturbi.

Le prove allergologiche praticate indicano che la maggior parte delle affezioni allergiche repertate sono da attribuire a streptomicine: infatti in dieci operai che lamentavano disturbi, sette han rivelato sensibilità cutanea verso questi antibiotici; in un solo caso, oltre alla streptomicina, a vitamina B_{12}.

A quest'ultimo caso si riferisce la seguente descrizione:

Caso 1: donna di 42 anni, operaia farmaceutica. Familiarità negativa per diatesi. Non significative manifestazioni patologiche nel passato. Da circa cinque anni lavora alla preparazione, raffinazione e titolazione del contenuto di B_{12} e streptomicine in brodi di cultura di germi e in particolare di streptomices (tipi griseus, venezuelae, olivaceus). Dopo circa quattro anni dall'inizio di questa attività, l'operaia ha cominciato a lamentare dopo esposizione a vitamine B_{12} e a streptomicine in soluzione e sotto forma pulverulenta, prurito alle sedi esposte ed endonasale, crisi sternutatorie, blefarospasmo, dispnea.

Il medico di fabbrica aveva evidenziato broncospasmo durante una visita medica praticata dopo qualche ora dall'inizio del lavoro. La sintomatologia era poi regredita in circa 24 ore dopo la sospensione del lavoro, per ricomparire con la ripresa della medesima attività: per questo si decideva di allontanarla definitivamente dal reparto adibendola ad altre mansioni senza che lamentasse successivamente altri disturbi.

Le prove allergologiche praticate hanno dato i seguenti risultati:

a) Prove intradermiche con inalanti, alimenti, micetì, pollini e batteri: si sono avute risposte positive verso i seguenti antigeni: polveri di ambiente, di Mite (dermatophagoides pteronissinus) aspergilli, monilie, penicilli, streptomices.

b) Le prove epicutanee praticate con i farmaci specifici si sono rivelate negative alle consuete letture. Le stesse, praticate per via intradermica, hanno dato risposta positiva verso streptomicine, vitamine B_{12} in tutte le formulazioni, dopo 20' dalla inoculazione; dopo circa un'ora la cute, sede della prova, era ritornata normale.

2° Gruppo: *soggetti che hanno usato streptomicine e/o cianocobalamine a scopi terapeutici.*

Trattasi di un gruppo di 60 persone di cui circa il 50 % avevano lamentato disturbi attribuiti all'uso dei farmaci; i restanti casi sono stati controllati per valutare l'eventuale presenza di sensibilità cutanee verso i suddetti medicinali.

I disturbi prevalenti erano il prurito più o meno diffuso, il senso di eccitazione e l'insonnia, la pirosi agli orifizi mucosi. In un caso (v. caso 2) si era avuto uno schock anafilattico ed asma.

Anche in questo gruppo le streptomicine si sono mostrate con capacità allergizzante superiore a quella della cianocobalamine (12 casi di allergia a streptomicine contro 2).

Tutte le persone avevano assunto prodotti contenenti i farmaci per via enterale o parenterale.

Nel gruppo dei soggetti senza disturbi, è da notare che in un caso si è avuta positività a streptomicine: in questo caso, come naturalmente anche

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TABELLA 2 - *Reazioni alle prove intradermiche (lettura dopo 20') (lettura dopo 24 e 48 ore: sempre negative).*

Sostanza	Caso 1	Caso 2	Caso 3
Streptomicina solfato	++	—	—
Diidrostreptomicina solfato	++	—	—
Estratto epatico in commercio	+	++	+
Estratto epatico bovino	+	+	+
Sieroalbumina di maiale	—	—	—
Sieroalbumina di bue	—	—	—
Tiamina	—	—	—
Riboflavina	—	—	—
Piridossina	—	—	—
Nicotinamide	—	—	—
Cobalamina in commercio	++	++++	+++
Cobalamina iperdepurata	++	++++	+++
Idrossicobalamina del commercio	++	+++	+++
Idrossicobalamina iperdepurata	++	+++	+++
Cianina B_{12}	++	+++	+++
Cobalto cloruro	—	—	—
Alcool benzilico	—	—	—
Citrinina	—	—	—
Urtiina	—	—	—

L'interpretazione delle risposte intradermiche è, come di consueto, la seguente: pomfo di mm. 5 di diametro medio = +; di mm. 10 = ++; di mm. 15 = +++; di oltre mm. 15 = ++++.

MARINKOVIC (1962), HOVDING (1968): il caso citato da ROY è stato mortale. Alcune reazioni allergiche di entità minore, di tipo reaginico (orticaria, dermatiti, asma) sono ancora riferite da BEUTORO (1952), GILLESPY (1955), UNGLEY (1955): a tipo ritardato sono invece le manifestazioni comparse dopo uso di cianocobalamine in soggetti in precedenza sensibilizzati a sali di cobalto e con progresse dermatosi (ROSTEMBERG e PERKINS, 1951). In questi casi gli AA. ipotizzavano la possibilità di reazione allergica al cobalto presente nella molecola della B_{12}.

Altri autori hanno infine cercato di provocare sperimentalmente una sensibilizzazione a B_{12}: TRAINA (1950), non ha ottenuto, usando cavie, lo scatenamento di shock dopo introduzione parenterale di ripetute dosi di vitamina; non così LIPTON e STEIGMANN (1963), che, usando cianocobalamide di origine fermentativa (da brodi batterici) ed estrattiva (dal fegato) emulsionata con adiuvante di Freund, ottenne reazioni a tipo anafilattico in cavie.

Nei tre casi descritti, la storia clinica ed i controlli allergologici indicherebbero che le manifestazioni subite siano da ascrivere ad ipersensibilità a vitamine B_{12}.

Con questi controlli si è potuto escludere la possibile sensibilizzazione ad additivi od impurità utilizzando prodotti iperdepurati e controllando i singoli componenti del prodotto in commercio quale l'alcool benzilico, citato come causa di orticaria (LEGERHOLM, 1958).

Nel caso 1 appare significativa la sensibilizzazione di tipo immediato per estratti di brodi di streptomices, per streptomicine, e vitamine B_{12}: questa polisensibilità mostrerebbe come le operazioni a cui l'operaia era addetta abbiano esposto al contatto e al successivo scatenamento della sindrome allergica.

Nel caso 2, in cui si ebbe uno schock anafilattico dopo inoculazione di un prodotto contenente B_{12}, si è avuta una chiara risposta cutanea a tipo immediato: la riduzione della reazione cutanea dopo meno di un'ora, fa-

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in tutti gli altri allergici, si è sconsigliato l'uso terapeutico o il contatto lavorativo con i suddetti farmaci. Ed ecco la descrizione dei due casi di ipersensibilità a vitamine B_{12}, del 2° gruppo):

Caso 2: donna di anni 60, insegnante elementare. Familiarità negativa per atopia. Da dieci anni soffre di bronchite cronica asmatiforme con crisi prevalentemente invernali. Nel 1967 praticava prove allergologiche per scarificazione senza la formulazione di una chiara diagnosi eziologica.

Nel settembre 1968, dopo pochi minuti dalla inoculazione intramuscolo di un preparato polivitaminico contenente anche estratto di fegato crudo, veniva colta da grave accesso asmatico e da collasso che richiedevano il ricovero ospedaliero. Le prove allergologiche praticate successivamente a questa grave crisi anafilattica han dato i seguenti risultati: *a*) prove intradermiche con inalanti, pollini, batteri, miceti: si sono avute risposte positive alla lettura dopo 20' dalla inoculazione per aspergilli, papularia, monilie, chaetomium, botrytis, micrococchi, pneumococchi, bacilli aerogenes, stafilococchi; *b*) prove con farmaci specifici: per precauzione sono state praticate per via epicutanea (con lettura dopo 20', 2, 24 e 48 ore) e, successivamente, vista la negatività di queste, per via intradermica. Si sono avute risposte positive verso estratti epatici e vitamine B_{12}. Nella tabella 2 darò i risultati più dettagliatamente; *c*) sono stati inoltre controllati altri antibiotici (penicilline, tetracicline, lincomicina, gentamicina, cloramfenicolo) con esito negativo. Dopo le prove con vitamina B_{12}, la paziente ha lamentato vivace prurito in sede di inoculo e diffusamente ed inoltre modesto senso di oppressione retrosternale. L'obbiettività, se si escludono gli evidenti ponfi di oltre 15 mm. di diametro medio con pseudopodi e intensa iperemia circostante, comparsi nella sede delle intradermiche, non ha rivelato clinicamente nulla di significativo: non si è inoltre avuta variazione della pressione arteriosa e della temperatura nei trenta minuti successivi alle iniezioni. Dopo circa un'ora le reazioni cutanee si erano notevolmente ridotte; erano scomparse dopo 24 ore.

Caso 3: uomo di 36 anni, insegnante e geografo. Familiarità negativa per diatesi. Nel 1966, a seguito di ingestione orale di antibiotici, compariva stomatite aftosa: successivamente, l'uso di polivitaminico, sempre per via orale, provocava prurito agli orifizi mucosi ed edema mucoso alla bocca ed al prolabio. Da tempo imprecisato soffre inoltre di Herpes labialis. Le prove allergologiche praticate han dato i seguenti risultati: *a*) prove intradermiche con inalanti, miceli, alimenti, batteri: negative alla lettura dopo 20', 24 e 48 ore; *b*) le prove verso antibiotici e chemioterapici (penicilline, tetracicline, oleandomicina, streptomicine, cloramfenicolo, eritromicina, lincomicina, sulfametoperazina) sono state praticate dapprima per via epicutanea con esito negativo e successivamente per via intradermica. Si è avuta risposta positiva alla prova praticata con tetraciclina HCl; *c*) le prove con vitamine hanno dato intensa positività cutanea di di tipo immediato per cianocobalamine (nella tabella al termine della descrizione dei casi verranno segnalati parzialmente i risultati). Anche in questo caso, i ponfi erano notevolmente ridotti dopo un'ora dall'inoculo per scomparire nelle 24 ore successive.

Nella tabella seguente sono date in conclusione le risposte ai prodotti controllati (tabella 2).

Discussione e conclusioni

La letteratura medica riferisce casi di allergia a vitamine B_{12} sufficientemente documentate. Così reazioni anafilattiche a tipo schock cutaneo o generalizzato sono ricordate da Young (1950), Dorodjelin (1961), Roy (1961),

rebbe pensare che solo ad anticorpi reaginici, o per lo meno in forma prevalente, sia da ascrivere tale affezione. A maggior chiarimento sarebbe opportuna la ricerca di altri anticorpi eventualmente presenti (con uso di tecniche atte a mostrare la presenza di anticorpi precipitanti o agglutinanti e ancora di altre frazioni con una immunoelettroforesi).

Nei casi controllati, non si è potuto con certezza escludere che vi sia stata esposizione ad un solo gruppo di sostanze (streptomicine o vitamine B_{12}); pertanto la ipotesi di poter accertare, con un controllo allergologico, la presenza di quantità di cobamidi tali da poter dare una sensibilizzazione, in preparati di streptomicine di origine fermentativa, non si è potuta chiarire. Vi è inoltre da segnalare che, accanto a un gruppo abbastanza numeroso a streptomicine, vi è un solo caso di ipersensibilità a B_{12} tra i soggetti esposti per ragioni di lavoro: questo mostrerebbe che, accanto alla buona penetranza dell'antigene streptomicina, vi è una scarsa capacità allergizzante della B_{12}: è pertanto da escludere che, se è piuttosto difficile che avvengano casi di allergia in lavoro dove l'esposizione può essere notevole, ciò avvenga per l'uso terapeutico di un farmaco ove la vitamina B_{12} può essere presente come impurità in quantità sicuramente minore. Sempre a proposito vi è infine da notare che in nessun caso tra gli esposti per terapia a questi prodotti si è avuta una doppia sensibilizzazione.

È ancora da segnalare come le diverse forme di introduzione (inalatoria, orale, parenterale) possono scatenare una allergia. Una ultima considerazione da fare è che a tutte le formulazioni di B_{12} attualmente in uso, i nostri pazienti hanno dato risposte cutanee pressochè sovrapponibili.

Per concludere resta da chiarire come vi possa essere una sensibilizzazione a vitamine B_{12} apportate al nostro organismo: è ipotizzabile che i nostri soggetti abbiano a creare un complesso B_{12}-proteina verso cui reagire con produzione di anticorpi (sintesi diversa in qualche modo da quella presente in soggetti normali). Questi complessi, indispensabili per l'uso della cianocobalamide nella eritropoiesi, sono stati dimostrati recentemente con Co^{57} da MELWYN (1968) sia nel caso di apporto terapeutico che alimentare di B_{12}. La tecnica usata non ha potuto differenziare comunque complessi diversi a seconda della provenienza. È una ipotesi da confermare e che potrebbe giustificare la possibilità di sensibilizzazione allergica verso altre vitamine (del gruppi B, K, D), sicuramente dimostrate ma non definite nella loro genesi.

RIASSUNTO

Si descrivono tre casi di manifestazioni allergiche a vitamine B_{12}, occorse in un gruppo di 110 persone: di questi 50 avevano lavorato alla produzione delle cianocobalamine, 60 le avevano assunte per terapia.

Si esclude la possibilità di allergie a solventi o ad impurità presenti nelle preparazioni in commercio.

In un caso, operaia addetta alla titolazione di vitamina B_{12} e streptomicine in brodi di cultura, si segnala la presenza di pulisensibilità verso ceppi di streptomices e streptomicine.

L'indagine ha messo in evidenza una modesta capacità allergizzante delle cobalamine rispetto alle streptomicine.

Si esclude la possibilità di poter analizzare la presenza come impurità di B_{12} in preparati di streptomicine di origine fermentativa con il metodo allergologico.

Si ipotizza come vi possa essere allergia alla molecola della vitamina B_{12} o ad un complesso B_{12}-proteina.

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SUMMARY

NAVA C. — *Allergic reactions to vitamins B_{12}.*

Three cases of allergic reactions to vitamins B_{12} are described, which occurred among a group of 110 subjects; out of these, 50 had been engaged in the production of cyanocobalamins, 60 had been treated with them. The possibility of allergies to solvents or to impurities present in the commercial preparations was ruled out. In a female worker engaged in the titration of vitamins B_{12} and streptomycins in culture media, a sensitization to Streptomyces strains and streptomycins was reported. The investigation showed a moderate allergy-inducing capacity of cobalamines in comparison to streptomycin. The possibility of analyzing by an allergologic method the presence of B_{12} as an impurity in preparations of streptomycins of fermentation origin was excluded. It is suggested as a hypothesis how allergy to the molecule of vitamin B_{12} or to a complex B_{12}-protein might occur.

ZUSAMMENFASSUNG

NAVA C. — *Allergische Erscheinungen durch Vitamin B 12.*

Es werden 3 Fälle von Überempfindlichkeit gegen Vitamin B 12 beschrieben, welche bei einer Gruppe von 110 Personen beobachtet wurden: Davon waren 50 Leute mit der Herstellung des Cyanocobalamins beschäftigt, bei 60 weiteren Personen war dieses als Medikament angewandt worden.

Die Möglichkeit, einer Allergie gegen ein Lösungsmittel oder gegen Verunreinigungen in den Handelspräparaten kann ausgeschlossen werden.

Bei einer Arbeiterin, welche das Vitamin B 12 und Streptomycin in flüssigen Kulturmedien zu titrieren hatte, bestand eine mehrfache Sensibilisierung gegen Kulturen von Streptomyces und Streptomycin.

Es besteht keine Möglichkeit, mit allergologischen Methoden Verunreinigungen in fermentativ gewonnenen Streptomycinpräparaten dafür verantwortlich zu machen.

Es wird angenommen, dass es sich hier um eine Allergie gegen das Vitamin B 12-Molekül oder gegen einen B 12-Eiweiss-Komplex handelt.

BIBLIOGRAFIA

BRIDGE P. D. (1952): Brit. Med., 7, 1, 690. — BORODULIN YU. D. (1961): Klin. Med. (Mosk), 39, 8, 139. — BORENSZTAJN D. (1954): Med. Dosw. mikrob., 6, 25. — BORENSZTAJN D., KURYLOWICZ (1953): Polskie arch. med., 23, 1032. — FABIANI P., ROUBERT J. (1961): Bull. Soc. Sci. Hyg. Aliment., 49, 258. — GILLESPY R. O. (1955): Lancet, 1, 1076. — HOVDING G. (1968): Brit. Med. J., 3, 102. — LAGERHOLM B., LOOHI A. and GENTILE H. (1958): Acta Allerg., 12, 295. — LIFTON M. M., STEIGMANN A. J. (1963): J. Allergy, 34, 362. — MARINKOVIC V. (citato da HOVDING) (1962): Srpski Arkn. ielok. lek., 90, 547. — MELWYN V. L. (1968): Atti del simposio internazionale sui fattori dell'eritropoiesi, Ed. Italseber, pag. 165. — ROSENBERG A. and PERKINS A. J. (1951): J. Allergy, 22, 466. — ROY A. N. (1961): J. Ind. med. Ass., 36, 262. — TRAINA V. (1950): Arch. Path., 49, 278. — UNGLEY C. C. (1955): Vitam. and Horm., 13, 137. — YOUNG W. C., ULRICH C. W. and FOUTS P. J. (1950): J. Amer. Med. Ass., 143, 893.

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VITAMIN B_{12} CONTENT OF LIVER AND SERUM IN MALIGNANT NEOPLASIA

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Elevated serum vitamin B_{12} levels have been observed in metastatic and primary malignant neoplasia of the liver in which the organ was extensively involved. The increased serum B_{12} has been attributed to necrosis and displacement of the liver cells by the invading tumor, as in damage secondary to virus invasion or carbon tetrachloride injury.[1] Studies with vitamin B_{12} labeled with radioactive isotope, however, demonstrated impaired hepatic uptake of the vitamin in only 2 of the 10 cases of metastatic liver neoplasia, despite very extensive involvement.[2]

The present study, employing previously described methods of bioassay of fresh liver biopsy material and serum,[3,4] was undertaken to clarify vitamin B_{12} relationships in malignant neoplasia of all types. Routine determinations of liver and serum B_{12} were carried out from January 1957 to May 1961, in all of the cases of neoplasia in which such a study was feasible.

Material

Thirty-eight patients exhibiting no clinical, histologic, or laboratory evidence of liver disease were the controls. Patients with malignant neoplasia were divided into the following groups: (1) carcinoma with liver metastases, 62 patients including 7 with hepatomas, and 5 in whom assays were made of tumor removed at surgery, (2) carcinoma without liver metastases, 49 patients, (3) leukemia, 6 patients, and (4) lymphoma, 21 patients, with 11 classified as Hodgkin's disease. In group 1, liver involvement was determined by positive biopsy, or evidence of liver metastases at surgery or autopsy in cases with negative biopsy. Absence of liver neoplasia in group 2 was established by taking biopsy specimens at surgery, by surgery or autopsy with negative findings after percutaneous biopsy, or by prolonged follow-up without clinical evidence of involvement after negative percutaneous biopsy. In groups 3 and 4, liver neoplasia could be diagnosed with certainty only with a positive biopsy.

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Bioassay Methods

Liver and serum samples were stored at −20°C. until assayed. Vitamin B_{12} was extracted from the liver and serum samples in the following manner: liver samples were weighed with a Cahn Electrobalance model M 10 which has an accuracy of ± 5 mμg. (Cahn Instrument Company, Paramount, Calif.), and autoclaved for 15 minutes at 120°C. After cooling, the liver samples weighing from 1 to 6 mg. wet weight were homogenized in a Potter-Elvehjem glass homogenizer with 5 ml. of 0.02 M sodium acetate buffer, pH 4.6, and transferred to covered centrifuge tubes. In the case of serum, 1 ml. of the serum was diluted with acetate buffer, pH 4.6. Liver and serum samples were then heated in a boiling water bath for 30 minutes to precipitate protein and liberate B_{12} from the bound form. After cooling and centrifugation, the clear supernatant was assayed for B_{12} with Bacto-B_{12} assay medium U.S.P. (Difco). *Lactobacillus leichmannii* ATCC 7830 was used as the test organism, and crystalline B_{12} was used as the standard. Because all of the samples were devoid of alkali-stable microbiologic activity, the B_{12} values obtained represent a true measure of "vitamin B_{12} activity." Values are expressed as milli-

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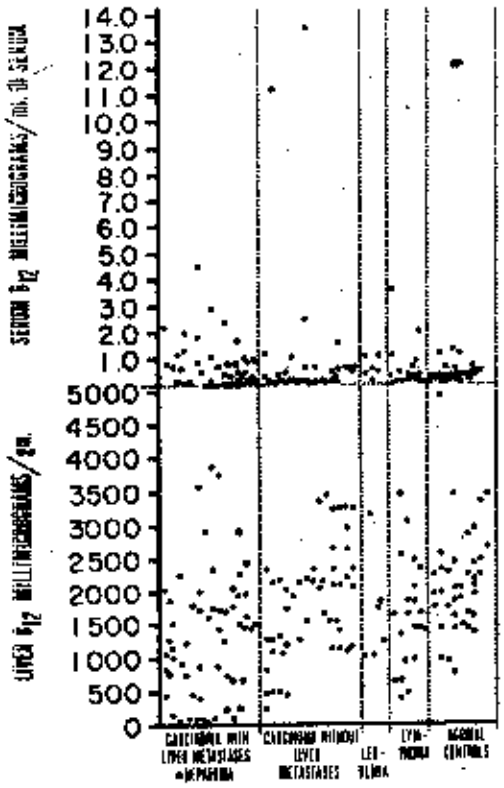

FIG. 1. Serum and liver vitamin B_{12} content in human malignant neoplasia and controls.

micrograms per gram for liver and millimicrograms per milliliter for serum.

Results

Controls. Extension of the control group to 38 patients resulted in little change in average values from previous studies.[3, 4] Liver B_{12} averaged 2105 mμg., and serum 0.4 mμg. As noted before, there was considerable range of values, with the great majority of liver assays in the 1400 to 2500 mμg. grouping (fig. 1). Serum levels varied less widely.

Group 1 (carcinoma with liver metastases). Average liver B_{12} was lower in this group than in any other, 1354 mμg. In the evaluation of these results, it was noted that all of the specimens which contained less than 1000 mμg. were heavily infiltrated with tumor, with the exception of two, which were both carcinomas of the stomach. Previous studies had led to the suspicion that most tumors might contain little B_{12}, and that tumor infiltration might be responsible for lower values obtained in such specimens.[5] Biopsy specimens were obtained at surgery in 5 patients directly from tumor involving liver. In 3 of these patients, specimens were also obtained from uninvolved portions of the liver as well. Results are shown in table 1. All of the pure tumor assays were extremely low. Uninvolved liver in 2 patients showed B_{12} within the control range, although below average normal value. In the third patient (case 5, table 1), the biopsy was markedly involved with desmoplastic reaction, few liver cells remaining. Average levels varied widely, and there were 6 patients in whom B_{12} was so low that it could not be measured accurately. Average serum value was 0.8 mμg., a definite elevation.

TABLE 1

Values for vitamin B_{12} in tumor and uninvolved liver from specimens removed at surgery

Case	Classification	Vitamin B_{12} levels: Liver tissue	Vitamin B_{12} levels: Tumor tissue
		mμg./gm. of tissue	
1	Adenocarcinoma of colon	1880	30
2	Adenocarcinoma of stomach	1500	10
3	Hepatoma.	Not obtained	30
4	Carcinoid	Not obtained	100
5	Adenocarcinoma of breast	100 (Marked desmoplasia, few cells)	10

Group 2 (carcinoma without liver metastases). There was a high average B_{12} content of 1844 mμg. in the uninvolved livers, which agrees closely with control levels. Conspicuous in this group were 5 instances of gastric carcinoma which made up all but 1 of the cases with values under 1000 mμg. The remainder of the liver levels were within the control range (fig. 1). Mean levels in serum were high, at 1.2 mμg., but it should be noted that this resulted to a great extent from two extremely high values.

Group 3 (leukemia). In this small group,

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the average liver content of B_{12}, 1665 mμg., was well within the control range despite leukemic infiltration in 1 of 6 patients. Although both cases of myelocytic leukemia showed elevations of serum B_{12}, the level was raised in one instance of lymphocytic leukemia as well. Average serum level was elevated to 0.7 mμg.

Group 4 (lymphoma). In lymphomas of all types, the average liver B_{12} content was 1628 mμg., comparable to values obtained in leukemia, and within control range. Although 3 specimens with tumor involvement showed low levels, 2 others were average. Serum values, with an average level of 0.6 mμg., were elevated in five instances. No tumor specimens were assayed.

Discussion

There is little information available on the status of vitamin B_{12} in human malignant neoplasia. In one study,[1] serum levels were found to be elevated in 16 of 18 patients with various malignant tumors extensively involving the liver. In 19 control patients, the serum levels were normal. These workers believed that the neoplastic process itself was not responsible for the rise of serum B_{12} levels, but that the increase was caused by necrosis and displacement of liver cells by the invading tumor, allowing the vitamin to escape into the blood. Our study with a larger number of patients both with and without liver neoplasia showed that there were indeed many who exhibited increase in serum vitamin B_{12}. The elevations appeared in certain cases of both groups, however, and were not invariable in either. There were more elevations (20 of 41 tested) in the group with neoplastic involvement of liver than in the group without (12 of 34 tested). However, there were 6 blood levels so low as to be nonmeasurable in the former, as compared with 2 in the latter.

The vitamin B_{12} content of the liver itself is definitely lowered in cases with metastatic liver disease, but low liver values do not necessarily coincide with high serum values, as might be expected if the responsible mechanism were "release" of B_{12} from liver into the serum. In carcinoma without liver neoplasia, average liver B_{12} levels differed little from the controls (1844 mμg. as compared to 2105 mμg.), yet there were numerous elevations of serum B_{12}. As in the group with liver involvement, high serum levels were, in some patients, accompanied by normal or higher than average liver values.

Serum vitamin B_{12} levels were also elevated in leukemia (3 of 6 cases), and lymphoma (5 of 21 cases). Again, average liver content was moderately reduced in both groups, but there was no evidence that lowered liver content and release of the vitamin into the serum was responsible for elevated values. Increased serum B_{12} has previously been noted in myelocytic leukemia,[5] but the mechanism has not been explained. This phenomenon now seems to occur in lymphocytic leukemia and lymphoma as well, but liver B_{12} values give no clue as to the cause.

Bioassays carried out on pure tumor removed from the liver during surgery revealed that in the five different varieties studied there is a very low vitamin B_{12} content. In 2 cases of this group, simultaneous biopsy specimens taken from uninvolved liver near the tumor revealed relatively normal values. In another instance, a biopsy specimen from what seemed to be normal liver revealed dense desmoplastic reaction. This area also showed very little B_{12}. When the liver is massively replaced by either tumor or desmoplasia, therefore, its vitamin B_{12} storage and metabolic function is largely obliterated. Our findings would lead us to conclude that this is predominantly a mechanical process with slight effect on the remaining liver cells, unless total hepatic circulation is severely compromised. This event is rare in neoplasia.

Our results tend to agree with those of other observers,[2] who noted elevated, normal, and decreased serum values in metastatic neoplasia of the liver. The relatively normal hepatic B_{12} content in uninvolved areas would also agree with their observations of normal uptake of radioactive Co^{60} B_{12} in 8 of 10 very extensively infiltrated livers. Whereas impaired uptake and storage may account for high serum values in some

patients it probably is not the mechanism involved in most instances, particularly not in those with minimal liver invasion.

Elevation and depression of serum vitamin B_{12} values may occur in all types of neoplasia for reasons not understood. The present study gives no clue as to the true mechanism, but it would seem to be more complicated than that of injury to the liver cells secondary to tumor invasion.

The livers of 5 patients with gastric carcinoma, uninvolved with tumor, contained very little vitamin B_{12}, with values of 500 mμg. or less. These 5 were remarkable as the only ones showing such low levels in this classification, in which the average value (1844 mμg.) was close to the average control level (2105 mμg.) and all of the others fell within the normal control range. This observation leads to the speculation that, in some cases of gastric carcinoma, impaired absorption of the vitamin may occur. Impaired absorption may be caused by a lack of, or an interference with, intrinsic factor. None of the 5 had clinical evidence of pernicious anemia, and the other 19 patients with gastric carcinomas in group 2 had normal, or slightly lowered B_{12} liver values. Thus, it would seem that the tumor invasion itself was not responsible for this finding, and that some precursor, or pernicious anemia-like condition is the more likely cause. Even in total gastrectomy a period of years is necessary to deplete B_{12} stores to the point of development of clinical pernicious anemia. Untreated pernicious anemia has been shown to be associated with almost no measurable liver B_{12},[3] so that it is possible that these 5 cases may represent transitional phases. We have found no such incidence of pernicious anemia among gastric cancer patients as would be anticipated from studies of hepatic B_{12} in this small series (5 of 24 cases). Further investigations are being carried out to test the validity of the concept of a subclinical type of pernicious anemia-like condition in gastric cancer. If confirmed, it may be possible to roughly estimate the duration of such a condition by the amount of liver vitamin B_{12}. Early recognition of such a state might aid in the diagnosis of gastric cancer.

Summary

1. Vitamin B_{12} content of human liver biopsy and serum specimens was determined by bioassay in 4 groups of patients with malignant neoplasia: (1) 62 with carcinoma involving the liver; (2) 49 with carcinoma without liver involvement; (3) 6 with leukemia; and (4) 21 with lymphoma. Thirty-eight patients without evidence of neoplasia or liver disease were used as controls.

2. Average liver values were definitely lowered in group 1, many of which were infiltrated with tumor. Pure tumor specimens in 5 cases showed extremely low values, which is a probable clue to low B_{12} content in infiltrated biopsy specimens, because uninvolved liver from group 1 contained relatively normal amounts. Group 2 had essentially normal liver content, except for 5 cases of gastric carcinoma. Average liver content in groups 3 and 4 was moderately reduced. Serum content in all of the groups varied considerably, ranging from very low to very high, although there were more elevations in group 1. There was no correlation between high serum and low liver values in group 1, casting doubt on the possibility that elevated serum B_{12} results from liver destruction secondary to neoplastic invasion.

3. None of the 5 cases of gastric carcinoma with extremely low liver content in group 2 showed clinical evidence of pernicious anemia, but reduced liver B_{12} in these patients may be the result of an absorption defect of an undetermined type.

4. Elevations of serum B_{12} occur in all types of malignant neoplasia, with or without liver involvement, but the mechanism is unknown.

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REFERENCES

1. Grossowicz, N., Hoffman, A., Aronovitch, J., Izak, G., and Rachmilewitz, M. Malig-

nant growth in the liver and serum vitamin B_{12} levels. Lancet, 1: 1116, 1957.

2. Glass, G. B. J., Boyd, L. J., and Ebin, J. Radioactive vitamin B_{12} in the liver. I. Hepatic uptake of $Co^{60}B_{12}$ in liver disease. J. Lab. Clin. Med., 48: 339, 1956.

3. Nelson, R. S., and Doctor, V. M. The vitamin B_{12} content of human liver as determined by bio-assay of needle biopsy material. Ann. Intern. Med., 49: 1301, 1958.

4. Nelson, R. S., and Doctor, V. M. Hepatic and serum vitamin B_{12} content in liver disease. Gastroenterology, 38: 188, 1960.

5. Beard, M. F., Putney, W. R., and Sanneman, E. H., Jr. Serum concentrations of vitamin B_{12} in patients suffering from leukemia. Blood, 6: 780, 1951.

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DISTRIBUTION OF VITAMIN B_{12} IN CELL FRACTIONS OF RAT BRAINS

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SUMMARY

Vitamin B_{12} activity by microbiological assay, and radioactivity at times between 6 h and 22 days after intramuscular injection of [^{58}Co]vitamin B_{12}, were measured in the subcellular fractions of the brains of 21 rats.

Radioactivity moved rapidly from the site of injection. At 6 h and 12 h after injection most radioactivity was found in the nuclear fractions. It then concentrated in the mitochondria fractions, at the expense of the nuclear fractions for the first 3 days, and thereafter at the expense of both the nuclear and microsome fractions. The proportion in the supernatant fractions was virtually constant throughout. After 8 days the radioactivity equilibrated with the total vitamin B_{12} activity.

Evidence is presented suggesting that the radioactivity measured was associated with vitamin B_{12}.

INTRODUCTION

Vitamin B_{12} appears to be essential for the correct functioning of the central nervous system of man. Subacute combined degeneration of the spinal cord is a well-known complication of the deficiency of vitamin B_{12} in pernicious anaemia, and this deficiency can cause cerebral manifestations[1]. It appeared, therefore, that information on the distribution of vitamin B_{12} in sub-cellular fractions of brain tissue might be a useful step towards elucidating the function of the vitamin in the metabolism of the brain. In this paper data are presented on the distribution of radioactivity in the cell fractions of rat brain tissue at various times after intramuscular injection of small amounts of [^{58}Co]vitamin B_{12}, compared with the vitamin B_{12} activity of the fractions as determined by microbiological assay.

MATERIALS AND METHODS

Animals

Male rats derived from stock of the Wistar strain were obtained from the War Department, Allington Farm, Porton Down, Salisbury, United Kingdom. They were fed without restriction on rat cake (modified diet 41 B based on diet 41 of BRUCE AND PARKES[2] and BRUCE[3], supplied by Oxo Ltd., Thames House, London, E.C.4.) and

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water. The 21 rats used in the main experiment weighed from 246 to 400 g (mean 339 g). They were given an intramuscular injection into a hind leg of 0.2–0.4 ml of an aqueous solution of [^{58}Co]vitamin B_{12}, and were killed at intervals up to 22 days after the injection. The 5 rats given $^{58}CoCl_2$ were similarly treated.

Chemicals

Two samples of [^{58}Co]vitamin B_{12} were purchased from the Radiochemical Centre, Amersham, Bucks (United Kingdom). Sample No. 1 had a specific activity of 7.6 $\mu C/\mu g$ vitamin B_{12}, and sample No. 2 a specific activity of 18 $\mu C/\mu g$. The radiochemical purity of these samples was found by the makers to be 98 % by isotope dilution analysis and 99 % by electrophoresis; less than 3 % of the radioactivity was due to ^{60}Co.

The $^{58}CoCl_2$ was obtained from the Radiochemical Centre as a carrier-free aqueous solution with a pH of about 6, and contained 100 μC radioactivity (less than 1 μg Co) in 10 ml. This solution (0.1–0.5 ml) was injected intramuscularly.

Other chemicals were of analytical grade.

Preparation of cell fractions

Whole brains, including the olfactory lobes and the medulla oblongata, were used. The rat was starved for 18 to 24 h and then lightly anaesthetized with ether. As much blood as possible was removed from the dorsal aorta; then the animal was killed by cardiac puncture. The roof of the skull was cut away with bone forceps and the brain freed from attachments with a spatula and placed immediately in ice-cold 0.25 *M* sucrose. Subsequent operations were performed at 0–5°.

After chilling for 2 min the brain was removed from the sucrose solution, blotted, weighed and homogenized in 10 ml 0.25 *M* sucrose for 1 min with an homogenizer of the Potter–Elvehjem type. The resulting suspension was diluted to a known volume, usually 14 ml, with 0.25 *M* sucrose, and contained about 0.13 g brain/ml. The time interval between killing the animal and completing the homogenization was 10 min. The homogenizing tube was made of Pyrex glass with smooth internal surface, and the polished Perspex pestle, driven at 1000 rev./min, had a diameter 0.13 mm less than the internal diameter of the tube.

The procedure for isolating the nuclear, mitochondria, microsome and supernatant fractions was based on that of HOGEBOOM[4]. It was found in 2 preliminary experiments that washing the sedimented fractions by re-suspending in 0.25 *M* sucrose and re-centrifuging did not affect the distribution and quantity of radioactivity or vitamin B_{12} activity. As more consistent respiratory activity was obtained in unwashed nuclear and mitochondria fractions, probably owing to the time factor involved, the results described in this paper were obtained from unwashed fractions. The relative centrifugal forces quoted are average values calculated for the mid-point of the centrifuge tubes. Fractions requiring more than 5000 × *g* for sedimentation were isolated at 2° in a No. 30 rotor of a Spinco centrifuge.

A measured volume of the homogenate, usually 7 ml, was layered on to an equal volume of 0.34 *M* sucrose and the nuclear fraction isolated by centrifuging for 10 min at 700 × *g*. The supernatant liquid, together with a layer of white material in suspension which was usually present immediately above the nuclear sediment, was removed with a pipette. The mitochondria fraction, which included the white material, was

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isolated by centrifuging for 10 min at 5500 × g. This white material presented technical difficulties in separation and its inclusion in the mitochondria layer is discussed below. The supernatant liquid was removed from the mitochondria sediment with a pipette and centrifuged for 45 min at 75000 × g to sediment the microsome fraction. As soon as they were isolated, the sedimented fractions were resuspended in a volume of 0.25 M sucrose equal to that of the homogenate from which they were obtained. The supernatant fraction, which consisted of the supernatant liquid remaining after sedimenting the microsome fraction, was diluted to twice this volume with 0.25 M sucrose.

An attempt was made to separate the white material from the brains of 2 rats. The brains were homogenized and the nuclear fractions isolated in the usual way. Most of the white material was separated in suspension from the supernatant liquid by centrifuging for 10 min at 1400 × g. The upper mitochondria-containing layer was removed with a pipette. The fraction containing the white material gave a positive reaction to the Liebermann–Burchard test and probably contained phospholipids, as judged by its solubility in organic solvents. It contained about 30 % of the nitrogen, 37 % of the phosphorus, 13 % of the succinic dehydrogenase activity and 15 % of the vitamin B_{12} activity of the mitochondria fractions prepared in the usual way, and was probably similar to fraction II-R_2 described by BRODY AND BAIN[5].

Characterization of fractions

The oxygen uptake of the whole homogenate, nuclear and mitochondria fractions, and occasionally of the microsome and supernatant fractions, was measured in duplicate in Warburg respirometers at 37° as previously described[6]. The first readings of the respirometers were usually taken 135 min after killing the rat, as soon as the mitochondria fractions were available, and readings were taken every 10 min for 1 h.

The succinic dehydrogenase activity of the various fractions was determined by the manometric method of SLATER[7].

Determination of nitrogen and phosphorus

Samples (0.5–1.0 ml) of the suspensions of the cell fractions were prepared for analysis by wet ashing with 1 ml concentrated H_2SO_4, 50 mg Na_2SO_4 and 5 mg HgO. The nitrogen content was determined by the micro-Kjeldahl procedure, and the phosphorus content by the method of BERENBLUM AND CHAIN[8].

Determination of vitamin B_{12} activity

The method was essentially the same as that previously described[4] to determine the vitamin B_{12} activity in cell fractions of rat livers. It was checked by measuring the recovery of added cyanocobalamin from homogenates of the brains of 10 rats. The mean recovery of 2 mμg cyanocobalamin added/ml homogenate was 1.96 mμg/ml with a standard deviation of 0.31 mμg, and the corresponding value for 4 mμg cyanocobalamin added/ml homogenate was 4.18 ± 0.70 mμg/ml.

To study the stability of the vitamin B_{12} activity of brain to alkali, extracts prepared from the whole homogenates of the brains of 6 rats were made alkaline (pH 12) with NaOH and heated at 100° for 30 min. The extracts were then cooled, neutralized with HCl, and suitable amounts of the solutions were added to the medium for assay.

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Paper chromatography

Extracts of the cell fractions were prepared as described in the method for determining vitamin B_{12} activity. The extracts were treated with activated charcoal, the charcoal washed twice with water and the adsorbed material eluted with hot acetone–water (75:25, v/v). The volume of the acetone extract was reduced by rotary evaporation under reduced pressure and the resulting concentrated solution chromatographed on paper as previously described[6]. Aqueous solutions of cyanocobalamin, [^{58}Co]vitamin B_{12} and $^{58}CoCl_2$ were chromatographed as standards.

Measurement of radioactivity

The radioactivity in samples (4 ml) of the suspensions of the cell fractions and in suitable areas of paper from paper chromatograms, was counted in a well-type scintillation counter having a thallium-activated sodium iodide crystal, as previously described[6].

The radioactivity in the whole animals, carcasses and hind legs was measured in a plastic phosphor well scintillation counter described by WARNER AND OLIVER[9].

RESULTS

[^{58}Co]Vitamin B_{12}

At 6 h after injection the nuclear fractions contained a mean of 52 % of the radioactivity of the whole homogenates (Table I). This value decreased to 27 % at 3 days after injection, and 15 % at 22 days. Conversely, the value in the mitochondria fractions increased from 26 % at 6 h after injection to 39 % at 3 days and 54 % at 22 days. The microsome fractions showed little change with time up to 3 days after injection but then decreased from 20 % at 3 days to 11 % at 22 days. During this period the values in the supernatant fractions remained practically constant. Less than 0.2 % of the dose of radioactivity was found in the whole brains 6 h after injection. This value increased to 0.34 % at 1 day and thereafter tended to increase slightly.

Vitamin B_{12} activity, and characterization of fractions

No detectable vitamin B_{12} activity was found in any of the brain extracts after treatment with alkali, whereas the serum of rats contains appreciable amounts of alkali-stable activity[10].

The vitamin B_{12} activity measured by microbiological assay, the nitrogen content, phosphorus content, oxygen uptake and succinic dehydrogenase activity of the cell fractions of the brains of the 21 rats are summarized in Table II. Of the fractions prepared, the mitochondria fractions contained the highest concentrations of each of the components. No respiratory or succinic dehydrogenase activity was observed in the microsome or supernatant fractions of rats Nos. 9, 13, 16, 19, 21 (Table I) and it was assumed that the corresponding fractions from the other rats also contained no measurable activity.

The ratios of vitamin B_{12} activity to nitrogen and to phosphorus of the homogenates and cell fractions of the brains of the 21 rats are summarized in Table III. In terms of nitrogen the ratio was higher in the mitochondria than in the whole homogenates. In terms of phosphorus the ratio was higher in the mitochondria and supernatant fractions than in the whole homogenates or the other fractions.

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TABLE I

DISTRIBUTION OF RADIOACTIVITY IN SUSPENSIONS OF BRAIN CELL FRACTIONS OF 21 ADULT MALE RATS AT VARIOUS TIMES AFTER AN INTRAMUSCULAR INJECTION OF [^{58}Co]VITAMIN B_{12}

Days after injection	Dose: Radioactivity (μC)	Dose: B_{12} (mμg)	Rat No.	Live weight (g)	Weight of brain (g)	Distribution of radioactivity in brain cell fractions as percent whole homogenate: Nuclear (%)	Nuclear Mean	Mitochondria (%)	Mitochondria Mean	Microsome (%)	Microsome Mean	Supernatant (%)	Supernatant Mean	Total of fractions (percent recovery) (%)	Total Mean	Percent dose in whole brain (%)	Percent dose in whole brain Mean
		55	1	392	1.94	51		25		19		16		111		0.15	
0.25	1*	55	2	371	1.89	51	52	25	26	16	17	20	16	112	111	0.16	0.16
		55	3	318	1.83	54		27		17		12		110		0.17	
		27.5	4	256	1.66	40		29		20		26		115		0.18	
0.5	0.5	27.5	5	268	1.79	51	46	23	28	21	19	20	24	115	117	0.19	0.19
		27.5	6	246	1.75	46		31		17		26		120		0.20	
		27.5	7	309	2.00	29		35		25		20		109		0.34	
1	0.5	27.5	8	300	1.73	26	28	34	33	22	21	15	17	97	99	0.32	0.34
		27.5	9	298	1.80	28		31		17		15		91		0.37	
		27.5	10	344	1.95	31		35		17		21		104		0.37	
3	0.5	27.5	11	397	2.03	25	27	38	39	19	20	19	18	101	104	0.31	0.3[illegible]
		27.5	12	353	1.82	26		43		24		15		108		0.27	
		66	13	396	2.04	19		43		23		25		110		0.56	
8	0.5	27.5	14	371	2.01	17	19	52	46	13	17	21	22	103	105	0.46	0.49
		27.5	15	312	2.16	21		44		16		20		101		0.44	
		66	16	367	1.62	21		46		13		23		103		0.40	
15	0.5	27.5	17	307	1.91	13	17	46	48	17	15	21	23	97	104	0.45	0.39
		27.5	18	400	1.83	18		51		16		26		111		0.33	
		66	19	398	1.95	15		56		10		19		100		0.50	
22	0.5	66	20	367	1.62	17	15	51	54	12	11	22	20	102	101	0.46	0.47
		66	21	344	1.86	14		55		12		19		100		0.45	

* These rats were given twice as much radioactivity because of the smaller uptake by the brain at this time after injection.

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TABLE II

VITAMIN B_{12} ACTIVITY, NITROGEN AND PHOSPHORUS CONTENT, OXYGEN UPTAKE AND SUCCINIC DEHYDROGENASE ACTIVITY, OF SUSPENSIONS OF CELL FRACTIONS OBTAINED FROM 1 g BRAIN, OF 21 ADULT MALE RATS

Mean values with standard deviations

Fraction	Vitamin B_{12} content		Nitrogen content		Phosphorus content		Oxygen uptake		Succinic acid dehydrogenase	
	(mμg)	Percent homogenate	(mg)	Percent homogenate	(mg)	Percent homogenate	($\mu lO_2/h$)	Percent homogenate	($\mu lO_2/h$)	Percent homogenate
Nuclear	3.7 ± 1.7	11.7 ± 3.0	2.9 ± 0.7	15.1 ± 3.8	0.44 ± 0.01	14.8 ± 4.4	91 ± 33	8.7 ± 2.7	293 ± 132	14.9 ± 6.4
Mitochondria	15.7 ± 3.3	49.5 ± 6.6	8.5 ± 0.8	43.8 ± 3.5	1.41 ± 0.01	47.0 ± 4.1	622 ± 49	59.4 ± 3.1	1717 ± 147	83.0 ± 4.3
Microsome	3.2 ± 1.1	10.0 ± 2.7	3.4 ± 0.6	17.6 ± 2.9	0.49 ± 0.09	16.4 ± 2.8	0*		0*	
Supernatant	5.2 ± 1.0	16.2 ± 1.9	4.6 ± 0.9	23.6 ± 4.2	0.39 ± 0.06	12.9 ± 1.8	0*		0*	
Total of fractions		87.4 ± 7.0		100.1 ± 5.6		91.2 ± 5.8		68.0 ± 3.3		97.8 ± 6.6
Wholehomogenate	31.8 ± 6.2		19.3 ± 0.8		3.00 ± 0.01		1049 ± 80		2055 ± 173	

* Activities measured in the fractions of 5 rats only.

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Animals given $^{58}CoCl_2$

One day after injection, loss of radioactivity was greater in animals receiving $^{58}CoCl_2$ than in animals given [^{58}Co]vitamin B_{12} (Table IV). The proportion of the dose of radioactivity was considerably less in all tissues measured, except liver, in the rats

TABLE III

RATIOS OF VITAMIN B_{12} ACTIVITY TO NITROGEN AND TO PHOSPHORUS IN THE SUSPENSIONS OF CELL FRACTIONS OF THE BRAINS OF 21 ADULT MALE RATS

Mean values with standard deviations.

Fraction	mμg vitamin B_{12} activity/mg nitrogen	mμg vitamin B_{12} activity/mg phosphorus
Nuclear	1.3 ± 0.3	8.6 ± 1.8
Mitochondria	1.9 ± 0.4	11.4 ± 2.9
Microsome	1.0 ± 0.3	6.6 ± 1.6
Supernatant	1.2 ± 0.3	13.8 ± 3.5
Whole homogenate	1.6 ± 0.3	10.8 ± 2.5

TABLE IV

DISTRIBUTION OF RADIOACTIVITY IN THE TISSUES 1 DAY AFTER INTRAMUSCULAR INJECTION, IN 3 ADULT MALE RATS, (MEAN WEIGHT: 240 g) GIVEN 1 μC $^{58}CoCl_2$ AND 3 SIMILAR RATS (MEAN WEIGHT: 302 g) GIVEN 0.5 μC [^{58}Co]VITAMIN B_{12}

Tissue	Percent dose of radioactivity present (mean values)	
	Rats given $^{58}CoCl_2$	Rats given [^{58}Co]vitamin B_{12}
Whole animals	22	81
Brain	0.02	0.34
Liver	4.2	3.7
Kidney	1.5	11.2
Heart	0.07	0.67
Spleen	0.03	0.61
Pancreas	0.12	0.75
Testes	0.08	1.31
Seminal vesicles	0.04	0.43
Stomach with contents	0.12	1.3
Small intestine with contents	0.5	4.3
Caecum with contents	0.87	3.0
1 ml serum	0.09	0.1
1 ml packed erythrocytes	0.01	0.02
Remainder of carcass	11	54

given $^{58}CoCl_2$. Similar results were obtained in a rat given 2 μC $^{58}CoCl_2$ and a rat given 5 μC. An attempt was made to measure the distribution of radioactivity in the intracellular fractions of the brains of these rats. Only in the rat given 5 μC $^{58}CoCl_2$ was there sufficient radioactivity to be measured. The results in the fractions were nuclear 16 %, mitochondria 45 %, microsome 19 %, supernatant 26 %, of the whole homogenate.

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DISCUSSION

The distribution of radioactivity in the sub-cellular fractions of the brains of rats given [^{58}Co]vitamin B_{12} intramuscularly depends on the time after injection (Table I). At first radioactivity was found mainly in the nuclear fraction. It then concentrated in the mitochondria fraction at the expense of the nuclear fraction and, after 3 days, at the expense of the microsome fraction also. This redistribution suggests that the particles are in a dynamic state. There was little change in the proportion of the dose in the whole brains between 1 and 22 days after injection. In contrast to the liver[8], the proportion of radioactivity in the supernatant fractions of brain remained practically constant throughout the 22 days.

If sufficient vitamin B_{12} was injected to over-saturate the animal, its distribution in the tissues might be affected. Our rats received a standard diet, 3 samples of which contained 10–15 μg vitamin B_{12}/kg by microbiological assay. The largest quantity of [^{58}Co]vitamin B_{12} injected (66 mμg) was about half the possible maximum daily intake[8]. Since on average the brains contained 0.34 % of the dose of radioactivity and 60 mμg vitamin B_{12} activity measured microbiologically, the amount of radioactive vitamin B_{12} in the brains was less than 1 % of the total. The route of entry of the vitamin may also affect its subsequent distribution. In 3 rats killed after 6 h, the legs receiving the injection of [^{58}Co]vitamin B_{12} contained 10 % of the radioactivity given, and the other hind legs 3 %. Corresponding values for 1 rat 3 days after injection were 3.2 % and 2.2 % respectively, and for 1 rat 15 days after injection 2.7 % and 1.9 % respectively. Since 6 h after injection the whole animals still contained 90–100 % of the dose, most of the injected [^{58}Co]vitamin B_{12} was distributed rapidly.

Direct association of radioactivity with vitamin B_{12} was partly confirmed by the different distribution obtained after injection of $^{58}CoCl_2$ (Table IV), and by paper chromatography. Radioactivity in extracts of brain and liver of rats at various times after injection of [^{58}Co]vitamin B_{12} was associated with the area on the paper where the vitamin B_{12} was located, whereas the radioactivity in similar extracts from rats given $^{58}CoCl_2$ was not associated with vitamin B_{12}.

As in the liver[8], the vitamin B_{12} activity measured microbiologically was concentrated in the mitochondria fractions (Table II), but the proportion in the microsome fractions was 6 times that in the corresponding liver fractions. The concentration of vitamin B_{12} activity in brain was about one third of that in liver. At least 8 days were required for the injected [^{58}Co]vitamin B_{12} to equilibrate with the vitamin B_{12} already present. The proportion of the dose of injected vitamin B_{12} in the brains was 15–20 times less than that in the livers of the same rats. This ratio agrees with that for total vitamin B_{12} content and suggests that the injected vitamin was distributed in the animal in a similar way to that of vitamin B_{12} absorbed from the diet.

The significance of these data depends primarily on the efficiency of separation and nature of the sub-cellular fractions. In addition to their separation at well-defined rates of centrifugation, the fractions were characterised by their oxygen uptake in a medium containing pyruvate, their succinic dehydrogenase activity, and their nitrogen and phosphorus contents. About 85 % of the oxygen uptake and succinic dehydrogenase activities of the separate fractions was found in the mitochondria fractions and the rest in the nuclear fractions (Table II). Since these activities are

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characteristic of mitochondria, the values indicate that the nuclear fractions contained 15% of the mitochondria. Although the sum of the oxygen uptakes of the fractions was only 68% of that of the whole homogenates, no evidence was found to suggest that the ether anaesthetic affected the respiratory activity. The difference may have been due to residual respiration of the homogenates and dilution of the enzymes of the fractions.

The nitrogen content of the mitochondria fractions was high compared with values reported by others[11]. This could be attributed to our method of separation. Thus inclusion of the white material already mentioned in the mitochondria fractions increased their nitrogen and phosphorus contents without greatly affecting their vitamin B_{12} content and succinic dehydrogenase activity. Whether the comparatively high concentration of vitamin B_{12} activity in the mitochondria fractions is associated only with mitochondria remains unproven. Washing the fractions with 0.25 *M* sucrose solution did not affect the quantity of vitamin B_{12} and so it must be strongly associated with the particles.

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REFERENCES

1 A. D. M. SMITH, *Brit. med. J.*, (1960) 1840.
2 H. M. BRUCE AND A. S. PARKES, *J. Hyg.*, 47 (1949) 202.
3 H. M. BRUCE, *J. Hyg.*, 48 (1950) 171.
4 G. H. HOGEBOOM, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, Inc., New York, 1955, p. 16.
5 T. M. BRODY AND J. A. BAIN, *J. Biol. Chem.*, 195 (1952) 685.
6 G. E. NEWMAN, J. R. P. O'BRIEN, G. H. SPRAY AND L. J. WITTS, in H. C. HEINRICH, *Vitamin B_{12} und Intrinsic Factor. 2. Europäisches Symposion, Hamburg*, 1961, Ferdinand Enke Verlag, Stuttgart, 1962, p. 424.
7 E. C. SLATER, *Biochem. J.*, 45 (1949) 1.
8 I. BERENBLUM AND E. CHAIN, *Biochem. J.*, 32 (1938) 295.
9 G. T. WARNER AND R. OLIVER, *Brit. J. Radiol.*, 35 (1962) 349.
10 M. A. BOOTH AND G. H. SPRAY, *Brit. J. Haematol.*, 6 (1960) 288.
11 H. MCILWAIN AND R. ROUNIGHT, in *Practical Neurochemistry*, J. and A. Churchill Ltd., London, 1962, p. 196.

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Vitamin B_{12} in the Rat Kidney. Evidence for an Association with Lysosomes[1, 2]

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The subcellular distribution of microbiological vitamin B_{12} activity and of radioactivity was studied in the rat kidney, 2–22 days after the administration of 20 mμg ^{57}Co-B_{12}. The subcellular distribution of radioactivity was the same as that of the microbiological B_{12} at all times. Over 50% of each was in the mitochondrial fraction at a greater concentration than in any other subcellular fraction.

Investigation of the distribution of microbiological vitamin B_{12} activity and of radioactivity among three mitochondrial subfractions, from 6–20 days after administration of a dose of ^{57}Co- or ^{58}Co-B_{12}, showed the distributions of the two to be the same at all times. Almost 50% of the B_{12} was in the lysosome-rich subfraction (M_3) at a concentration per mg protein about six times greater than in any other mitochondrial subfraction or in any of the subcellular fractions. Density-gradient centrifugation confirmed the association of B_{12} with lysosomes.

The M_3 subfraction was unable to take up ^{57}Co-B_{12} *in vitro*. Using an M_3 fraction from a rat previously administered ^{57}Co-B_{12}, it was shown that on incubation at 37° radioactivity was released with similar kinetics to the release of lysosomal enzymes.

It has been known for some time that the kidney of the rat appears to have somewhat unique properties in its metabolism of vitamin B_{12} compared to other tissues. Not only does it contain more total vitamin B_{12}, at a higher concentration than other tissues (1, 2), but it also acts as the major initial site of accumulation for newly administered vitamin B_{12} (2–4), particularly in conditions of sufficiency of the vitamin (1, 4, 5) and especially when cyanocobalamin is the form administered (6, 7). In addition, the peculiar turnover of vitamin B_{12} within the kidney has suggested a special role of the kidney in the metabolism of this vitamin (4, 5).

The precise role of vitamin B_{12} in the kidney is unknown. It may be simply stored there or it may have a functional involvement. The demonstration of methylmalonyl coenzyme A isomerase activity in kidney (8, 9) together with the nature of the dynamics of the vitamin there (1, 4, 5) suggests that both functional activities and storage take place. Further evidence as to the role of vitamin B_{12} in the kidney may be derived from a study of its precise subcellular localization within the renal tissue. Such investigations with rat liver (10, 11), mouse liver (12), rat brain (13), and rat heart (9) have shown there to be a common concentration of vitamin B_{12} in the mitochondrial fraction separated from each tissue, although some contradictory findings have been reported (2, 14). In the case of rat kidney three independent investigations have not found any particular association of vitamin B_{12} with mitochondrial fractions (11, 15, 16).

In the experiments reported in this paper

[1] This work was initiated while Peter Newmark was the recipient of a Mary Goodger Scholarship and continued with support from the Medical Research Council.

[2] Throughout this paper the term vitamin B_{12} is used nonspecifically to cover the whole group of cobalamins.

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the subcellular localization of vitamin B_{12} in rat kidney has been reinvestigated. Evidence for a specific association of vitamin B_{12} with a lysosome-rich subcellular fraction was obtained and some properties of the associated vitamin B_{12} were investigated.

MATERIALS AND METHODS

The animals were Wistar albino rats, usually 4-6 months old. After starving overnight the abdomen was opened under ether anesthesia, as much blood as possible was removed from the dorsal aorta and the kidneys were then removed into chilled medium. The animals were then killed.

Homogenization. At all stages, unless mentioned, tissue fractions were maintained at 0–4°. Extensive preliminary investigations (9) of modifications of standard techniques established a reasonably satisfactory technique for obtaining effective homogenization of kidney tissue with consistent maintenance of the integrity of the subcellular particles, in particular the mitochondria. Homogenization of the tissue in a medium of 0.25 M sucrose, 1 mM EDTA, pH 7.4 was carried out with a micro Waring-type blender (total capacity 8 ml) driven at 20,000 rpm for 30 sec. Precooling and operation of the apparatus in a cold room minimized the temperature rise to 15°.

Subcellular fractionation. Subcellular fractionation was carried out by differential centrifugation. The homogenate (10% w/v) was centrifuged at 700*g* for 3 min and the supernatant fluid removed. After two washings of the nuclear sediment, each with recentrifugation at 700*g* for 3 min, the three supernatant fluids were pooled and recentrifuged at 700*g* for 1 min to give a supernatant S_1 and a sediment which was added to the nuclear sediment. S_1 was centrifuged at 10,000*g* for 10 min to give a supernatant S_2 and a mitochondrial sediment. S_2 was centrifuged at 114,000*g* for 60 min to give a microsomal sediment and the soluble supernatant fraction. Sediments were resuspended, by means of a hand-operated Potter-Elvehjem homogenizer, in the homogenization medium described earlier to give the corresponding fraction.

The procedure used to separate the mitochondrial fraction into its subfractions was similar to that of Shibko and Tappel (17). It consisted of the individual resuspension in the homogenization medium of the three visibly distinct layers of the mitochondrial sediment, as produced by centrifugation of S_1 at 10,000*g* for 10 min. The top fluffy layer (M_1) was first removed and resuspended by swirling with medium. The large, fawn-colored middle layer (M_2) was then separated from the bottom, dark-brown pellet (M_3) by careful direction of a jet of medium from a Pasteur pipette. M_1 and M_2 were resuspended either with a Pasteur pipette or with a very loose, hand-operated Potter–Elvehjem type homogenizer.

For further separation of lysosomes from mitochondria, density-gradient centrifugation was used. One milliliter of an M_2 or M_3 fraction was layered on top of a 4-ml linear, 30-80% sucrose gradient and centrifuged at 165,000*g* for 80 min in a swing-out rotor. The bottom of the centrifuge tube was then pierced and the contents collected dropwise into 10 successive fractions.

Characterization of fractions. Mitochondria were characterized by their respiratory activity which was measured manometrically at 25° with α-ketoglutarate as substrate. The reaction mixture consisted of 0.5 ml of tissue fraction, 30 μmoles α-ketoglutarate, pH 7.4, 30 μmoles potassium phosphate buffer, pH 7.4, 6 μmoles magnesium chloride, 5 μmoles ADP, and 500 μmoles sucrose in a final volume of 3 ml. The tissue fraction was incubated in the reaction mixture for 10 min to allow temperature equilibration before the zero time reading was taken. Thereafter readings were taken every 10 min for at least 60 min. During this period oxygen uptake increased almost linearly with time. Results are expressed as QO_2 (μlO_2 consumed/hr/mg protein).

Lysosomes were characterized by acid phosphatase and β-glucuronidase activity. Acid phosphatase was determined either by measurement of inorganic phosphate release from β-glycerophosphate at pH 5.0 (18) or by measurement of *p*-nitrophenol release from *p*-nitrophenyl disodium orthophosphate at pH 4.8 (19). In both cases the reaction was carried out for 10 min at 37° and in the presence of 0.1% Triton X-100 (to ensure total release of enzyme activity). For the measurement of inorganic phosphate the Technicon autoanalyzer procedure based on the method of Fiske and Subbarow (20) was used. For the estimation of *p*-nitrophenol, the absorbancy at 405 mμ was measured, having removed any particulate matter by centrifugation if necessary.

β-Glucuronidase activity was determined by measurement of phenolphthalein release from phenolphthalein glucuronic acid (sodium salt, Sigma) at pH 5.2 (21). The reaction was carried out in the presence of 0.1% Triton X-100 and was continued for 60 min at 37° during which time the rate of release of phenolphthalein was found to be constant. For the estimation of phenolphthalein the absorbancy at 545 mμ was measured at alkaline pH, having removed any particulate matter by centrifugation.

Protein was determined by the biuret method (22) usually directly on the tissue fractions but

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... fractions from sucrose-density gradients, on their TCA-precipitated proteins redissolved in ... sucrose, 1 mM EDTA, pH 7.4. This medium ... also to be used in the setting up of standard curves for the method.

Administration and measurement of vitamin B_{12}. Radioactive cyanocobalamin, either ^{57}Co- or ^{58}Co-labeled, was obtained from the Radiochemical Centre, Amersham and stored in the dark at 4°. The various batches ranged in their specific activity from 17.7–168.0 μCi/μg. The dose for *in vivo* experiments with rats was 1 μCi (5.9–56.5 mμg). The doses were administered intramuscularly, in a volume of 0.25–0.4 ml into the right hind leg of rats under light anesthesia.

The radioactivity of whole animals or carcasses was measured either in a plastic phosphor-well scintillation counter (23) or else in a Packard liquid scintillation detector (model 446 Armac). Samples of tissue fractions were counted either in a scintillation counter using a Harwell scaler (type 1000B) or else a Packard autogamma spectrometer (model 52.3). Counting time was usually 10 or 20 min.

Total tissue vitamin B_{12} was assayed microbiologically with *Lactobacillus leichmanii* using a method developed for serum (24) but later applied to tissues (10, 13). Since the EDTA of the tissue medium was found to depress the growth response of the microorganism, due allowance had to be made for this in constructing assay calibration curves.

RESULTS

Subcellular distribution and recovery of mitochondrial and lysosomal enzyme activities. In six experiments the kidney homogenate had a mean respiratory activity (α-ketoglutarate substrate) of 6300 μl O_2/hr/g fresh tissue, with a QO_2 of 27.7 (Table I). The much higher QO_2 of the mitochondrial fraction confirmed that the mitochondria were concentrated in that fraction. About 40% of the respiratory activity of the homogenate was recovered in the mitochondrial fraction although the total recovery in all the fractions was only 54.4 ± 6.9%. Reconstitution of the mitochondrial fraction with the soluble supernatant fraction in two experiments increased the mean recovery of the respiratory activity of the homogenate in it from 46.3 to 86.2%. This suggested that a soluble constituent contributed in some way to the respiration of the mitochondria. Whatever may be the effect, the finding that reconstitution of the full respiratory activity was so easy indicated that the mitochondria could not be greatly damaged.

In four experiments the subcellular distribution of two lysosomal enzymes, acid phosphatase and β-glucuronidase, was also determined (Table I). Both enzymes were concentrated in the mitochondrial fraction indicating that this fraction also contained the lysosomes.

Subcellular localization of vitamin B_{12}. Six rats were used to determine the subcellular distribution of the total microbiological vitamin B_{12} activity and of a small dose of ^{58}Co–cyanocobalamin at various times after its intramuscular administration. The microbiological vitamin B_{12} activity was predominantly associated with the mitochondrial fractions, with only very small amounts associated with microsomal fractions (Table II). The distribution of ^{58}Co–cyanocobalamin among the subcellular fractions was almost identical to that of the microbiological vitamin B_{12} activity at all times after injection (Table II). Even such variations as are shown were closely paralleled by the microbiological vitamin B_{12} distribution when these figures were considered for individual experiments. It thus appeared that the ^{58}Co–cyanocobalamin had equilibrated with the tissue vitamin B_{12} as regards distribution within 2 days of administration and that this distribution was maintained throughout the period studied, despite the rapid loss of total ^{58}Co–cyanocobalamin from the kidney over this period (Table II). The concentration of the microbiological vitamin B_{12} (mμg B_{12}/mg protein) and the relative concentration of the radioactive B_{12} (per cent of homogenate radioactivity/per cent of homogenate protein) was calculated for each fraction. These results were combined with those from four other similar experiments in which rats were administered 6–17 mμg ^{57}Co–cyanocobalamin and the kidney subcellular distribution of radioactivity, but not of microbiological B_{12}, was measured 6–29 days later. The results show that both microbiological and radioactive vitamin B_{12} concentration were greatest in the mitochondrial fractions (Table III).

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TABLE I

PROTEIN CONTENT AND ENZYME SPECIFIC ACTIVITIES OF HOMOGENATES AND SUBCELLULAR FRACTIONS OF RAT KIDNEY[a]

		Whole homogenate	Subcellular fractions			Recovery of homogenate activity (%)
			Nuclear	Mitochondrial	S_1	
Protein content (mg/g fresh tissue)	(10)	232.0 ± 29.1	48.7 ± 7.6	89.3 ± 6.0	94.4 ± 7.0	96.3 ± 4.3
QO_2 (α-ketoglutarate) (μl O_2/hr/mg protein)	(6)	27.7 ± 4.3	21.8 ± 12.0	75.6 ± 8.3	17.3[b]	54.4 ± 6.9
Acid phosphatase (p-nitrophenyl phosphate) (mμmoles/min/mg protein)	(4)	39.7 ± 13.1	40.3 ± 11.6	60.1 ± 15.4	49.2 ± 8.2	112.0 ± 29.0
β-glucuronidase (phenolphthalein glucuronic acid) (mμmoles/min/mg protein)	(4)	0.28 ± 0.23	0.30 ± 0.28	0.51 ± 0.24	0.18 ± 0.10	123.3 ± 27.1

[a] The methods used are all described in the text. Results are expressed as means ± standard deviation and the number of determinations is given in parentheses in the first column.

[b] One determination only.

TABLE II

SUBCELLULAR DISTRIBUTION OF MICROBIOLOGICAL AND RADIOACTIVE B_{12} IN RAT KIDNEY[a]

B_{12} measured	Days after injection	B_{12} content of homogenate (mμg/g fresh tissue)	Recovery of homogenate B_{12} in fractions (%)				Total recovery (%)
			Nuclear	Mitochondrial	Microsomal	Soluble supernatant	
Radioactive	2 (1)	5.4[b]	22.5	49.3	2.9	19.8	94.3
	8 (1)	4.9[b]	23.3	60.8	2.4	16.1	102.6
	15 (2)	3.8[b]	20.8	54.0	2.4	17.2	95.3
	22 (2)	2.9[b]	19.0	52.3	2.3	20.5	94.1
Microbiological	—[c] (6)	3610 ± 304	19.5 ± 0.6	55.3 ± 5.8	2.2 ± 0.3	18.3 ± 5.4	95.4 ± 7.2

[a] Rats were given 20 mμg ^{60}Co-cyanocobalamin by intramuscular injection. All methods are described in the text. The number of experiments is given in parentheses in the second column.

[b] These figures are calculated from the total counts of the kidney homogenate and the specific activity of the injected dose.

[c] The microbiological B_{12} levels are the means ± standard deviations measured in the same six animals in which the distribution of radioactivity was determined.

Partial purification of lysosomes and the determination of their vitamin B_{12} content. The mitochondrial sediment produced by centrifuging S_1 at 10,000*g* for 10 min possessed three visibly distinct layers or subfractions (M_1, M_2, and M_3) which were investigated for their mitochondrial and lysosomal enzyme activities. The enzyme specific activities are shown in Table IV. The respiratory specific activity was greatest in M_2 whereas the specific activities of acid phosphatase and β-glucuronidase were greatest in M_3. Thus M_2 is relatively rich in mitochondria and M_3 in lysosomes.

The distribution of microbiological vitamin B_{12} activity and of radioactivity in the mitochondrial subfractions was investigated 6-29 days after administration of ^{57}Co- or ^{60}Co cyanocobalamin (Table V). Over this time period there appeared to be

TABLE III

CONCENTRATION OF MICROBIOLOGICAL AND RADIOACTIVE VITAMIN B_{12} IN RAT KIDNEY HOMOGENATES AND SUBCELLULAR FRACTIONS[a]

		Homogenate	Subcellular fractions			
			Nuclear	Mitochondrial	Microsomal	S_1
Microbiological B_{12} concentration (mμg B_{12}/mg protein)	(6)	17.1 ± 1.4	10.2 ± 0.9	22.8 ± 2.1	2.5 ± 0.3	10.6 ± 2.6
Radioactive B_{12} relative concentration $\left(\frac{\text{\% homogenate radioactivity}}{\text{\% homogenate protein}}\right)$	(10)	—	1.08 ± 0.19	1.56 ± 0.18	0.18 ± 0.01	0.71 ± 0.08

[a] Rats were given 6–20 mμg ^{60}Co- or ^{58}Co-cyanocobalamin 2–29 days before the experiment. All methods are described in the text. Results are expressed as means ± standard deviation, and the number of experiments is given in parentheses in the first column.

TABLE IV

PROTEIN CONTENT AND ENZYME SPECIFIC ACTIVITIES OF MITOCHONDRIAL FRACTIONS AND SUBFRACTIONS OF RAT KIDNEY[a]

		Whole mitochondrial fraction	Subfractions of mitochondrial fraction			Recovery of mitochondrial fraction activity (%)
			M_1	M_2	M_3	
Protein content (mg/g fresh tissue)	(10)	82.0 ± 13.1	22.7 ± 5.6	44.0 ± 5.6	10.1 ± 4.8	98.7 ± 7.4
Q_{O_2} (α-ketoglutarate) (μl O_2/hr/mg protein)	(6)	38.5 ± 6.4	12.2 ± 7.3	37.9 ± 10.8	6.7 ± 8.0	62.4 ± 5.0
Acid phosphatase (β-glycerophosphate) (μg P_i/10 min/mg protein)	(2)	5.2	2.9	9.3	32.9	141.0
Acid phosphatase (p-nitrophenyl phosphate) (mμmoles/min/mg protein)	(5)	51.2 ± 16.2	41.9 ± 13.5	44.9 ± 15.2	117.1 ± 45.5	102.2 ± 19.1
β-glucuronidase (phenolphthalein glucuronic acid) (mμmoles/min/mg protein)	(3)	0.56	0.29	0.46	1.40	80.7

[a] The methods used are all described in the text. Results are expressed as means; standard deviations are included when justified by the number of determinations, as given in parentheses in the first column.

significant changes in the distribution of radioactivity. This and the similarity of the distribution of radioactivity to that of the total vitamin B_{12} (Table V), suggests that equilibration had taken place within 6 days of administration. The concentration of the microbiological vitamin B_{12} (mμg B_{12}/mg protein) and the relative concentration of the radioactive B_{12} (per cent homogenate radioactivity/per cent homogenate protein) was calculated for each subfraction and the results show that both were about six times as high in M_3 as in any other subfraction (Table VI).

TABLE V

DISTRIBUTION OF MICROBIOLOGICAL AND RADIOACTIVE VITAMIN B_{12} IN SUBFRACTIONS OF THE RAT KIDNEY MITOCHONDRIAL FRACTION[a]

B_{12} measured	Days after injection	B_{12} content of mitochondrial fraction (mμg/g fresh tissue)	Recovery of mitochondrial B_{12} in subfractions (%)			Total recovery (%)
			M_1	M_2	M_3	
Radioactive	6 (1)	2.7[b]	4.5	41.2	45.7	91.3
	15 (2)	1.6[b]	5.6	43.4	43.5	92.6
	22 (2)	1.5[b]	9.0	39.7	48.4	96.0
	25 (1)	0.7[b]	3.8	43.1	48.9	95.8
	29 (1)	0.4[b]	3.0	48.6	54.5	106.1
Microbiological	— (3)	2010	7.1	38.9	46.9	92.2

[a] Rats were given between 6 and 20 mμg ^{57}Co- or ^{58}Co-cyanocobalamin by intramuscular injection. A methods are described in the text. The number of experiments is given in parentheses in the seco column.

[b] These figures are calculated from the total counts of the mitochondrial fraction and the specif activity of the injected dose.

[c] The microbiological B_{12} levels are the means measured in one of the 15-day, and both of the 22-da animals in which the distribution of radioactivity was determined.

TABLE VI

CONCENTRATION OF MICROBIOLOGICAL AND RADIOACTIVE VITAMIN B_{12} IN RAT KIDNEY MITOCHONDRIA FRACTIONS AND SUBFRACTIONS[a]

		Mitochondrial fraction	Mitochondrial subfractions		
			M_1	M_2	M_3
Microbiological B_{12} concentration (mμg B_{12}/mg protein)	(3)	24.4	6.2	17.7	92.9
Radioactive B_{12} relative concentration $\left(\frac{\%\ \text{homogenate radioactivity}}{\%\ \text{homogenate protein}}\right)$	(7)	—	0.31 ± 0.07	1.23 ± 0.21	7.13 ± 1.97

[a] Rats were given 6–20 mμg ^{57}Co- or ^{58}Co-cyanocobalamin 6–29 days before the experiment. A methods are described in the text. Results are expressed as means; standard deviations are include where justified by the number of experiments, as given in parentheses in the first column.

Further purification of lysosomes by means of density-gradient centrifugation. The lysosome-rich M_3 subfractions separated from mitochondrial fractions of rats given ^{57}Co- or ^{58}Co-cyanocobalamin were further purified by density-gradient centrifugation. Fractions were removed from the gradient and analyzed for their acid phosphatase and β-glucuronidase activity and for their protein and radioactive vitamin B_{12} content. Figure 1 shows the results for a typical experiment in which 9.8 mμg of ^{57}Co-cyanocobalamin had been intramuscularly administered to a rat 15 days before investigation. The radioactivity was concentrated in the same fraction as the lysosomal enzymes which was derived from a major, high-density band, well separated from the other bands. Identical density gradient centrifugation of M_3 fractions gave a relatively minor band in the high-density region, with very much larger bands corresponding to the two lowest density bands shown in Fig. 1.

In vitro uptake and release of ^{57}Co-cyanocobalamin by lysosomes. The lysosome-rich M_3 subfraction separated from a stock rat was tested for its ability to take up ^{57}Co-cyanocobalamin during *in vitro* incubation. Tubes containing 1.2 mg protein of an M_3

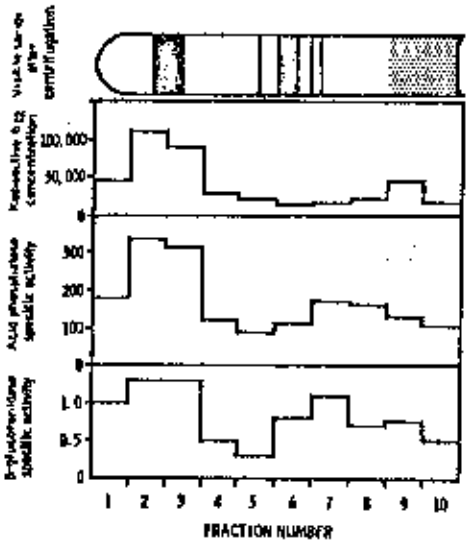

FIG. 1. Concentration of radioactivity and specific activity of acid phosphatase and β-glucuronidase in the fractions obtained after density-gradient centrifugation of a rat kidney M_3 (lysosome-rich) mitochondrial subfraction. The rat was given 0.5 mμg ^{57}Co-cyanocobalamin 15 days prior to the experiment. The M_3 fraction was obtained and centrifuged on a density gradient as described in Materials and Methods. After centrifugation four distinct bands were visible as illustrated at the top of this figure; the stippled area indicates the volume originally occupied by the sample of M_3. The total contents of the gradient were collected into 10 successive fractions after piercing the bottom of the tube and each fraction was assayed for acid phosphatase, β-glucuronidase, protein, and ^{57}Co-radioactivity. Enzyme specific activities are calculated as nμmoles substrate used/min/mg protein; radioactive B_{12} concentration as counts/30 min/mg protein.

fraction together with 6 μμg ^{57}Co–cyanocobalamin, in a total volume of 1 ml of 0.25 M sucrose, 1 mM EDTA, pH 7.4, were incubated either at 37° for 30 min or at 0° for 30, 60, or 240 min. After centrifugation at 10,000*g* for 15 min the radioactivity in the pellets was determined. Uptake was negligible (2–3%) in all cases.

An M_3 fraction was prepared from a rat previously given ^{57}Co–cyanocobalamin by the routine resuspension of the M_3 pellet in homogenization medium. The release of radioactivity from it into the soluble supernatant fluid during incubation at 37° over

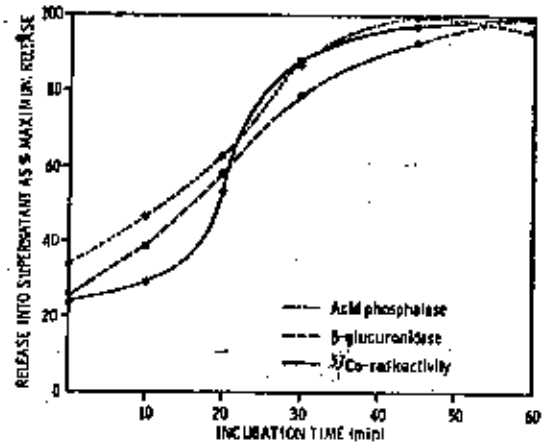

FIG. 2. The release of ^{57}Co-radioactivity, acid phosphatase, and β-glucuronidase from a rat kidney M_3 (lysosome-rich) mitochondrial subfraction, during incubation at 37°. The M_3 fraction (4.2 mg protein) was shaken in a water bath and samples were removed at 0, 15, 30, 45, and 60 min. Each sample was immediately cooled to 4° and then centrifuged at 10,000*g* for 15 min. The supernatant fractions were analyzed for enzyme activity and radioactivity. The total enzyme activities and total radioactivity were determined on a zero-time sample which was disrupted with Triton X-100 and not centrifuged. Maximum release (60 min) of radioactivity was 97% of the total, maximum release (45 min) of acid phosphatase was 67% of total, and maximum release (60 min) of β-glucuronidase was 75% of total.

60 min was compared with the release of acid phosphatase and β-glucuronidase activities. The results are shown in Fig. 2 and are expressed in terms of the release into soluble form over a given time as a percentage of the maximum released activity. Although this maximum appeared to be reached by 60 min in each case only the radioactivity showed 100% release. A maximum of about 70% of either enzyme was released, the other 30% presumably being membrane bound (Fig. 2 legend).

DISCUSSION

Previous investigations of the subcellular distribution of radioactive cyanocobalamin in the kidney have given inconsistent results (11, 15, 16), but in no case has the mitochondrial fraction (presumably also including intact lysosomes) unequivocably contained the greatest amount of radioactivity.

In the experiments reported here, however, the mitochondrial fraction contains at least 50% of both the microbiological and the radioactive vitamin B_{12}. In the case of the radioactive B_{12} the discrepancy between these and previous results could be due to the different routes of administration of the radioactive cyanocobalamin or due to the different amounts administered, but this could not account for the discrepancy in the microbiological B_{12} distribution. A more likely explanation for both discrepancies is the efficiency of the different methods used for homogenization and subcellular fractionation of the kidney. We have previously shown that the method used for homogenization of a tissue (heart muscle) can greatly affect the apparent subcellular distribution of vitamin B_{12} inasmuch as a homogenization method that inadvertently damaged subcellular particles led to a false redistribution of the vitamin (9). The results of enzyme distribution given here at least show that in this study there was a reasonable recovery of intact mitochondria and lysosomes in one fraction (the mitochondrial fraction). The earlier investigators (11, 15, 16) did not report the data necessary to judge the efficiency of homogenization and subcellular fractionation in their experiments.

The present demonstration of a mitochondrial concentration of microbiological vitamin B_{12} is very similar to that in several other tissues of the rat (9–11, 13) and in mouse liver (12). The subcellular distribution of radioactive cyanocobalamin has equilibrated with that of endogenous vitamin B_{12} within 2 days of administration. This is considerably more rapid than in other rat tissues where it takes 2–3 weeks (2, 10, 13). The fact that the subcellular distribution of radioactivity does not change, even while the total radioactivity of the kidney falls by about 50% during the first 3 weeks after administration, must mean either that there is equal loss from each subcellular fraction, or that the loss is accompanied by continual redistribution of radioactivity.

Further separation of the kidney mitochondrial sediment led to a partial separation of lysosomes from mitochondria. The success of this separation is shown by the distribution of enzymes (Table IV and Fig 1) and is similar to that reported by other workers using essentially similar techniques (17, 25, 26). The distribution studies reported here also indicate that a lysosome-rich fraction contains at least 25% of the total kidney vitamin B_{12} at a concentration (mμg/ml protein) about six times greater than in any other fraction, and that it contains a similar concentration of radioactivity after intramuscular radioactive cyanocobalamin administration.

Further support for the localization of vitamin B_{12} in the kidney lysosomes comes from the comparison of release of radioactivity with the release of lysosomal enzymes during incubation of the M_3 subfraction at 37° for up to 60 min, conditions under which lysosomes are known to be labile (17). The release of all three activities showed similar kinetics (Fig 2) suggesting that their origin was from the same subcellular particle.

There are two main routes by which vitamin B_{12} could reach kidney lysosomes. One is the direct route to the renal tissue via the blood supply. The other is via the tubular reabsorption of any vitamin B_{12} that has passed through the glomerular filter. In man, free cyanocobalamin apparently is not reabsorbed, as shown by the success with which glomerular filteration rates have been measured with it (27, 28). However, with autoradiographic studies after administration of radioactive cyanocobalamin, Salan using rats (29) and Flodh, using mice (30), have shown that there is a specific concentration of radioactivity in the kidney proximal tubule, suggesting its tubular reabsorption. If so, then this is presumably reabsorption of protein-bound B_{12} rather than of the free vitamin. Consistent with this possibility are the observations that tubular reabsorption of proteins takes place in the proximal convoluted tubules of the rat (31, 32) and the fact that the rat serum protein that binds vitamin B_{12} *in vitro* is of low molecular weight (33), similar to that of man (molecular weight 37,000) (34) and therefore could relatively easily, perhaps freely (35), traverse the glomerular filter of the rat.

Studies of the reabsorption of proteins by kidney proximal tubule cells of animals

including the rat have shown that the process involves micropinocytosis of the proteins into vacuoles which have the characteristics of lysosomes (25, 31, 36). Filtered proteins to which vitamin B_{12} was bound presumably could be reabsorbed similarly, thus accounting for the lysosomal concentration of vitamin B_{12} demonstrated in this paper.

If vitamin B_{12} does reach kidney lysosomes by pinocytosis of a protein-B_{12} complex, it would be worth reinvestigating the possibility of an analogous mechanism of uptake of intrinsic factor-bound vitamin B_{12} by the ileal epithelial cells (37). A similar failure of a lysosome-mediated uptake of protein by both kidney and intestinal epithelial cells could account for the pathological association of proteinuria and a congenital, selective, intrinsic factor-B_{12} complex malabsorption (Imerslund-Gräsbeck disease) occasionally found in children (38). However, recent evidence suggests that lysosomes are not involved in the ileal absorption of the vitamin in the guinea pig (39).

Whether vitamin B_{12} is found in the kidney lysosomes as an adjunct to the conservation of serum proteins by tubular reabsorption, and whether it has a functional role there, remain to be seen.

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REFERENCES

1. Dryden, L. P., and Hartman, A. M., *J. Nutr.* **90**, 382 (1966).
2. Gräsbeck, R., Ignatius, R., Järnefelt, J., Lindén, H., Mali, A., and Nyberg, W., *Clin. Chim. Acta* **6**, 56 (1961).
3. Rosenblum, C., Chow, B. F., Condon, G., and Yamamoto, R. S., *J. Biol. Chem.* **198**, 915 (1952).
4. Harte, R. A., Chow, B. F., and Barrows, L., *J. Nutr.* **49**, 669 (1953).
5. Okuda, K., *J. Nutr.* **77**, 131 (1962).
6. Uchino, H., Ukyo, S., Yagiri, Y., Yoshino, T., and Wakisaka, G., *Ann. N.Y. Acad. Sci.* **112**, 844 (1964).
7. Heinrich, H. C., and Gabbe, E. E., *Ann. N.Y. Acad. Sci.* **112**, 871 (1964).
8. Beck, W. S., Flavin, M., and Ochoa, S., *J. Biol. Chem.* **229**, 997 (1957).
9. Newmark, P. A., D. Phil. thesis, University of Oxford (1967).
10. Newman, G. E., O'Brien, J. R. P., Spray, G. H., and Witts, L. J., *in* "Vitamin B_{12} und Intrinsic Factor. 2. Europäisches Symposion" (H. C. Heinrich, ed.), p. 424. Enke, Stuttgart (1962).
11. Strength, D. R., Alexander, W. F., and Wack, J. P., *Proc. Soc. Exp. Biol. Med.* **102**, 15 (1950).
12. Swendseid, M. E., Bethell, F. H., and Ackermann, W. W., *J. Biol. Chem.* **190**, 701 (1951).
13. Newman, G. E., O'Brien, J. R. P., Spray, G. H., Williams, D. L., and Witts, L. J., *Biochim. Biophys. Acta* **64**, 438 (1962).
14. Rachmilewitz, M., Stein, Y., Stein, O., Aronovitch, J., and Grossowicz, N., *Haematol. Lat.* **2**, 297 (1959).
15. Barrows, C. H., and Chow, B. F., *Proc. Soc. Exp. Biol. Med.* **95**, 517 (1957).
16. Tan, C., Hansen, H. J., and Miller, O. N., *Fed. Proc.* **25**, 430 (1966).
17. Shibko, S., and Tappel, A. L., *Biochem. J.* **95**, 731 (1965).
18. Appelmans, F., Wattiaux, R., and de Duve, C., *Biochem. J.* **59**, 438 (1955).
19. Linhardt, K., and Walter, K., *in* "Methods of Enzymatic Analysis" (Bergmeyer, H. V., ed.) second printing, revised, p. 783. Verlag Chemie/Academic Press, Weinheim/New York (1965).
20. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.* **66**, 375 (1925).
21. Gianetto, R., and de Duve, C., *Biochem. J.* **59**, 433 (1955).
22. Gornall, A. G., Bardawill, C. J., and David, M. M., *J. Biol. Chem.* **177**, 751 (1949).
23. Warner, G. T., and Oliver, R., *Brit. J. Radiol.* **35**, 349 (1962).
24. Spray, G. H., *Clin. Sci.* **14**, 661 (1955).
25. Maunsbach, A. B., *J. Ultrastruct. Res.* **15**, 197 (1966).
26. Straus, W., *J. Biophys. Biochem. Cytol.* **2**, 513 (1956).
27. Nelp, W. B., Wagner, H. N., and Reba, R. C., *J. Lab. Clin. Med.* **63**, 480 (1964).
28. Weeke, E., *Scand. J. Clin. Lab. Invest.* **21**, 139 (1968).
29. Salmi, H. A., *Ann. Acad. Sci. Fennicae, Ser. A5*, 103, (1963).
30. Flodh, H., *Acta. Radiol. Suppl.* 284 (1968).

31. Straus, W., *J. Biophys. Biochem. Cytol.* 3, 1037 (1957).
32. Fisher, E. R., *Exp. Mol. Pathol.* 3, 304 (1964).
33. Newmark, P. A., Unpublished observations.
34. Hom, B., Oleson, H., and Lous, P., *J. Lab. Clin. Med.* 68, 958 (1966).
35. Venkatachalam, M. A., Karnovsky, M. J., and Cotran, R. S., *J. Exp. Med.* 130, 381 (1969).
36. Straus, W., *J. Histochem. Cytochem.* 15, 37[illegible] (1967).
37. Wilson, T. H., *Physiologist* 6, 11 (1963).
38. Gräsbeck, R., Gordin, R., Kantero, I., and Kuhlbäck, B., *Acta Med. Scand.* 167, 2[illegible]9 (1960).
39. Peters, T. J., Quinlan, A., and Hoffbrand, A. V., *Clin. Sci.* 37, 568 (1969).

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THE MECHANISM OF UPTAKE OF VITAMIN B_{12} BY THE KIDNEY OF THE RAT *IN VIVO*

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SUMMARY

1. The purpose of this investigation was to study the appearance and significance of protein-bound [^{57}Co]vitamin B_{12} in the rat kidney 0.25–4.0 h after intramuscular injection of [^{57}Co]cyanocobalamin.

2. The total amount of [^{57}Co]vitamin B_{12} in the kidney increased over the time period studied.

3. A study of the subcellular distribution of the [^{57}Co]vitamin B_{12} demonstrated that the radioactivity of the lysosome-rich fraction and the soluble supernatant increased with time at the expense of the mitochondria-rich fraction and the microsomal fraction.

4. Initially most of the [^{57}Co]vitamin B_{12} of both the mitochondria-rich and lysosome-rich fractions was protein bound, but with increasing time the bound proportion fell markedly.

5. Gel filtration on Sephadex G-200 indicated the identity of the major vitamin B_{12}-binding protein in the kidney fractions, the serum and the urine.

6. These data are consistent with the theory that vitamin B_{12}, bound to a low-molecular-weight protein of rat serum, passes through the glomerular filter into the tubules where most of the B_{12}–protein complex is then taken into the renal tissue by a process of endocytosis, with a subsequent release of the B_{12} from the complex taking place in the lysosomes.

INTRODUCTION

When cyanocobalamin is given to a rat of normal vitamin B_{12} status by intramuscular injection the kidney rapidly accumulates a greater percentage of the administered vitamin than any other tissue[1]. In an earlier communication we demonstrated that the [^{57}Co]vitamin B_{12}* ([^{57}Co]B_{12}) present in the renal tissue 6 days after intramuscular administration of [^{57}Co]cyanocobalamin ([^{57}Co]CNB_{12}) to rats had the same subcellular distribution as the endogenous B_{12}, both being concentrated in a lysosome-rich subcellular fraction[2]. It was suggested that this association of vitamin

* Throughout this paper the term vitamin B_{12} (B_{12}) refers non-specifically to the whole group of cobalamins.

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B_{12} with lysosomes implied that its uptake by the kidney cells was by a process of pinocytosis and that this could involve tubular reabsorption of a complex of vitamin B_{12} with its specific binding protein in the serum. Further studies of subcellular distribution in the first 4 days after injection of [^{57}Co]B_{12} confirmed the rapid association of radioactivity with the lysosome-rich fractions and showed that whereas 1 h after injection about 20% of the [^{57}Co]B_{12} in the fraction was bound to protein, after 24 h less than 2% remained bound[3].

The experiments reported in the present paper were designed to substantiate the finding of protein-bound [^{57}Co]B_{12} in the kidney of the rat and to investigate both the time course of its appearance and the origin of the protein.

MATERIALS AND METHODS

Adult female Wistar Albino rats were used for all experiments except those involving collection of urine for which 100–150 g male rats of the same strain were used. [^{57}Co]CNB_{12} (50–300 μC/μg) was obtained from the Radiochemical Centre Amersham, and 1 μC doses were given to animals by intramuscular injection of the hind leg.

The animals were starved overnight prior to injection and starvation was continued until the end of the experiment. Serum was obtained from blood taken from the dorsal aorta of animals under ether anaesthesia, prior to removing the kidneys and killing the animals.

Homogenisation and fractionation of the kidneys was carried out in 0.25 M sucrose, 1 mM EDTA (pH 7.4) as described previously[2], with separation of the three layers of the mitochondrial sediment into the M_1, M_2 (mitochondria-rich) and M_3 (lysosome-rich) fractions[3]. Enzymatic characterisation of these fractions has been described[2].

Tissue fractions were prepared for gel filtration by sonication[3] which led to release of about 90% of the [^{57}Co]B_{12} into a non-sedimentable form. Urine and serum samples for gel filtration were untreated except for the removal of any precipitate by centrifugation. Gel filtration was carried out on Sephadex G25, medium or G200 (Pharmacia) at 4° in glass columns. Sample volume was 2.0 ml mixed with 0.5 ml 80% w/v sucrose. For G200 the method of HOM *et al.*[4] was used with 85 cm × 2.5 cm columns eluted with 0.1 M Tris (pH 8.0) containing 1 M NaCl; the void volume was 180 ml and free vitamin B_{12} was eluted starting at 405 ml. For G25 the same eluting buffer was used as for G200 but at a flow rate of 1.5 ml/min through a 25 cm × 2.5 cm column. The void volume was 48 ml and free vitamin B_{12} was eluted starting at 69 ml. Column effluents were monitored for absorbancy at 254 nm by an LKB 4701A Uvicord. Fraction volume was 3.0 ml.

^{57}Co radioactivity was measured in a Packard Autogamma Spectrometer. Recovery of radioactivity in gel filtration experiments was always greater than 85%. Microbiological vitamin B_{12} activity was assayed with *Euglena gracilis* using appropriate standards[5]. Protein was determined by a biuret method[6].

RESULTS

Subcellular distribution and concentration of [^{57}Co]B_{12}

The subcellular distribution of [^{57}Co]B_{12} was determined in duplicate experiments at 0.25, 0.5, 1 and 4 h after the intramuscular injection of 7.8–8.7 ng of [^{57}Co]-

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TABLE I

SUBCELLULAR DISTRIBUTION OF [^{57}Co]B_{12} IN RAT KIDNEY

Rats were given 7.8–8.7 ng, 1 μC of [^{57}Co]CNB_{12} by intramuscular injection. From 0.25–4.0 h later, as shown, the kidneys were removed and homogenised in 0.25 M sucrose, 1 mM EDTA (pH 7.4). Subcellular fractionation of the homogenate was then carried out and the ^{57}Co radioactivity of each fraction was determined. In addition, the protein content of homogenates and M_2 and M_3 fractions were determined. The mean results from duplicate experiments at each time interval are given. The figures in parentheses are the relative concentrations of [^{57}Co]B_{12} (% of homogenate [^{57}Co]B_{12}/% of homogenate protein).

Time after injection (h)	*% [^{57}Co]B_{12} dose in kidney*	*Recovery of homogenate [^{57}Co]B_{12} in fractions (%)*						*Total recovery (%)*
		Nuclear	*M_1*	*M_2*	*M_3*	*Microsomal*	*S_3*	
0.25	7.8	24.4	10.2	24.9 (1.2)	10.3 (2.1)	19.7	3.7	93.2
0.5	12.5	24.3	8.2	23.2 (1.3)	12.3 (3.1)	17.7	7.1	92.8
1.0	13.4	22.9	7.4	17.6 (1.0)	17.1 (4.5)	17.1	9.8	91.9
4.0	19.0	25.2	3.0	18.3 (1.0)	24.1 (7.1)	7.8	11.6	90.0
Control*	—	6.0	4.8	1.2	0.3	0.4	87.0	99.8

* For the control experiment a kidney homogenate from an untreated rat was incubated with 1.0 ng [^{57}Co]CNB_{12} for 10 min at 4° before subcellular fractionation.

CNB_{12} during which period the proportion of the injected dose appearing in the kidneys increased from 7.8% at 0.25 h to 19.0% at 4 h. The results are shown in Table I where the distribution is expressed as the percentage recovery of the total radioactivity of the homogenates in each fraction.

Whereas the percentage of [^{57}Co]B_{12} in the nuclear fraction remained constant, that in the M_3 (lysosome-rich) and S_3 (soluble supernatant) fractions increased with time at the expense of the M_1, M_2 (mitochondria-rich) and microsomal fractions. In addition the relative concentration of [^{57}Co]B_{12} (percentage of homogenate [^{57}Co]B_{12}/percentage of homogenate protein) in the M_2 and M_3 fraction was determined and it is seen that whereas the relative concentration of [^{57}Co]B_{12} decreased slightly with time in the M_2 fraction, it increased markedly in the M_3 fraction (Table I). A control experiment in which a rat kidney homogenate was incubated for 10 min at 4° with an amount of [^{57}Co]CNB_{12} equivalent to that found in the kidney 1 h after intramuscular injection showed that there was little [^{57}Co]B_{12} associated with any of the particulate fractions (Table I).

Protein-bound [^{57}Co]B_{12} in the M_2 and M_3 fractions

The proportions of protein-bound and free [^{57}Co]B_{12} in the supernatants obtained after sonication of M_2 and M_3 fractions prepared at 0.25–4.0 h after intramuscular injection of [^{57}Co]CNB_{12}, were determined by gel filtration on Sephadex G-200 and/or G-25. The proportion of protein-bound [^{57}Co]B_{12} decreased with time in both fractions and at all times the M_2 fraction contained a greater proportion of bound [^{57}Co]B_{12} than the M_3 fraction (Table II). On Sephadex G-200 the bound [^{57}Co]B_{12} always appeared as a single symmetrical peak just preceding the free [^{57}Co]B_{12} (Fig. 1) and therefore of low molecular weight. An example of a control experiment in which the supernatants of a sonicated M_2 or M_3 fraction were incubated

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TABLE II

PROPORTION OF PROTEIN BOUND [^{57}Co]B_{12} IN TWO SUBCELLULAR FRACTIONS OF RAT KIDNEY

M_2 (mitochondria-rich) and M_3 (lysosome-rich) subcellular fractions from the experiments described in Table I were sonicated and centrifuged. The supernatants were then subjected to gel filtration on Sephadex G-200 and/or G-25 in order to separate protein bound [^{57}Co]B_{12} from free [^{57}Co]B_{12}. The proportion of the former as a percentage of the total [^{57}Co]B_{12} was determined in duplicate experiments at each time interval and mean results are presented. The type of Sephadex used is not distinguished because it did not affect the results. In all experiments the total recovery of [^{57}Co]B_{12} was greater than 85%.

Time after injection (h)	*Proportion of protein-bound* [^{57}Co]B_{12} (%)	
	M_2	M_3
0.25	90.0	78.8
0.5	70.0	44.1
1.0	53.3	29.4
4.0	29.7	10.5
Control*	0.5	0

* For the control experiments M_2 and M_3 fractions were prepared from an untreated rat and sonicated and centrifuged. The supernatants were then incubated with 40–200 pg of [^{57}Co]B_{12} for 30 min at 37° and then the proportion of protein-bound [^{57}Co]B_{12} was determined with Sephadex G-200.

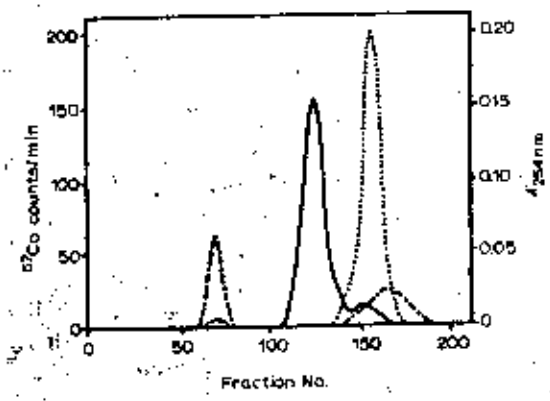

Fig. 1. Elution pattern from Sephadex G-200 of [^{57}Co]B_{12} in rat M_3 (mitochondria-rich) fractions either obtained 0.25 h after intramuscular injection of [^{57}Co]CNB_{12} (———), or obtained from an untreated rat and then incubated with [^{57}Co]CNB_{12} for 30 min at 37° *in vitro* (·····). Protein content ($A_{254\,nm}$) is also shown (–·—·–).

in vitro for 30 min at 37° with 40 pg [^{57}Co]B_{12} and then subjected to Sephadex G-200 gel filtration is also incorporated into Fig. 1 and it can be seen that apart from a trace eluted in the void volume, there was no protein-bound radioactivity.

Microbiologically active vitamin B_{12} in M_2 and M_3

In several of the experiments in which M_2 and M_3 were subjected to gel filtration on Sephadex G-200 the fractions eluted from the columns were assayed microbiologically for their total vitamin B_{12} content. Similar results were found in all

cases with one small peak of protein-bound vitamin B_{12} eluting in a position similar to the protein-bound [^{57}Co]B_{12} but with most of the activity eluting as free B_{12}. An example is shown in Fig. 2.

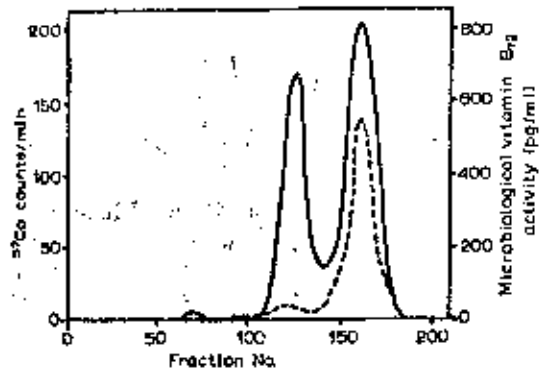

Fig. 2. Elution pattern from Sephadex G-200 of [^{57}Co]B_{12} (———), and microbiological vitamin B_{12} activity (------), in a rat kidney M_2 (lysosome-rich) fraction obtained 1 h after intramuscular injection of [^{57}Co]CNB_{12}.

Protein-bound [^{57}Co]B_{12} in serum

Sephadex G-200 gel filtration of serum obtained from rats 0.25 h and 4 h after intramuscular injection of 8.7 ng [^{57}Co]CNB_{12} showed that all the [^{57}Co]B_{12} was protein-bound. Fig. 3 shows the results of gel filtration of a 0.25 h sample. Most of the [^{57}Co]B_{12} was bound to a low molecular weight protein eluting after the third (albumin) peak of protein and in the same position as the binder in M_2 and M_3, but smaller amounts of radioactivity were also bound to higher molecular weight proteins. At 4 h relatively greater amounts of [^{57}Co]B_{12} were bound to the higher molecular weight proteins.

Protein-bound [^{57}Co]B_{12} in urine

On two occasions urine was collected continually for 5 h after the intramuscular

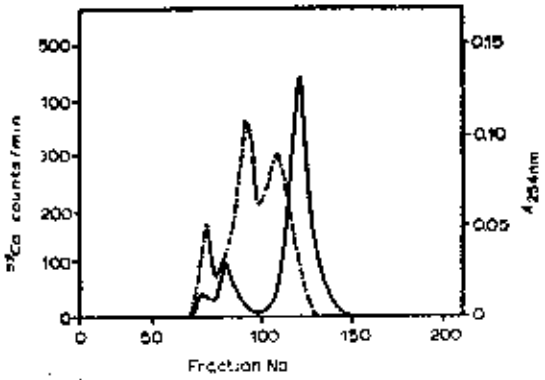

Fig. 3. Elution pattern from Sephadex G-200 of [^{57}Co]B_{12} (———), and protein ($A_{254\,nm}$; ------), in 2.0 ml rat serum obtained 0.25 h after intramuscular injection of [^{57}Co]CNB_{12}. The total radioactivity represents 0.9% (70 pg) of the injected dose.

injection of 8.7 ng [^{57}Co]CNB_{12}. In both cases about 0.4 % of the injected radioactivity was detected in the urine. Sephadex G 200 gel filtration showed it to be partly free and partly bound to a low-molecular-weight protein which was eluted in the same position as both the binder in the renal tissue fractions and the major binder in the serum. A small, but variable amount (8–24 pg/ml) of low molecular weight protein-bound radioactivity could also be demonstrated after the addition of 80–100 pg [^{57}Co]B_{12} *in vitro* to 5 h collections of urine from untreated rats.

DISCUSSION

We noted in an earlier study that 1 h after injection of [^{57}Co]CNB_{12} into rats the kidney M_3 (lysosome-rich) fraction appeared to contain a lower per cent of the radioactivity of the homogenate than at 24 h and over[3]. The present results (Table I) confirm and extend this to show that the radioactivity of the M_3 fraction increases from 10.3 % at 0.25 h to 17.1 % at 1 h, until by 4 h the radioactivity (24.1 %) and its relative concentration (7.1) have attained their maximum, equilibrium values as found previously at 24 h and over[3]. Initially, during the same period, the percentage of radioactivity in the M_2 (mitochondria-rich) fraction is greater than that of the M_3 fraction though still at a lower relative concentration (Table I). There is also a notably high initial localisation of [^{57}Co]B_{12} in the microsomal fraction. Although these figures could mean that the [^{57}Co]B_{12} first enters the mitochondria and microsomes and only then is transferred to lysosomes, rather than being absorbed by endocytosis (pinocytosis) as previously suggested[2,3] the latter appears to be a much more likely physiological process, particularly in view of the evidence (discussed below) that vitamin B_{12} is absorbed by the kidney as a complex with serum protein. An alternative explanation of the initial M_3 localisation can be based on the studies of STRAUS[7,8] on the uptake of intravenously injected horseradish peroxidase by rat kidney. He found that the concentration of this protein in his microsomal fraction (P Dr III) was greatest from 10 min to 1 h, in his mitochondria-rich fraction (M Dr II) at 1 h and in his nuclei/lysosome-rich fraction (N Dr I) only at 10 h after injection. Straus explained these results as the uptake of peroxidase into small phagosomes (endocytotic vacuoles), co-sedimenting with microsomes, which were then converted via intermediate size phagosomes, co-sedimenting with mitochondria, into large phagosomes (secondary lysosomes) co-sedimenting with nuclei[7], and substantiated this by microscopical evidence[8].

The changes in the subcellular distribution of [^{57}Co]B_{12} with time (Table I) are sufficiently similar to the horseradish peroxidase results to suggest that the same conclusions may be drawn as to their significance. That is, [^{57}Co]B_{12} is first taken up into small endocytotic vacuoles (sedimenting in the microsomal fraction) which then, as a result of fusion with primary lysosomes, increase in size so that they sediment with the mitochondria in the M_2 fraction and ultimately as secondary lysosomes in the M_3 fraction (compare STRAUS[7] who co-sedimented lysosomes with nuclei in N Dr I). Although we have no direct evidence that it is the secondary lysosomes of the M_3 fraction with which the vitamin B_{12} is associated, rather than with contaminating mitochondria or other subcellular particles, we have previously reported experiments that substantiate this contention[2]. These experiments demonstrated that [^{57}Co]B_{12} remained associated with the lysosomal enzymes on further purification of M_3 frac-

tions by density gradient centrifugation and that [^{57}Co]B_{12} was released from M_2 fractions with similar kinetics to two lysosomal marker enzymes.

The present study also demonstrated that the levels of [^{57}Co]B_{12} in the S_2 fraction (soluble supernatant) rose throughout the time period studied (Table I) and still had not attained their 24 h equilibrium level of 18 %[3] by 4 h. This increase may also be a reflection of the increasing concentration of [^{57}Co]B_{12} in the lysosomes, since any destruction of these relatively labile particles during homogenisation etc. will lead to redistribution of their [^{57}Co]B_{12} into S_2.

The proportion of protein-bound radioactivity in both the M_2 and M_3 fractions approached 100 % at the shortest time interval studied (0.25 h) after intramuscular injection of [^{57}Co]CNB_{12} (Table II). The protein-bound proportion fell with increasing time so that by 4 h more than 70 % of the [^{57}Co]B_{12} in both fractions was free (Table II) and we have previously demonstrated that by 24 h more than 97 % is free in the M_2 fractions[3]. These findings substantiate our previous suggestion[3] that the initial vitamin B_{12}–protein complex appearing in the renal tissue is subsequently broken down to release free B_{12}, probably by means of a lysosomal enzyme. The finding of a greater proportion of free [^{57}Co]B_{12} in M_3 than in M_2 at all times (Table II) corroborates this suggestion of lysosomal release of the vitamin. The identification of the last peak of radioactivity, eluted from M_2 and M_3 fractions on Sephadex G-200, as free [^{57}Co]-B_{12} rather than bound to the 254 nm absorbing material in the same peak (Fig. 1), was established by the elution of this radioactivity on Sephadex G-25 in the same position as authentic free [^{57}Co]CNB_{12}.

The evidence in this paper is also consistent with our previous hypothesis that the protein-bound [^{57}Co]B_{12} detected in the kidney results from the uptake of the intact complex of [^{57}Co]B_{12} with a vitamin B_{12}-binding protein of the serum[2,3]. Thus although there appears to be a protein that binds a small proportion of the endogenous vitamin B_{12} of the kidney M_2 and M_3 fractions (Fig. 2), it must be fully saturated with endogenous vitamin B_{12} because it is incapable of binding any [^{57}Co]-CNB_{12} added *in vitro* (Fig. 1). Consequently the [^{57}Co]B_{12}–protein complex found in the M_2 and M_3 fractions following injections of rats with [^{57}Co]CNB_{12} *in vivo* (Table II) is almost certainly derived from the serum. Additional evidence for this is the fact that both this renal tissue protein-bound [^{57}Co]B_{12} and the major component of the serum protein-bound [^{57}Co]B_{12} are eluted from Sephadex G-200 in the same position (Figs. 1 and 3), suggesting their identity. Their position of elution is similar to that of the low molecular weight, vitamin B_{12}-binding protein of human serum[4], sometimes known as transcobalamin II, which appears to be the primary vitamin B_{12} transport protein in man[9]. It was not possible to substantiate further the identity of the vitamin B_{12} binding protein of the renal tissue and serum of the rat by means of carboxymethyl cellulose column chromatography[10] because the tissue binder always precipitated during dialysis against the starting buffer.

The major vitamin B_{12}-binding protein in rat serum has a relatively low molecular weight as shown by its late elution from Sephadex G-200 (Fig. 3) and, as we have previously pointed out[2,3], this could allow for passage of the B_{12}–protein complex through the glomerular filter followed by uptake of the vitamin B_{12}–protein complex in the proximal convoluted tubules, where considerable endocytosis of proteins is known to occur[11]. The detection of a [^{57}Co]B_{12}–protein complex in rat urine either *in vivo* or *in vitro*, that eluted from Sephadex G-200 in the same position

as the major complex in rat serum and the renal tissue fractions, substantiates this possibility. Cooper has recently claimed that the low molecular weight B_{12} binder of serum detected *in vitro* actually exists *in vivo* as a polymer of higher molecular weight[13] and in support of this he was unable to detect any protein bound radioactive B_{12} either in the tubular fluid[11] or the urine* of the dog after intravenous injection of free radioactive vitamin B_{12}. This apparent discrepancy between species may be related to the fact that a very high proportion of the total urinary protein of the rat is β-globulin[13], a class to which the low molecular weight B_{12}-binding protein of serum belongs[14].

The preliminary findings of PLETSCH AND COFFEY[15,16] on the uptake of intravenously injected [^{57}Co]CNB_{12} by rat liver bear many resemblances to our earlier findings with the kidney[2,3], and those presented here. Initially [^{57}Co]B_{12} was concentrated in the liver microsomal fraction but later significant amounts were found in the lysosomes[15]. Part of the microsomal and the lysosomal [^{57}Co]B_{12} was bound to a protein which had similar characteristics to the B_{12}-binding protein in the serum[15]. Such similarities between these data for the liver and ours for the kidney suggest that endocytosis of a protein-B_{12} complex may be a mechanism of vitamin B_{12} uptake common to all tissues. The low molecular weight B_{12}-binding protein of serum may well act as a transport system by stimulating pinocytosis. The relatively basic nature of this protein[17] would support such a role[18] although direct attempts to prove it in tissue culture experiments have been unsuccessful[19].

ADDENDUM

After submission of this paper a full account of part of the work of PLETSCH AND COFFEY[20] became available. These authors have studied the uptake of [^{57}Co]CNB_{12} by rat liver 5 min–72 h after intracardiac administration, whilst confirming the general finding that the liver of the rat takes up much less B_{12} than the kidneys. As with the kidney, radioactivity was at first concentrated in the microsomal fraction and later passed into the soluble supernatant. However, in contrast to the kidney, radioactivity accumulated in the mitochondrial fraction with time whilst remaining more or less constant in the partially purified lysosome fraction. Preinjection of rats with Triton WR-1339 facilitated the separation of a much purer lysosome fraction and led to the demonstration of a transient (0.5-h peak) accumulation of [^{57}Co]B_{12} in the lysosomes although it is not clear to what extent this might have been a consequence of the Triton WR-1339 treatment. Further refined techniques made it possible to show that considerable [^{57}Co]B_{12} was initially associated with plasma membrane fragments in the microsomal fraction.

The interpretation placed on these results is the same as we have previously put forward for ours[2,3] and substantiate here: namely that vitamin B_{12} (as a complex with transcobalamin II) is taken up from circulation into tissues by a process of pinocytosis. The differences in the ultimate subcellular localisation of the vitamin B_{12} in the two tissues may reflect different roles for the vitamin.

* B. A. Cooper, personal communication.

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REFERENCES

1 H. C. Heinrich and E. E. Gabbe, *Ann. N.Y. Acad. Sci.*, 112 (1964) 871.
2 P. Newmark, G. F. Newman and J. R. P. O'Brien, *Arch. Biochem. Biophys.*, 141 (1970) 121.
3 P. Newmark, in H. R. V. Arnstein and R. J. Wrighton, *The Cobalamins, a Glaxo Symp., Greenford, 1970*, Churchill Livingstone, Edinburgh–London, 1971, p. 79.
4 B. L. Hom, H. Olesen and P. Lous, *J. Lab. Clin. Med.*, 68 (1966) 958.
5 B. B. Anderson, *J. Clin. Pathol.*, 17 (1964) 14.
6 A. G. Gornall, C. J. Bardawill and M. M. David, *J. Biol. Chem.*, 177 (1949) 751.
7 W. Straus, *J. Cell Biol.*, 12 (1962) 231.
8 W. Straus, *J. Cell Biol.*, 21 (1964) 295.
9 C. A. Hall, *Br. J. Haematol.*, 16 (1969) 429.
10 C. A. Hall and A. E. Finkler, *J. Lab. Clin. Med.*, 65 (1965) 459.
11 A. B. Maunsbach, in J. T. Dingle and H. B. Fell, *Lysosomes in Biology and Pathology*, Vol. 1, North Holland Publishing Co., Amsterdam–London, 1969, p. 115.
12 B. A. Cooper, *Blood*, 34 (1969) 856.
13 D. Mendel, *J. Physiol.*, 148 (1959) 1.
14 C. Lawrence, *Blood*, 33 (1969) 899.
15 Q. Pletsch and J. W. Coffey, *Fed. Proc.*, 28 (1969) 628.
16 Q. Pletsch and J. W. Coffey, *Fed. Proc.*, 30 (1971) 240. Abs.
17 A. E. Finkler and C. A. Hall, *Arch. Biochem. Biophys.*, 120 (1967) 79.
18 H. J.-P. Ryser, *Bull. Swiss Acad. Med. Sci.*, 24 (1968) 363.
19 H. J.-P. Ryser, *J. Cell Biol.*, 27 (1965) 92A.
20 Q. Pletsch and J. W. Coffey, *J. Biol. Chem.*, 246 (1971) 4619.

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Renal Failure and Serum Vitamin B_{12} Concentrations

By

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The significance of unusually high serum vitamin B_{12} levels is in most instances obscure. It is known, however, that an elevated serum B_{12} concentration can occur in some blood dyscrasias, hepatic disorders and renal failure (3). Except in very typical cases of myeloproliferative diseases, the finding of a high B_{12} content in the serum is of limited diagnostic value. The phenomenon is of some theoretical interest, however. During the years 1961—1963, we have had the opportunity to follow the serum vitamin B_{12} values in patients with severe acute and chronic renal failure. The results differ in some respects from those of other workers and will therefore be reported.

Material and Methods

The series studied comprised 14 patients with acute tubular necrosis and 20 patients with chronic renal failure. Because of a pronounced renal insufficiency all patients in the acute group and 15 patients in the chronic group were subjected to hæmodialysis. Seven subjects in the latter group had renal failure of moderate degree, and therefore no dialysis was performed. The primary causes of the acute tubular necrosis are given in Table I and the diagnoses of the patients with chronic renal disease are evident from Table II. The plasma creatinine values are included in the tables in order to give an idea of the degree of renal failure. No other clinical data or results of laboratory investigations will be presented, because no correlation between them and the serum B_{12} concentrations could be detected.

The control series consisted of 636 vitamin B_{12} determinations performed during the years 1956—1963 on healthy persons and hospital patients not suffering from renal diseases or other conditions known to affect the serum vitamin B_{12} level. These controls were collected within the regions of the cities of Vasa and Helsingfors.

The serum vitamin B_{12} was assayed microbiologically with *Euglena gracilis* strain z, according to the method of Hutner, Bach and Ross (2). The vitamin B_{12} level in the serum was determined before and after dialysis, and in some patients the assay was repeated 2—3 times.

Results

The microbiological vitamin B_{12} activities in the investigated sera are seen in Tables I and II. In both the acute and

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the chronic group of renal failure a slight increase in the serum vitamin B_{12} concentrations during haemodialysis was observed in some cases. This elevation was not significant, however. The mean serum vitamin B_{12} level in the chronic group was 396.2 ± 54.2 pg/ml and in the acute group 529.8 ± 88.0. The corresponding value for the controls was 351.8 ± 7.3. Thus a highly significant difference in the serum vitamin B_{12} concentrations between the control group and the patients with acute renal failure was found ($t = 4.20$, $p < 0.001$). No significant difference was seen between the controls and the group with chronic renal insufficiency.

There was no correlation between the vitamin B_{12} content of the serum and the plasma creatinine values or any other parameter of renal insufficiency.

Table I. Serum vitamin B_{12} concentrations and plasma creatinine values before and after haemodialysis in patients with acute tubular necrosis. The diagnoses refer to the primary cause of the renal damage.

Case No.	DIAGNOSIS	Serum B_{12} pg/ml		Plasma creatinine mg/100 ml	
		Before dialysis	After dialysis	Before dialysis	After dialysis
1	Mercury chloride intoxication	488	604	18.9	7.1
		524	560	19.1	9.3
2	Premature separation of placenta.		282	15.0	7.6
	Caesarean section	446	541	20.3	7.2
		384	376	20.0	9.3
3	Crush syndrome	672	672	17.5	11.8
		984	984	19.1	9.8
4	Gall-bladder disease	644	720	13.6	8.2
5	Tumour of the left kidney. Nephrectomy	324		13.2	
6	Tumour of the right kidney.	192	168	16.9	11.2
	Nephrectomy	432	420	21.3	11.3
7	Cerebral contusion. Multiple fractures	2184	1888	17.0	9.6
8	Sequels after abortion	447	512	17.0	10.2
		560	683	20.0	7.6
9	Electroconcussion. Burns	400	512	15.5	13.4
		420	400	13.4	14.3
		410	460	18.0	9.6
10	Premature separation of placenta	680	720	16.3	7.5
		420	400	15.9	9.3
11	Ileus	142	177	14.5	8.6
12	Pulmonary infarction	400	420	16.4	10.8
13	Sequels after endarterectomy	240	170	15.5	8.1
14	Poisoning with industrial solvents	262	348	4.0	4.0
	Mean	529.8 ± 88.0			

Discussion

Many explanations may be advanced to account for elevated serum B_{12} concentrations in renal failure. Matthews and Beckett (3) discuss, *inter alia*, the possibility of an increased serum vitamin B_{12} binding capacity, failure of utilization of vitamin B_{12} by the bone marrow, failure of excretion of the vitamin and a possible release of vitamin B_{12} from necrotizing renal tissue. As long as our knowledge of the role of the kidneys in vitamin B_{12} metabolism is incomplete we may accept them all, at least as contributory factors in raising the serum vitamin B_{12} level in patients with renal failure. However, we want to make some comments on this and on our own results.

The excretion of vitamin B_{12} by the kidneys is limited, the daily excretion

Table II. Serum vitamin B_{12} concentrations and plasma creatinine values before and after haemodialysis in patients with chronic renal failure.

Case No.	DIAGNOSIS	Serum B_{12} pg/ml		Plasma creatinine mg/100 ml	
		Before dialysis	After dialysis	Before dialysis	After dialysis
15	Chronic glomerulonephritis	232	240	28.0	14.0
16	Chronic pyelonephritis	540	640	10.6	6.8
17	Chronic glomerulonephritis	452	588	11.9	5.7
		444	280	14.5	6.5
18	Chronic glomerulonephritis	248	244	15.0	6.5
19	Subchronic glomerulonephritis	216	180	18.0	10.2
20	Chronic pyelonephritis	400	460	14.5	5.9
21	Subacute pyelonephritis	171	152	12.1	6.8
	Chronic interstitial nephritis	444	412	17.5	8.0
22	Subacute glomerulonephritis	540	180	11.9	5.8
		128	128	13.2	8.0
		200	130	19.7	13.0
23	Chronic glomerulonephritis	290	290	17.0	9.0
		580	1096	17.0	7.7
24	Chronic glomerulonephritis	236	260	28.0	16.0
25	Malignant hypertension	214	242	21.9	15.2
26	Chronic interstitial nephritis	348	340	18.0	9.8
27	Chronic nephritis	720	640	9.5	4.3
28	Renal tuberculosis	484		7.4	
29	Chronic pyelonephritis	305		10.4	
30	Chronic pyelonephritis	1496		4.4	
31	Renal tuberculosis	653		4.0	
32	Chronic pyelonephritis	260		2.4	
33	Chronic pyelonephritis	364		1.4	
34	Chronic pyelonephritis	155		8.0	
	Mean	396.2 ±54.2			

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amounting to 0–275 ng (1). But the main elimination route is the biliary and intestinal tract, which can probably compensate for a moderate vitamin B_{12} overload caused by a disturbed renal excretion mechanism. Such a compensatory mechanism may be insufficient, however, for the elimination of an acute excess in the serum vitamin B_{12} content, for instance after parenteral administration of large amounts of B_{12} or endogenous vitamin released after tissue damage. In such cases even a slightly reduced renal excretion capacity may lead to considerably elevated serum values. This is probably what happens in acute tubular necrosis. A contributory mechanism may be an inability of the injured renal tissue to incorporate the circulating vitamin. Such damage to a possible B_{12} acceptor mechanism is in accordance with the low B_{12} contents in liver tissue observed in acute hepatitis and cirrhosis of the liver, and may perhaps also explain the high serum concentrations found by Matthew and Beckett in chronic renal failure (3). In our patients with chronic renal failure the mean serum vitamin B_{12} levels did not differ significantly from those of the controls. This discrepancy between the two series is difficult to explain. The different techniques used for the B_{12} determination cannot account for the divergent results. One explanation is perhaps the very great variations in the serum values seen in patients with renal failure. In five persons with chronic renal insufficiency (not included in this material) we have found considerable variations in the serum levels from time to time in the same patient. The differences are of such a magnitude that they cannot be accounted for only by the error of the assay method. An extreme example is a 39-year-old male patient with renal tuberculosis, and a plasma creatinine level varying between 7.4 and 10.4. His serum vitamin B_{12} values determined at about 3-week intervals were as follows: 484, 240, 395, 124, 653, 416, 10,000, 896, 1,064, 2,008 pg/ml. The most obvious explanation for these variations is the variability of the disease itself with its possible influence on the intestinal vitamin B_{12} absorption, the general health and the dietary habits of the patients with varying periods of inadequate dietary vitamin B_{12} supply. Rath and coworkers (4), for instance, have shown that absorption of radioactive vitamin B_{12} in patients with renal diseases may be even more efficient than in normal subjects. In this connexion it is also important to remember that antibiotics may cause a transient rise in the serum vitamin B_{12} concentrations. (5).

In the case of chronic renal failure the interpretation of the results is difficult and the difference between our results and those of Matthew and Beckett may be insignificant.

From the above discussion we conclude that the only type of renal failure in which a plausible explanation for the increased serum B_{12} levels may be found is acute tubular necrosis.

Summary

The serum vitamin B_{12} concentrations in 20 patients with chronic renal failure and 14 patients with acute tubular necrosis were determined and compared with those of 636 controls. The average serum vitamin B_{12} level of the acute

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group was significantly higher than that of the controls. No difference in the serum vitamin B_{12} concentrations was found between the chronic group and the control group. Serum vitamin B_{12} values measured before and after haemodialysis did not differ significantly from each other. The probable cause for the raised serum vitamin B_{12} values in acute tubular necrosis is release of the vitamin from injured tissue and, possibly, damage to tissue vitamin B_{12} acceptors.

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References

1. Heinrich, H. C.: Klin. Wschr. *32*, 867, 1954.
2. Hutner, S. H., Bach, M. K., & Ross, G. I. M.: J. Protozool. *3*, 101, 1956.
3. Matthews, D. M. & Beckett, A. G.: J. clin. Path. *15*, 456, 1962.
4. Rath, C. E., Mc Curdy, P. R., Schreiner, G. E. & Duffy, B. J.: Proc. 6th Internat. Congress Soc. Haematology, Boston. Grune & Stratton, New York 1958, p. 329.
5. Ueda, K., Nishimura, M. & Sekino, J.: in Vitamin B_{12} und Intrinsic Factor II, (H. C. Heinrich, ed.), Enke, Stuttgart 1962, p. 549.

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Urinary Aminoimidazolecarboxamide in the Rat as Influenced by Dietary Vitamin B_{12}, Methionine and Thyroid Powder [1,2,3]

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ABSTRACT The objectives of this investigation were to 1) determine whether aminoimidazolecarboxamide (AIC), like formiminoglutamic acid, was elevated in the urine of rats fed a vitamin B_{12}-methionine-deficient diet; 2) assess the value of thyroid powder supplementation in enhancing the development of vitamin B_{12} deficiency; and 3) study the effect of supplementary methionine on AIC excretion. Vitamin B_{12}-supplemented controls averaged about 20 μg of urinary AIC/day. Rats fed the vitamin B_{12}-deficient diet for 6 weeks or more excreted at least twice as much. Thyroid powder did not alter urinary AIC excretion, nor did it enlarge the difference in weight gain between vitamin B_{12}-supplemented and deficient animals. Injection of 10 μg of vitamin B_{12} or supplementation of the diet with 2% of DL-methionine lowered AIC excretion of vitamin B_{12}-deficient rats to the level of vitamin B_{12}-supplemented controls, but had no effect on the AIC excretion of the control animals. The results of this investigation are consistent with the theory that vitamin B_{12} influences folic acid metabolism at the level of methionine biosynthesis.

In humans, megaloblastic anemia may occur with deficiencies of either folic acid or vitamin B_{12}. When this anemia is caused by a vitamin B_{12} deficiency alone (such as pernicious anemia), temporary remission can be induced by the administration of large doses of folic acid, which suggests that the 2 vitamins interact metabolically (1). Furthermore, formiminoglutamic acid (FIGLU), a metabolite dependent upon tetrahydrofolic acid (THF) for its degradation to glutamic acid in histidine catabolism (2), is elevated in the urine of either vitamin B_{12}- or folic acid-deficient rats (3) and chicks (4) fed low methionine diets. FIGLU excretion is lowered in such animals by feeding a methionine supplement. The same appears to occur in humans (5).

These clinical and nutritional observations, in conjunction with the knowledge that both vitamin B_{12} and folic acid cofactors participate in methionine biosynthesis in higher animals, have led to the hypothesis (6–8) that tissue levels of various THF cofactors are regulated at the site of methionine biosynthesis. According to this theory, in the event of dietary deficiencies of both vitamin B_{12} and methionine, there exists a functional folic acid deficiency (THF "trapped" as 5-methyl-THF) which can be overcome by either methionine or vitamin B_{12}. If this is true, then a methionine-vitamin B_{12} deficiency should affect all folic acid cofactor-dependent reactions as it does FIGLU metabolism.

Aminoimidazolecarboxamide (AIC) is the hydrolysis product of AIC-ribotide, an intermediate of purine biosynthesis which is dependent upon 10-formyl-THF for eventual conversion to inosinic acid (9). Clinical data (10–12) indicate that pernicious anemia is accompanied by elevated urinary AIC. Dietaries of the patients studied are not available, and hence evaluation of the influence of methionine intake on AIC excretion has not been possible. The present study was therefore

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[3] Portions of these data were presented at the VIIth International Congress of Nutrition, Hamburg, August, 1963. (Stokstad, E. L. R., S. M. Oace and C. Kutzbach 1967 Proceedings of the VIIth International Congress of Nutrition, vol. 5. F. Vieweg and Son, Braunschweig, p. 583.)
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undertaken to investigate the influence of dietary methionine on the AIC excretion of vitamin B_{12}-deficient rats. Since a variety of thyroid-active substances have been used to aggravate vitamin B_{12} deficiency in rats (13,14), the effects of thyroid powder on growth and AIC excretion of vitamin B_{12}-deficient and supplemented rats were also studied. Our results are in accordance with, and lend additional support to, the theory that vitamin B_{12} and methionine regulate folic acid metabolism at the level of methionine biosynthesis.

MATERIALS AND METHODS

Male, 21-day-old weanling rats of the Sprague-Dawley strain [1] were housed individually in screen-bottomed metabolism cages in a temperature- and light-controlled room. Animals were assigned to dietary groups by pair-weighing, so that the initial group-average weights were always about 50 g. Both food and water were allowed ad libitum. Fresh food was given every 2 to 3 days; water bottles were changed twice weekly.

The diets (table 1) were made in batches of 20 kg and stored in a refrigerator. Thyroid powder and DL-methionine were added at the expense of glucose and soy protein, respectively. Two separate vitamin mixes (with and without vitamin B_{12}) were prepared for incorporation into the respective diets. At certain points, 10 μg of vitamin B_{12} was administered by intraperitoneal injection in one milliliter of physiological saline.

TABLE 1

Composition of basal low methionine diet [1]

	g/kg diet
Soy assay protein [2]	200
Glucose monohydrate [3]	704
Corn oil [4]	40
Salts [5]	35
Fat-soluble vitamin mix [6]	10
Water-soluble vitamin mix [7]	10
Choline chloride [8]	1

[1] Supplements: 3 g thyroid powder replaced 3 g glucose; 5 or 20 g methionine replaced 5 or 20 g protein.
[2] General Biochemicals, Inc., Chagrin Falls, Ohio; provided 1.8 g methionine/kg diet.
[3] Cerelose, Corn Products Company, Argo, Illinois.
[4] Mazola, Corn Products Company.
[5] Johnson et al. (15).
[6] In corn oil, provided /kg diet: vitamin A acetate, 16,000 IU; vitamin D, 2000 IU; and α-tocopheryl acetate, 50 mg.
[7] In glucose, provided in mg/kg diet: thiamine·HCl, 18; riboflavin, 15; nicotinic acid, 50; pyridoxine·HCl, 15; Ca pantothenate, 50; menadione, 10; folic acid, 5; biotin, 2; vitamin B_{12} (when added), 0.05.
[8] Nutritional Biochemicals Corporation, Cleveland.

Urinary AIC was determined by color development with Bratton and Marshall (16) reagents for diazotizable amines and subsequent spectrophotometric analysis. Since AIC differs from many aromatic amines present in urine in that it can not be acetylated, pretreatment of urine with acetic anhydride achieves greater specificity (17). Indican also interferes with AIC determination, and this can be quantitatively removed by an anion-exchange resin (18). Urine specimens were collected through screened funnels into flasks containing 2 ml of 1 N H_2SO_4 as a preservative. A 24-hour sample was diluted to 25 ml with distilled water, adjusted to pH 2 with concentrated H_2SO_4 or NaOH, and filtered by gravity through Whatman no. 1 filter paper. Filtered urine (10 ml) was applied to a column (I.D., 8 mm) which had been fitted with a glass wool plug and contained 1.5 g of anion-exchange resin (AG2-X8)[9]. Urine was eluted by gravity and the resin was washed with 2 ml of 0.01 N H_2SO_4, bringing the total eluate volume to 12 ml. An aliquot of the eluate (4 ml) was treated with 0.1 ml of acetic anhydride and, after 30 minutes of occasional vigorous shaking, acetylation was stopped with 3 ml of 0.36 N H_2SO_4. One milliliter of each of the following reagents was then added with automatic syringe-type pipettes: 1) 0.1% sodium nitrite; 2) 2 minutes later, 0.5% ammonium sulfamate; and 3) 30 seconds later, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (color reagent). The identical procedure (after column treatment) was followed with 4-ml aliquots of standard solutions that contained from 0.1 to 15 μg of AIC (linear range). Distilled water replaced the color reagent in urine blanks. After 20 to 60 minutes, samples were read in one-centimeter cuvettes at 540 mμ with a Beckman-DB spectrophotometer. The timing and reagent concentrations reported here are those which were found to yield maximal color development and stability under our laboratory conditions. Duplicate analyses agreed within 5% and recovery of AIC

[9] Berkeley Pacific Laboratories, Berkeley, California
[9] Bio-Rad Laboratories, Richmond, California.

that was added to the urine before column treatment was greater than 95%.

A preliminary growth study with 8 rats per group was conducted to assess the ability of the basal vitamin B_{12}- and methionine-deficient diet to support nearly normal growth while still allowing a growth response to both vitamin B_{12} and methionine. In the second experiment, the basal diet alone and the basal diet plus vitamin B_{12}, thyroid powder, or both, were fed. Growth, food intake and AIC excretion were measured and the influences of vitamin B_{12} injection and methionine feeding on AIC excretion were studied. Food intake was recorded for the first 10 weeks of the study.

RESULTS

In the preliminary growth study, 4 groups of 8 rats were fed the basal diet with modifications as shown in table 2. The group average weights at the end of 10 weeks and the average daily gain during the 10-week feeding trial are also presented. Vitamin B_{12} supplementation elicited a significant growth response ($P < 0.05$)[7] in the absence of, but not in the presence of, the methionine supplement. Methionine supplementation supported a highly significant increase ($P < 0.01$) in body weight as compared with that of rats fed either of the diets without added methionine.

The diet modifications and the 10-week weight gain, food intake and feed efficiency data for rats whose AIC excretion was later studied are presented in table 3. Vitamin B_{12}-deficient animals (groups 1 and 3) gained less weight and ate less food than their respective vitamin B_{12}-supplemented controls (groups 2 and 4). The retarded growth of vitamin B_{12}-deficient rats cannot be totally accounted for by depressed appetite since their feed efficiency ratios are lower as well. Diets containing thyroid powder supported poorer growth and enhanced food consumption as compared with these measurements of rats fed the respective diets without added thyroid powder. All differences in weight gain, food intake and feed efficiency attributable either to vitamin B_{12} status (1 vs. 2 and 3 vs. 4) or to thyroid powder status (1 vs. 3 and 2 vs. 4) are significant at the 5% level.

TABLE 2

Effect of vitamin B_{12} and methionine on growth

Group	Supplements		Body wt, 12 weeks[1]	Daily gain[1]
	Methionine	Vitamin B_{12}		
	g/kg diet	μg/kg diet	g	g
G1	—	—	305 ± 40[2]	3.6
G2	—	50	353 ± 98	4.3
G3	6	—	424 ± 36	5.3
G4	6	50	421 ± 27	5.3

[1] Experimental diets fed 10 weeks.
[2] SD.

After several weeks (as early as 6 weeks, but only representative data at 9 and 11 weeks are reported), the average daily AIC excretion of vitamin B_{12}-deficient rats was approximately twice that of the vitamin B_{12}-supplemented controls (table 4). This was true whether or not thyroid powder was present in the ration. If the AIC excretion data were adjusted for differences in body weight, this would further spread the already significant differences between supplemented and deficient groups. However, in control rats, AIC excretion is unrelated to age (5–20 weeks) or to body weight (100–500 g) and ranges from 15 to 25 μg/day. Therefore, experimental AIC excretion is most appropriately reported as micrograms per day, not corrected for differences in body sizes among dietary groups.

Groups 1 and 2 were then subdivided to study the effects of vitamin B_{12} injection (1a and 2a) and methionine feeding (1b and 2b) on AIC excretion. During the second day after injection with vitamin B_{12}, most of the previously vitamin-deficient rats of group 1a excreted similar amounts of AIC as the continuously supplemented rats of group 2a. All were within control range 5 days after, and the 10 μg dose of vitamin B_{12} still effectively prevented an increase in urinary AIC even 10 days after injection. Reduction of AIC excretion by vitamin B_{12} injection was somewhat delayed in the vitamin-deficient, thyroid-fed animals of group 3. However, 3 days after injection, groups 3 and 4 experienced similar AIC excretion rates and, again, this ef-

[7] Significance of differences between means assessed by Student's t test.

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fect was still in evidence at 10 days post-injection.

The vitamin B_{12}-deficient and sufficient rats of groups 1 and 2, respectively, that had not been injected with vitamin B_{12} were fed diets containing 2% added methionine for 3 days. Urinary AIC was determined daily during methionine feeding and for 3 days after return to the original diets (table 5). During the second and

TABLE 3

Effect of vitamin B_{12} and thyroid powder on weight gain and food intake

Group	Thyroid supplement	Vitamin B_{12} supplement	Wt[1] gain	Food[1] intake	Feed[1] efficiency
	g/kg diet	*μg/kg diet*	*g*	*g*	*g/kg food*
1 (9)[2]	—	—	220 ± 38[3]	1101 ± 115	199 ± 21
2 (10)	—	50	278 ± 24	1256 ± 96	222 ± 13
3 (5)	3	—	162 ± 24	1268 ± 86	127 ± 12
4 (5)	3	50	230 ± 24	1481 ± 131	155 ± 9

[1] During 10 weeks of feeding.
[2] Numbers in parentheses indicate number of rats/group.
[3] SD.

TABLE 4

Urinary AIC of vitamin B_{12} supplemented and deficient rats fed diets with or without thyroid powder and the effect of injection of 10 μg of vitamin B_{12} on aminoimidazolecarboxamide (AIC) excretion

Group[1]	Supplements		Weeks fed diets		Days after vitamin B_{12} injection[1]				
	Vitamin B_{12}	Thyroid	9	11	1	2	3	8	10
	μg/kg	*g/kg*	*μg AIC/24 hr*		*μg AIC/24 hr*				
1a	—	—	42 ± 14[2]	52 ± 26	40 ± 17	24 ± 12	—	16 ± 2	23 ± 5
2a	50	—	20 ± 2	22 ± 2	24 ± 4	19 ± 4	—	17 ± 1	19 ± 2
	P value[4]		**	**	*	ns	—	ns	ns
3	—	3	40 ± 9	46 ± 11	34 ± 10	31 ± 8	22 ± 4	—	23 ± 3
4	50	3	23 ± 3	24 ± 4	26 ± 7	22 ± 1	22 ± 1	—	25 ± 4
	P value[4]		**	**	*	**	ns	—	ns

[1] Groups 1a and 2a were injected during week 13; groups 3 and 4 were injected during week 12.
[2] Five rats/group. Groups 1a and 2a are part of groups 1 and 2 (table 3).
[3] SD.
[4] Probability that AIC excretions of vitamin B_{12} supplemented and deficient groups are not significantly different. Calculated by Student's t test. ** = significant ($P < 0.05$); * = borderline significance ($0.05 < P < 0.1$); ns = not significant ($P > 0.1$).

TABLE 5

Temporary depression in aminoimidazolecarboxamide (AIC) excretion of vitamin B_{12}-deficient rats by methionine supplementation

Group[1]	Vitamin B_{12}	Weeks fed diets before methionine feeding[2]		Days during methionine feeding[3]			Days after methionine feeding[2]		
		12	14	1	2	3	1	2	3
	μg/kg	*μg AIC/24 hr*		*μg AIC/24 hr*			*μg AIC/24 hr*		
1b	—	40 ± 12[4]	40 ± 11	31 ± 12	24 ± 2	26 ± 4	35 ± 11	38 ± 11	37 ± 8
2b	50	23 ± 2	20 ± 3	21 ± 2	21 ± 5	23 ± 2	21 ± 1	22 ± 2	22 ± 3
	P value[5]	**	**	*	ns	ns	**	**	**

[1] Groups 1b ($n = 5$) and 2b ($n = 4$) were those animals from groups 1 and 2 (table 3) that had not been injected with vitamin B_{12} (table 4).
[2] For 14 weeks before and 3 days after methionine feeding, rats were fed the basal, low methionine diet (table 1) with or without vitamin B_{12} as indicated.
[3] During methionine feeding, 20 g of DL-methionine replaced 20 g of soy protein/kg diet.
[4] SD.
[5] Probability that AIC excretion of vitamin B_{12} supplemented and deficient groups is not significantly different. Calculated by Student's t test. ** = significant ($P < 0.05$); * = borderline significance ($0.05 < P < 0.1$); ns = not significant ($P > 0.1$).

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third days of methionine feeding, AIC excretion of vitamin B_{12}-deficient animals (group 1b) decreased to that of the vitamin-supplemented controls (group 2b). Immediately upon removal of the methionine supplement, the urinary AIC of group 1b animals returned to its elevated pre-methionine level. AIC excretion of vitamin B_{12}-supplemented rats was unaffected by methionine feeding.

DISCUSSION

In formulation of the basal diet, 2 factors were considered. First, the diet had to be low in methionine as well as vitamin B_{12} to demonstrate the effects of vitamin B_{12} deficiency on folic acid metabolism (6). Second, the diet should be adequate in other nutrients (as indicated by good growth rates) so that measurements of control animals are representative of a normal metabolic situation. Silverman and Pitney (3) limited methionine by using a low protein (9% casein) diet. However, even their vitamin-sufficient ration resulted in very poor growth rates, frequently less than one gram per day. Soy protein, which is low in methionine, can be incorporated as 20% of a diet and still provide less methionine than a 9% casein diet. By using soy protein, then, the basal diet satisfied the requirement of the laboratory rat for crude protein, as well as for fat, vitamins (except vitamin B_{12}) and minerals as established by the NRC (19).

Male, Sprague-Dawley rats, when fed diets adequate in all known nutrients, are expected to attain a weight of about 350 g at 10 weeks after weaning (19). This represents an average gain of approximately 4.4 g/day. The data in table 2 show that our basal diet fulfills the requirements for this study. It is deficient in methionine and vitamin B_{12} (as evidenced by a growth response to these nutrients when added) and also supports very good growth.

Whether the hyperthyroid state had any specific effect on the severity of vitamin B_{12} deficiency is equivocal. In the presence of thyroid powder, vitamin B_{12} stimulated growth to approximately the same absolute amount (68 g in 10 weeks) as it did in rats fed diets containing thyroid powder (58 g in 10 weeks). Still, owing to their smaller body size, this absolute growth response represents a much greater percentage benefit from vitamin B_{12} in hyperthyroid rats, i.e., vitamin B_{12}-supplemented thyroid-fed rats gained 42%, whereas the non-thyroid gained only 26% more weight than their deficient counterparts, respectively. Perhaps this indicates greater depletion of vitamin B_{12} in hyperthyroid rats. If this is so, however, it is not manifest in AIC excretion. Vitamin B_{12} deficiency doubled urinary AIC, regardless of the presence of thyroid powder. The average AIC excretions of hyper- and euthyroid rats of similar vitamin B_{12} status were not significantly different, even when expressed on a body weight basis. However, injection of vitamin B_{12} lowered the AIC excretion rate more slowly in thyroid-fed, vitamin B_{12}-deficient rats than in rats fed the basal diet without thyroid powder. The poor health (as evidenced by diarrhea, watery eyes and nose and dull, matted fur) of the thyroid-fed animals as compared with the apparently good health of the euthyroid rats, also prevents any definitive evaluation of their respective vitamin B_{12} status.

In our experiments, methionine supplementation at 2% of the diet was as effective in lowering AIC excretion of vitamin B_{12}-deficient rats as injection of vitamin B_{12} itself. This parallels the changes in FIGLU excretion with these dietary manipulations in rats (3) and chicks (4).

While our investigation was in progress, McGeer et al. (20) reported that methionine supplementation did not lower AIC excretion of vitamin B_{12}-deficient rats. Their control diet consisted of grains and other natural foodstuffs, and their vitamin B_{12}-deficient ration was a commercially prepared[a] defined diet which differed from the control diet in numerous aspects other than their respective vitamin B_{12} content. Elevated AIC excretion could have been due to a dietary factor other than lack of vitamin B_{12}. This contention is supported by the fact that even addition of vitamin B_{12} to their deficient ration did not significantly reduce AIC excretion. Since our investigation deals with the effect of methionine on urinary AIC which is responsive to vitamin B_{12} status, the report of

[a] Nutritional Biochemicals Corporation, Cleveland.

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McGeer et al. does not constitute contradictory evidence.

Recent biochemical studies offer an explanation at the enzyme level for our observations on the influence of dietary vitamin B_{12} and methionine on AIC excretion. The tissue concentration of S-adenosylmethionine increases after injection of methionine (21). This compound is a potent inhibitor of methylenetetrahydrofolate reductase (22), the enzyme which catalyzes the irreversible (23) reduction of methylene-THF to methyl-THF. Vitamin B_{12} deficiency greatly depresses the level of cobamide-containing methyltetrahydrofolate-homocysteine transmethylase (24), the enzyme which catalyzes the release of THF from methyl-THF during methionine biosynthesis. Thus, methionine feeding, by elevating S-adenosylmethionine concentration, would inhibit formation of methyl-THF, whereas vitamin B_{12} supplementation would enhance utilization of methyl-THF. Either way, THF is released (from "trapped" methyl-THF) and the metabolic blocks causing the elevated excretion of AIC and FIGLU would be abolished.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. Herbert, V. 1959 The Megaloblastic Anemias. Grune and Stratton, New York.
2. Tabor, H., and L. Wyngarden 1959 The enzymatic formation of formiminotetrahydrofolic acid, 5,10-methenyltetrahydrofolic acid, and 10-formyltetrahydrofolic acid in the metabolism of formiminoglutamic acid. J. Biol. Chem., 234: 1830.
3. Silverman, M., and A. J. Pitney 1958 Dietary methionine and the excretion of formiminoglutamic acid by the rat. J. Biol. Chem., 233: 1179.
4. Fox, M. R. S., W. J. Ludwig and M. C. Baroody 1961 Effects of vitamin B_{12} and methionine on excretion of formiminoglutamic acid by the chick. Proc. Soc. Exp. Biol. Med., 107: 723.
5. Herbert, V., and L. W. Sullivan 1963 Formiminoglutamicaciduria in humans with megaloblastic anemia: Diminution by methionine or glycine. Proc. Soc. Exp. Biol. Med., 112: 304.
6. Noronha, J. M., and M. Silverman 1962 On folic acid, vitamin B_{12}, methionine and formiminoglutamic acid metabolism. In: Vitamin B_{12} und Intrinsic Factor, ed., H. C. Heinrich. F. Enke, Stuttgart, p. 728.
7. Buchanan, J. M. 1964 The function of vitamin B_{12} and folic acid coenzymes in mammalian cells. Medicine, 43: 697.
8. Weissbach, H., and H. Dickerman 1965 Biochemical role of vitamin B_{12}. Physiol. Rev., 45: 80.
9. Schulman, M. P. 1961 Purines and pyrimidines. In: Metabolic Pathways, vol. 2, ed., D. M. Greenberg. Academic Press, New York, p. 399.
10. Luhby, A. L., and J. M. Cooperman 1962 Aminoimidazolecarboxamide excretion in vitamin-B_{12} and folic-acid deficiencies. Lancet, 2: 1381.
11. Herbert, V., R. R. Streiff, L. W. Sullivan and P. L. McGeer 1964 Deranged purine metabolism manifested by aminoimidazolecarboxamide excretion in megaloblastic anemias, hemolytic anemia and liver disease. Lancet, 2: 45.
12. Middleton, J. E., R. F. Coward and P. Smith 1964 Urinary excretion of A. I. C. in vitamin-B_{12} and folic-acid deficiencies. Lancet, 2: 256.
13. Bethell, J. J., and H. A. Lardy 1949 Comparative effectiveness of vitamin B_{12}, whole liver substance and extracts high in APA activity, as growth promoting materials for hyperthyroid animals. J. Nutr., 37: 495.
14. Lewis, U. J., D. V. Tappan, U. D. Register and C. A. Elvehjem 1950 Effect of carbohydrate on growth response to vitamin B_{12} in the hyperthyroid rat. Proc. Soc. Exp. Biol. Med., 74: 568.
15. Johnson, R. R., P. Bouchard, J. Tinoco and R. L. Lyman 1967 Fatty acid changes in liver and plasma lipid fractions after safflower oil was fed to rats deficient in essential fatty acids. Biochem. J., 105: 343.
16. Bratton, A. C., and E. K. Marshall, Jr. 1939 A new coupling component for sulfanilamide determination. J. Biol. Chem., 128: 537.
17. Stetten, M. R., and C. L. Fox, Jr. 1945 An amine formed by bacteria during sulfonamide bacteriostasis. J. Biol. Chem., 161: 333.
18. McGeer, P. L., E. G. McGeer and M. C. Griffin 1961 Excretion of 4-amino-5-imidazolecarboxamide in human urine. Can. J. Biochem. Physiol., 39: 591.
19. National Research Council, Committee on Animal Nutrition 1962 Nutrient requirement of laboratory animals, publ. 990. National Academy of Sciences — National Research Council, Washington, D. C., p. 51.
20. McGeer, P. L., N. P. Son and D. A. Grant 1965 Excretion of 4(5)-amino-5(4)-imidazolecarboxamide and formimino-L-glutamic acid in folic acid and vitamin B_{12}-deficient rats. Can. J. Biochem., 43: 1367.
21. Baldessarini, R. J. 1966 Alterations in tissue levels of S-adenosylmethionine. Biochem. Pharmacol., 15: 741.
22. Kutzbach, C., and E. L. R. Stokstad 1967 Feedback inhibition of methylene-tetrahydrofolate reductase in rat liver by S-adenosylmethionine. Biochim. Biophys. Acta, 139: 217.

2019

23. Katzen, H. M., and J. M. Buchanan 1965 Enzymatic synthesis of the methyl group of methionine. VIII. Repression-derepression, purification and properties of 5,10-methylenetetrahydrofolate reductase from *Escherichia coli*. J. Biol. Chem., 240: 825.

24. Kutzbach, C., E. Galloway and E. L. R. Stokstad 1967 Influence of vitamin B_{12} and methionine on levels of folic acid compounds and folate enzymes in rat liver. Proc. Soc. Exp. Biol. Med., 124: 801.

1916

J. Nutr. 72:99-104

Effect of Feeding D-Sorbitol on the Intestinal Absorption of Vitamin B_6 and Vitamin B_{12} in Rats[1]

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D-Sorbitol can enhance the absorption of orally administered vitamin B_{12} in man (Chow et al., '57) and in animals (Greenberg et al., '57). It can also increase the absorption of iron by rats (Herndon et al., '58). Morgan and Yudkin ('57) reported that adding D-sorbitol to an adequate diet, deficient in the vitamins of the B group only, will bring about the normal growth rate of weanling rats. The latter finding may be explained either on the basis of increase in intestinal synthesis, enhancement of absorption or more efficient utilization of these vitamins. It is, therefore, of interest to determine whether prolonged feeding of dietary D-sorbitol could affect the absorption and excretion of vitamin B_{12} and B_6. The results of such studies are reported in this paper.

EXPERIMENTAL

Experimental animals. Young adult rats of the McCollum strain from our stock colony were used. One half of them was fed ad libitum a basal diet (table 1) low in vitamin B_6 and B_{12}, with or without supplementation of these vitamins, and were housed in individual cages with a large-mesh wire screen bottom. The other half was given the same diet except that D-sorbitol was added to the basal diet in amounts to replace a part of sucrose. The animals were offered these diets for 12 weeks. On the 7th days of the 4th and 12th weeks, they were placed in individual metabolism cages. Urine specimens were collected for 24 hours and assayed for vitamin B_6 activity.

Determination of vitamin B_{12}. Vitamin B_{12} concentrations were determined in plasma and liver tissue with Skeggs' media (Skeggs et al., '50) with *Lactobacillus leichmannii* 4797 as the test organism. The technique has been described elsewhere (Yamamoto et al., '57).

Determination of vitamin B_6. Vitamin B_6 activity in urine and liver was meas-

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TABLE 1
Composition of the basal diets

Ingredients	Diet 1	Diet 2	Diet 3
	%	%	%
Vitamin-free casein	21	20	—
Soybean	—	—	62
Sucrose	70	71	30
Corn oil[1]	5	5	4
Salt mixture no. 4[2]	4	4	4
Vitamins[3]	$-B_6$, $-B_{12}$	$-B_6$	$-B_{12}$
Thyroid powder	—	—	0.1

[1] Mazola, Corn Products Refining Company, New York.
[2] Hegsted et al. ('41).
[3] Each kilogram of diet contained the following in milligrams: thiamine, 2.0; riboflavin, 3.0; pyridoxine, 10; Ca pantothenate, 20; niacin, 50; inositol, 100; biotin, 0.1; PABA, 250; folic acid, 0.2; choline chloride, 1,000; vitamin B_{12}, 0.05; α-tocopherol, 23; menadione, 2.1; and, vitamin A, 1,200 and vitamin D, 170 units.

cured using *Neurospora sitophilia* 299 as the test organism (Stokes et al., '43).

Measurement of vitamin B_{12} absorption. Adult female rats from our stock colony (average weight, 200 gm) were fasted overnight and then given by mouth one of the following treatments of radioactive vitamin B_{12}[1] labeled with cobalt^{60}: (a) a single dose of 50 mμg or (b) two doses of 1,000 mμg with a 24-hour interval. The feed was returned to the rats 4 hours after the administration of the test dose. The animals were killed 4 days after the treatment (a), or two days after the treatment (b), and the radioactivities in the liver and kidneys were measured. Feces from rats receiving treatment (a) were collected for 4 days, and all specimens were processed for radioactivity measurement according to the procedures described elsewhere (Okuda et al., '59).

Growth study. Weanling rats of the McCollum strain were raised in individual cages and fed ad libitum a basal diet deficient either in vitamin B_6 or B_{12}, with or without supplementation of D-sorbitol. They were weighed weekly.

RESULTS

Effects of D-sorbitol on the growth of rats fed a vitamin B_6-deficient diet. In order to determine whether supplementation of D-sorbitol improves growth rate, two groups of 8 weanling rats were fed basal diet 2 with or without supplementation of 10% of D-sorbitol. A comparison of the growth curves of such groups (fig. 1) clearly demonstrates that the addition of D-sorbitol improved the growth rate of weanling rats fed a vitamin B_6-deficient diet. The difference in the body weight between the two groups became statistically significant after three weeks of feeding, and its magnitude increased progressively. Although the animals were not pair-fed, the feed consumption of both groups was measured daily and found to be practically the same. These data con-

[1] The specific activity was 1 μc per μg; kindly supplied by Merck and Co., Inc.

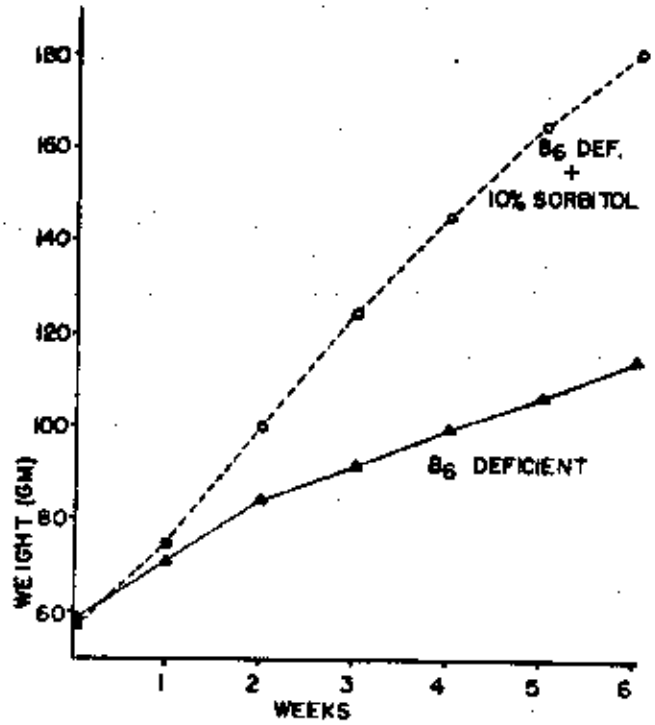

Fig. 1 Effect of D-sorbitol on the growth of weanling rats fed a vitamin B_6-deficient diet.

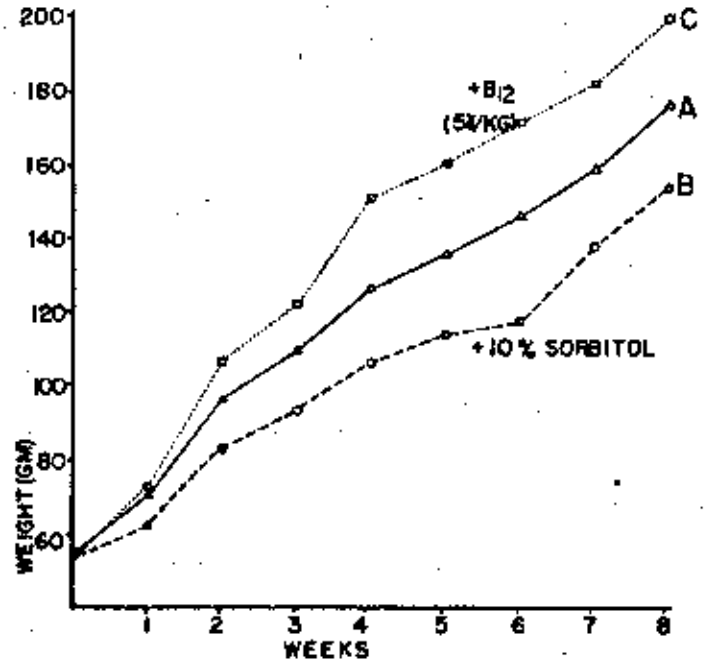

Fig. 2 Effect of D-sorbitol on the growth of weanling rats fed a soybean diet deficient in vitamin B_{12}.

firm essentially the findings of Morgan and Yudkin ('57).

Effect of D-*sorbitol on the growth of rats fed a soybean diet supplemented with desiccated thyroid.* To study the effect of dietary D-sorbitol on the growth of rats fed a vitamin B_{12}-deficient diet, three groups (8 each) of weanling rats were fed basal diet 3 which consisted of a soybean protein containing 0.1% of desiccated thyroid and which was deficient in vitamin B_{12} with (group B) or without (group A) D-sorbitol. Desiccated thyroid was added to the diet to make the animals even more responsive to vitamin B_{12} therapy (Emerson, '49). The third group (C) received the same diet (diet 3) supplemented with 5 µg of vitamin B_{12} per kg of diet. The growth curves of such animals in a typical experiment (fig. 2) demonstrate that sorbitol did not improve the growth of the weanling rats receiving this diet. In fact, there was a possibility of retarded growth, particularly during the first week of the experimental diet (group B diet) although the difference in mean body weights at the end of 8 weeks of feeding between groups A and B was not statistically significant. The group which received vitamin B_{12} in the diet had the highest mean body weight throughout the entire experimental period.

After 8 weeks of feeding, the animals in groups A and B were tested for their ability to absorb radioactive vitamin B_{12} by feeding a single dose of 50 mµg. The results of the study (table 2, study 1) show a reduction of absorption in the sorbitol-treated animals as evidenced by a significant decrease in kidney radioactivity.

Urinary excretion and liver concentrations of vitamin B_6. In order to determine whether D-sorbitol enhances the intestinal absorption of vitamin B_6, and if it does, whether such an effect is limited to vitamin B_6 which has been added to the diet, the following experiment was carried out. Twenty-four young adult female rats were divided into 4 groups: group A, received the basal diet 1 without vitamin B_6 and B_{12}; group B, the same diet supplemented with vitamin B_6 and B_{12}; group C, the basal diet in which

TABLE 2

Absorption of radioactive vitamin B_{12} after prolonged feeding of D-sorbitol

Study (Weeks of Feeding)	Group[1]	Dosage	Fecal test		Organ uptake	
			Recovery	Absorption	Liver	Kidneys
			mμg	mμg	mμg	mμg
1 (6)	A B_{12}-deficient	50 mμg × 1	26.5 ± 2.71[2]	23.5	4.92 ± 0.54	3.23 ± 0.29
	B B_{12}-deficient + sorbitol	50 mμg × 1	32.3 ± 0.49	17.7	3.76 ± 0.25	2.18 ± 0.15
2 (12)	A B_{12}-deficient	50 mμg × 1	24.1 ± 0.90	25.9	4.63 ± 0.15	3.25 ± 0.14
	C B_{12}-deficient + sorbitol	50 mμg × 1	32.6 ± 1.39	17.4	2.60 ± 0.32	1.72 ± 0.19
	B B_{12}-supplemented	1000 mμg × 2			37.9 ± 7.4	70.4 ± 15.1
	D B_{12}-supplemented + sorbitol	1000 mμg × 2			15.3 ± 2.1	27.4 ± 3.5

[1] Six rats in each group.
[2] Standard error of the mean.

sucrose was partially replaced by D-sorbitol, and group D, the basal diet which was supplemented with vitamin B_6 and B_{12} and containing D-sorbitol. The content of sorbitol in the diets for groups C and D was 10% during the first 6 weeks of feeding and was subsequently increased to 20% during the second 6-week period in order to make the effect of sorbitol more marked. The 24-hour excretion of vitamin B_6 in the urine collected during the last day of the 4th week was measured and found to be 1.04 ± 0.04 μg in group A and 6.52 ± 0.32 μg in group C (table 3), demonstrating a marked increase in excretion ($P < 0.001$).

Effect of prolonged feeding of D-sorbitol on absorption of vitamin B_{12}. To study the effect of prolonged feeding of sorbitol on vitamin B_{12} absorption, another set of animals in the various groups similar to those described above (table 3) was given a vitamin B_{12} absorption test at the end of a 12-week feeding period. The animals were fasted overnight to eliminate the effect of any sorbitol remaining in the gastrointestinal tract, and were given radioactive vitamin B_{12} by mouth on the following morning. When the dose of 50 mμg was fed to groups A and C, and the absorption was estimated from the radioactivity in fecal matter and organs, a significantly poorer absorption was demonstrated in group C in comparison with group A (see table 2, study 2). An even greater difference was obtained between groups B and D which received two doses of 1,000 mμg; the uptake of radioactivity was 37.9 ± 7.4 mμg, and 15.3 ± 2.1 mμg in the liver and 70.4 ± 15.1 mμg and 27.4 ± 3.5 mμg in the kidneys, respectively. The decrease in absorption of vitamin B_{12} after 12 weeks of dietary sorbitol also is reflected in a lowering of vitamin B_{12} concentration in the plasma and liver (table 3, last two columns).

DISCUSSION

The data presented here demonstrate clearly that supplementation of a vitamin B_6-deficient diet with D-sorbitol brings about an increase in the urinary excretion and in the liver concentration of vitamin B_6 in young adult rats. These results may be interpreted as evidence that the absorption of vitamin B_6 was increased by the

[1] Herndon, J. F., S. M. Greenberg, E. J. Van-Loon, J. K. Mathues and E. T. Parmelle 1959 Activities of D-sorbitol on the growth of rats deficient in pyridoxine, vitamin B_{12}, biotin or pantothenic acid. Federation Proc., *18*: 529 (abstract).

TABLE 3

Effects of prolonged feeding of D-sorbitol on urinary excretion and liver content of vitamin B_6 and on liver and plasma vitamin B_{12} concentrations

Group[1]	Diet	Av. body weight		Urinary excretion of B_6		B_6 in liver	B_{12} in liver	B_{12} in plasma
		Initial	12 weeks	4 weeks	12 weeks			
		gm	gm	μg/24 hours	μg/24 hours	μg/gm wet tissue	mμg/gm wet tissue	mμg/ml
A	$-B_6-B_{12}$	153	202	1.04 ± 0.04[2]	1.45 ± 0.03	5.8 ± 0.5	160 ± 32	0.107 ± 0.009
B	$+B_6+B_{12}$	150	209	12.20 ± 1.65	14.15 ± 3.51	9.9 ± 0.4	333 ± 53	0.732 ± 0.032
C	$-B_6-B_{12}$ + sorbitol	152	200	6.52 ± 0.32	8.88 ± 1.12	9.9 ± 0.6	151 ± 13	0.086 ± 0.010
D	$+B_6+B_{12}$ + sorbitol	153	218	15.52 ± 1.72	19.35 ± 2.71	10.4 ± 0.3	278 ± 34	0.574 ± 0.030

[1] Six rats in each group.
[2] Standard error of the mean.

sorbitol supplementation. This finding is in harmony with the observation that the growth rate of weanling rats fed a vitamin B_6-deficient diet was improved by the supplementation of 10% of D-sorbitol. This confirms the report by Herndon et al.[4]

The effects of D-sorbitol on vitamin B_{12} absorption under similar conditions were different from those on vitamin B_6. Unlike the latter vitamin, prolonged feeding of D-sorbitol results in apparent reduction of vitamin B_{12} absorption with concomitant decreases in the plasma and liver concentrations of vitamin B_{12} in a manner similar to prolonged dietary deprivation of B_6, (Hsu et al., '57; Ranke et al., '60). In distinct contrast with the growth-promoting effect of sorbitol in B_6 deficiency, sorbitol did not improve and might even have retarded the growth of the weanling rats fed a vitamin B_{12}-deficient soybean diet supplemented with desiccated thyroid. It should be pointed out that such thyroid-treated animals may be different from the ones deprived of vitamin B_{12} in the diet. The influence of sorbitol on the utilization of intestinal vitamin B_6 is apparently different from that on the utilization of vitamin B_{12}. No single mechanism would account for two diversified effects of sorbitol on these vitamins. The effect of sorbitol on vitamin B_6 may be explained on the basis of increased synthesis of vitamin B_6 by intestinal microorganisms, but an effect of sorbitol to increase the concentrations of available vitamin B_6 in the lumen without increasing synthesis cannot be ruled out.

On the surface, our observation of the reduced absorption after prolonged feeding of sorbitol seems to contradict the reported observations on increased absorption when vitamin B_{12} was co-administered with D-sorbitol by mouth to normal rats and human volunteers. Chow et al. ('58) observed a greater increase of vitamin B_{12} levels in plasma in the human subjects receiving 50 μg of vitamin B_{12} and 18 gm of liquid sorbitol as compared with that in control subjects receiving vitamin B_{12} alone after a long period of administration. However, these diametrically opposite results may be explained on the basis of the difference in the physical states of the two mixtures of vitamin B_{12}

and sorbitol. Our unpublished data indicate that sorbitol enhanced absorption of vitamin B_{12} only when a large dose (1,000 mcg) of vitamin B_{12} was used and that without the cecum, this effect cannot be shown in rats. The markedly enlarged ceca of the sorbitol-dosed animals as observed suggest such a possible role of cecum in absorption of vitamin B_{12}. It should be pointed out that, in the present study, the absorption of vitamin B_{12} was measured under a condition in which sorbitol was not present in the lumen when the test dose of vitamin B_{12} was administered. Such a condition would permit one to estimate the effect of prolonged feeding of sorbitol on the absorption capacity of the intestinal wall, rather than a direct effect of sorbitol on absorption of B_{12} or interaction between the two components. The involvement of intrinsic factor in the observed reduction of absorption of vitamin B_{12} seems unlikely, because the absorption of a large dose of vitamin B_{12} has been shown repeatedly not to depend on intrinsic factor (Doscherholmen and Hagen, '57; Okuda, '60). The absorption study using such a dose also demonstrated the reduction of absorption by sorbitol (table 2, group B vs D).

SUMMARY

The effects of D-sorbitol in the diet on the utilization of vitamin B_6 and B_{12} were studied in rats. The following conclusions were drawn:

1. The addition of D-sorbitol to a diet deficient in vitamin B_6 increased the urinary excretion and liver concentrations of vitamin B_6 in adult rats, and markedly improved the growth rate in weanling rats.

2. After feeding D-sorbitol at the level of 10 to 20% in the diet for 8 to 12 weeks, the intestinal absorption of orally-fed radioactive vitamin B_{12} was reduced.

3. The addition of D-sorbitol to a diet deficient in vitamin B_{12} did not improve the growth of weanling rats.

These observations demonstrate a multiple effect of D-sorbitol on vitamins in the intestine. The possible mechanism for the increased utilization of vitamin B_6 by the dietary D-sorbitol has been discussed.

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LITERATURE CITED

Chow, B. F., P. Meier and S. M. Free, Jr. 1957 Absorption of vitamin B_{12} enhanced by D-sorbitol. Am. J. Clin. Nutrition, 5: 30.

Chow, B. F., H. Prystowsky, A. E. Hellegers and V. Wong 1958 Studies on the absorption of vitamin B_{12} in human pregnancy with especial reference to the effect of D-sorbitol. Am. J. Obst. Gyn., 75: 91.

Doscherholmen, A., and P. S. Hagen 1957 A dual mechanism of vitamin B_{12} plasma absorption. J. Clin. Invest., 36: 1551.

Emerson, G. 1949 Growth-promoting activity of vitamin B_{12} in rats receiving thyroid substance. Proc. Soc. Exp. Biol. Med., 70: 392.

Greenberg, S. M., J. F. Herndon, E. G. Rice, E. T. Parmelee, J. J. Galesich and E. J. VanLoon 1957 Enhancement of vitamin B_{12} absorption by substances other than intrinsic factor. Nature, 180: 1401.

Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. G. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., 138: 459.

Herndon, J. F., E. G. Rice, R. G. Tucker, E. J. VanLoon and S. M. Greenberg 1956 Iron absorption and metabolism. III. The enhancement of iron absorption in rats by D-sorbitol. J. Nutrition, 64: 615.

Hsu, J. M., and B. F. Chow 1957 Effect of pyridoxine deficiency on the absorption of vitamin B_{12}. Arch. Biochem. Biophys., 72: 322.

Morgan, T. B., and J. Yudkin 1957 The vitamin-sparing action of sorbitol. Nature, 180: 543.

Okuda, K., J. A. Wider and B. F. Chow 1959 The effect of intrinsic factor on the hepatic uptake of vitamin B_{12} following intravenous injection. J. Lab. Clin. Med., 54: 535.

Okuda, K. 1960 Vitamin B_{12} absorption in rats, studied by a "loop" technique. Am. J. Physiol., July.

Ranke, B., E. Ranke and B. F. Chow 1960 The interrelationship between vitamin B_6 and B_{12} deficiencies in rats. J. Nutrition, 71: 411.

Skeggs, H. R., H. M. Nepple, K. A. Valentik, J. W. Huff and L. D. Wright 1950 Observations on the use of *Lactobacillus leichmannii* 4797 in the microbiological assay of vitamin B_{12}. J. Biol. Chem., 184: 211.

Stokes, J., A. Larsen, C. R. Woodward and J. W. Foster 1943 A neurospora assay for pyridoxine. Ibid., 150: 17.

Yamamoto, R. S., K. Okuda and B. F. Chow 1957 Effect of carbontetrachloride injury on plasma and liver vitamin B_{12} levels. Proc. Soc. Exp. Biol. Med., 94: 497.

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Intestinal absorption and concurrent chemical changes of methylcobalamin

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Intestinal absorption of $^{14}CH_3$- and/or ^{57}Co-methylcobalamin [CH_3-B_{12}] was studied with regard to chemical changes. It was found that absorption of a physiologic dose of CH_3-B_{12}-^{57}Co in the rat was the same as that of cyanocobalamin [CN-B_{12}] in quantity and speed, but more radioactivity accumulated in liver after 8 hours, indicating a gradual conversion of CH_3-B_{12} to hydroxocobalamin and cobamide coenzyme. Schilling test with CH_3-B_{12}-^{57}Co yielded urinary excretion about 1/3 that with CN-B_{12}-^{57}Co, and similar changes of CH_3-B_{12} in human body were suggested. In the rat, expiration of $^{14}CO_2$ following oral administration of $^{14}CH_3$-B_{12} was small, as contrasted by quick and massive evolution of $^{14}CO_2$ when photolyzed $^{14}CH_3$-B_{12} was given. With supraphysiologic doses, more than 60 per cent of ^{14}C disappeared from the gastrointestinal tract in 2 to 3 hours, suggesting instability of free CH_3-B_{12}, and there was a distinct difference in tissue distribution between ^{14}C and ^{57}Co following administration of $^{14}CH_3$-B_{12}-^{57}Co. It seems, therefore, that cleavage of -CH_3 in vivo is different in mechanism from that in vitro or after photolysis. Paper chromatography of digested ileal mucosa obtained after oral administration of a small dose of CH_3-B_{12}-^{57}Co, demonstrated unchanged CH_3-B_{12} and some other cobamides. It is concluded that in the alimentary tract, free CH_3-B_{12} is labile and loses CH_3 progressively. However, in a physiologic situation where intrinsic factor is operative, CH_3-B_{12} may be partially protected by it from such chemical changes.

Methylcobalamin (CH_3-B_{12}) was first prepared by Smith and co-workers[1] in a series of synthesis of alkylcobalamins, and shortly thereafter it was found to be the cofactor in bacterial synthesis of methionine from homocysteine.[2] Subsequent studies by Lindstrand and Ståhlberg[3, 4] demonstrated its presence in human blood and tissues. Thus, vitamin B_{12} in foods consists mainly of 5'-deoxyadenosylcobalamin (DAB_{12}) and CH_3-B_{12}.[2, 5]

In the structure of CH_3-B_{12}, a methyl group is attached to the cobalt atom of the corrin ring, and this rare cobalt-carbon linkage is unstable and readily undergoes photolysis.[6] When the methyl group is detached, the cobalamin moiety is converted to hydroxocobalamin (OH-B_{12}).[7] The behavior and metabolism of this naturally occurring cobamide have not as yet been understood. The

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Table I. Speed of absorption of orally administered CH_3-B_{12}-^{57}Co in comparison with

Time after administration	*4 hrs.*		*8 hrs.*	
Cobamide	*CN*	*CH_3*	*CN*	*CH_3*
Liver	3.1 ± 0.3	3.2 ± 0.5	4.3 ± 0.4	4.9 ± 0.
Small intestine				
Wall	8.2 ± 1.1	7.6 ± 0.9	5.2 ± 1.1	6.1 ± 0.
Washing	28.1 ± 6.6	25.5 ± 8.8	8.1 ± 1.2	7.6 ± 1.
Small content	23.5 ± 5.7	28.2 ± 6.4	26.4 ± 4.6	30.1 ± 3.
Feces	0	0	0	0
(Calculated absorption)	48.4	46.3	63.9	62.9
Blood (10 ml.)	—*	—*	—*	—*

Figures are per cent of dose, mean ± standard error.
*Too small to be quantitative.

present communication is concerned with a study in man of intestinal absorption of ^{57}Co-labeled CH_3-B_{12} and investigation into its structural integrity during and after absorption in rats using $^{14}CH_3$- and ^{57}Co-labeled CH_3-B_{12}, which enabled separate determination of the labile methyl and the stable cobalamin moieties.

Materials and methods

Cobamides. Labeled CH_3-B_{12} preparations were kindly provided by Prof. S. Fukui, Kyoto University Faculty of Engineering Chemistry, Kyoto, and the specific activities of $^{14}CH_3$-B_{12} and CH_3-B_{12}-^{57}Co were 0.037 μCi per microgram and 4 μCi per microgram, respectively. They were kept at -20° C. under tin-foil cover, and a strict precaution was received to avoid light. Containers and syringes were covered, and all handling was done in the dark. The purity of the preparations dissolved in distilled water was checked with a 12 by 150 mm. column of carboxymethyl (CM) cellulose, eluting first with distilled water and then with 0.05 M acetic acid. Effluents were collected 5 ml. per tube. Under these conditions, pure CH_3-B_{12} was eluted with water in a single peak at tube No. 9. After switch to acetic acid, OH-B_{12} was eluted with the peak at tube No. 5. When studied with these procedures, the purity of $^{14}CH_3$-B_{12} was found to be almost 100 per cent, and that of CH_3B_{12}-^{57}Co more than 95 per cent. Cyanocobalamin (CN-B_{12}-^{57}Co) of a specific activity of 3 μCi per microgram was also used for comparison.

Animals and absorption measurement. Adult rats of the Wistar strain that had been on a commercial chow diet were used. They were fasted overnight, fed a test dose of ^{57}Co-cobamide via a stomach tube, and absorption was calculated from the radioactivity recovered from the intestinal content or feces. For the collection of feces, metabolism cages were used.

Intrinsic factor. Rat intrinsic factor concentrate (IFC) was prepared briefly as follows.[8] The glandular portion of rat stomach was scraped, homogenized with a physiologic saline, and the supernatant was taken after centrifugation at 3,000 r.p.m. for 20 minutes. Hog IFC, WES No. 942, was kindly supplied by Dr. L. Ellenbogen, of Lederle Laboratories, Pearl River, N. Y. It had a B_{12} binding capacity of approximately 0.5 μg per milligram as measured by the exhaustive dialysis method.[9]

Photolysis. Materials in solutions were exposed to 2 fluorescent lamps of 40 watts at a distance of 30 cm. for 2 hours. When $^{14}CH_3$- and ^{57}Co-labeled CH_3-B_{12} were exposed to light and then examined with a CM-cellulose column, no CH_3-B_{12} was detected; there was a complete shift of the peak of ^{57}Co to the position identical with that of OH-B_{12} and ^{57}Co was eluted in the void volume. The absorption spectra recorded with a 27 μg per milliliter solution of crystalline CH_3-B_{12} in 0/03 M phosphate buffer of pH 7.0, before and after photolysis, were typical of that of CH_3-B_{12} and of OH-B_{12}.

Measurement of ^{14}C and $^{14}CO_2$. Rats were placed in a specially designed glass apparatus for trapping CO_2, and phenethylamine was used for absorption of CO_2. Tissues were dried in an oven of 120° C., and burned in a combustion chamber containing O_2 and phenethylamine.

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$CN\text{-}B_{12}\text{-}^{57}Co$ in the rat; the dose is 10 ng.

24 hrs.		48 hrs.	
CN	CH_3	*CN*	CH_3
6.4 ± 0.8	6.8 ± 1.2	5.8 ± 1.2	9.3 ± 1.1
4.6 ± 0.5	5.2 ± 0.7	3.6 ± 0.4	4.8 ± 0.5
1.8 ± 0.2	1.9 ± 0.3	0.6 ± 0.1	0.8 ± 0.1
15.7 ± 5.1	12.6 ± 3.2	3.2 ± 0.5	2.8 ± 0.4
13.6 ± 4.3	16.1 ± 3.6	26.7 ± 3.6	27.3 ± 4.9
68.9	69.4	60.5	61.1
0.86 ± 0.21	0.92 ± 0.36	0.51 ± 0.16	0.43 ± 0.14

Liquid scintillation counting was carried out in a scintillator consisting of dioxane, naphthalene, PPO, and POPOP as previously described.[10] Urine and plasma were added directly to the scintillator and measured with corrections for quenching. In experiments in which two different labels were used, correction for gamma had to be made in beta counting. To this end, a correction curve was drawn to relate liquid scintillation counting with gamma counting using graded doses of aqueous $CH_3\text{-}B_{12}\text{-}^{57}Co$. Under the conditions used, the corrections were within 3 per cent of the beta counts.

Measurement of ^{57}Co. Weighed tissues were dissolved in 20 per cent NaOH in a water bath, made up to 5 ml., and counted in a well-type gamma counter. Feces were collected for 6 days, softened with water, and homogenized after addition of concentrated sulfuric acid, and 5 ml. aliquots were counted in duplicate.

Paper chromatography. A specimen containing ^{57}Co was applied on a filter paper, Toyo No. 50, and was developed by the ascending method for 20 hours using a solvent consisting of sec-butanol, glacial acetic acid, and water, 100:3:5. The paper was dried, cut in 1 cm. strips and they were counted after dissolving in 5 ml. H_2SO_4.

Results

Absorption of $CH_3\text{-}B_{12}$ vs. $CN\text{-}B_{12}$. Aqueous solution of 10 ng., a physiologic dose, of CH_3- and $CN\text{-}B_{12}\text{-}^{57}Co$ in 1 ml. was fed by mouth to 2 groups of 12 rats, and they were killed after 4, 8, 24, and 48 hours. The liver, kidneys, pancreas, lungs, brain, heart, skeletal muscle, stomach, small intestine, cecum, and colon were removed. The small intestine was washed with 50 ml. of saline, the cecal content was collected, and colonic content was added to feces. Table shows the decreases of radioactivity from the intestine that served for the calculation of absorption, and tissue absorption represented by the liver. There was practically no difference in quantitative absorption nor in time relation. There was a gradual yet significant increase of radioactivity in the liver, greater in animals receiving $CH_3\text{-}B_{12}$ compared with $CN\text{-}B_{12}$.

In a similar experiment, 3 different doses of CH_3 and $CN\text{-}B_{12}\text{-}^{57}Co$ were fed to 4 rats each, and feces was collected for 6 days. The absorption calculated from fecal recovery was 67.2 ± 4.1 per cent (standard error) for $CN\text{-}B_{12}$ and 68.4 ± 5.2 per cent for $CH_3\text{-}B_{12}$ with a 5 ng. dose, 63.3 ± 4.8 per cent and 61.8 ± 4.1 per cent, respectively, with a 20 ng. dose, and 48.4 ± 6.8 per cent and 49.8 ± 5.9 per cent with a 100 ng. dose. There was no difference in organ and tissue uptakes between the 2 groups except for the difference in the liver uptake of radioactivity as in the preceding study, i.e., a greater uptake in the $CH_3\text{-}B_{12}$ group.

Table II. Urinary excretion following oral administration of CH_3-B_{12}-^{57}Co in comparison with CN-B_{12}-^{57}Co (Schilling test)

Cobamide (0.5 μg)	*No. of subjects*	*Flushing dose*	*Excretion in 24 hour urine (%)*
CN-B_{12}-^{57}Co	25	CN-B_{12}	25.3 ± 3.8
CH_3-B_{12}-^{57}Co	9	CN-B_{12}	7.2 ± 0.9
CH_3-B_{12}-^{57}Co	5	CH_3-B_{12}	6.8 ± 2.5

Table III. Binding of $^{14}CH_3$-B_{12} by hog IFC and effect of photolysis

Preparation of mixture	*Dialysis*	*c.p.m. in bags*	*Per cent relative to control*
$^{14}CH_3$-B_{12}* + IFC†	–	10,800	100
$^{14}CH_3$-B_{12}* + IFC†	+	9,280	86
$^{14}CH_3$-B_{12}* + IFC† (photolysis)	+	320	3
$^{14}CH_3$-B_{12}* (Photolysis) + IFC†	+	220	2

*0.5 μg.
†1.0 mg.

Absorption of CH_3-B_{12}-^{57}Co in man. Absorption of 0.5 μg of CH_3-B_{12}-^{57}Co was measured by the Schilling test in 14 subjects with no major diseases affecting the gastrointestinal function. For the flushing, 1 mg. of nonradioactive CN-B_{12} was injected intramuscularly in 9 subjects and 1 mg. of CH_3-B_{12} in 5 subjects. The results were compared with those obtained in 25 subjects using the same dose of CN-B_{12}-^{57}Co (Table II). The 24-hour urinary excretion following oral administration of CH_3-B_{12}-^{57}Co was about a third that for CN-B_{12}, regardless of the type of B_{12} used for the flushing.

Binding of CH_3-B_{12} by IFC and plasma, and stability of the complex. The capacity of CH_3-B_{12} to combine with IF and transcobalamin was studied in comparison with CN-B_{12}. Four graded doses of 2, 5, 10, and 20 ng. of CH_3-B_{12}-^{57}Co and CN-B_{12}-^{57}Co were mixed with 0.01 mg. of hog IFC in 1 ml. saline, and the amounts bound to IFC were measured after 48 hours of exhaustive dialysis against saline. There was practically no difference between the two types of cobamide quantitatively. The experiment was repeated with rat IFC of a comparable binding power, and with 1 ml. of human serum, and the results were the same. When photolyzed ($h\nu$) CH_3-B_{12}-^{57}Co was used, there was an increasing binding with increasing doses, as contrasted by a leveling off in the case of CN- and CH_3-B_{12}.

Our previous study with IF-DAB_{12}[10] showed that the label in 5′-deoxyadenosyl moiety is still attached to IF after photolysis. It seemed of interest, therefore, to examine the behavior of CH_3-B_{12} under the same conditions. $^{14}CH_3$-B_{12} (0.5 μg) and 1 mg. of hog IFC were mixed in different orders with or without exposure to light, and dialyzed (Table III). When the mixture was exposed to light, the radioactivity was completely dialyzed, while the unexposed mixture was not. The ^{14}C label after detachment from the cobalamin structure by photolysis was not bound to IFC. Thus, binding by IF did not afford protection from photolysis of the CH_3 group.

Table IV. Evolution of $^{14}CO_2$ following oral administration of $^{14}CH_3$-B_{12} to the rat and recovery of ^{14}C after 7 hours *

Hours after administration	*$^{14}CO_2$ evolved (per cent of ^{14}C given)*	*Localization*	*Recovery of ^{14}C after 7 hours (%)*
1	0.45 ± 0.06	Urine in 7 hours	0.91 ± 0.12
2	0.36 ± 0.03	Liver	0.74 ± 0.10
3	0.51 ± 0.07	Kidneys	0.30 ± 0.06
4	1.45 ± 0.31	Cecal content	36.0 ± 8.6
5	2.29 ± 0.42		
6	1.70 ± 0.35		
7	1.45 ± 0.21		
Total in 7 hours	8.25 ± 1.43		

* Figures are averages of 4 rats, mean ± standard error.

$^{14}Co_2$ evolution following oral $^{14}CH_3$-B_{12}. Four rats were fed 3.4 μg of $^{14}CH_3$-B_{12}, immediately placed in CO_2-trapping apparatuses, and $^{14}CO_2$ expiration was measured for 7 hours. After 7 hours, they were killed, and ^{14}C in major organs and fecal material as well as in the 7-hour urine collection was measured (Table IV). The evolution of $^{14}CO_2$ was very small for the first 3 hours, after which there was some increase, and the 7-hour total was 8.25 per cent of the radioactivity originally given. About 36 per cent of the administered ^{14}C was recovered from the cecal content after 7 hours, but very little ^{14}C was found in the liver and kidney. When the same amount of hv $^{14}CH_3$-B_{12} was fed to another group for comparison, 18 per cent of ^{14}C was expired in the first hour, and the total in 7 hours was 45 per cent.

To explore the fate of the label in the methyl group after reaching the cecum, 1.7 μg of $^{14}CH_3$-B_{12} was injected into the cecum transserosally during laparotomy. The evolution of $^{14}CO_2$ was 1.5 per cent in the first hour, and after 4 hours 84 per cent of ^{14}C was recovered from the cecal content. These data indicate that $^{14}CH_3$ of $^{14}CH_3$-B_{12} was slowly but continuously absorbed from the small intestine as well as from the cecum. In contrast, ^{14}C in the photolysis product of $^{14}CH_3$-B_{12} was quickly absorbed and expired as $^{14}CO_2$.

Stability of $^{14}CH_3$-B_{12} in the small intestine. $^{14}CH_3$-B_{12} and CH_3-B_{12}-^{57}Co in a dose of 2 μg were administered orally to 4 rats each, and they were killed 2 at a time after 1 and 2 hours. The stomach, small intestine, and cecum were removed, and double ligatures were placed at the pylorus, at positions 1/3 and 2/3 of the small intestine, and at the ileal end. The digestive tract was separated at the ligatures, and ^{14}C and ^{57}Co in each portion were determined. It is evident from Fig. 1, which shows the result at 1 hour, that when $^{14}CH_3$-B_{12} was fed, about 43 per cent of the label was recovered or 57 per cent was absorbed in contrast to less than 10 per cent absorption of ^{57}Co. No radioactivity was recovered from the cecum. It is obvious that when a large dose was fed, the $^{14}CH_3$ group was detached to a significant extent in the upper gastrointestinal tract. The result at 2 hours was not much different, except for further but slow decreases in the recovery of radioactivity.

To eliminate the influence of gastric juice and pancreatic enzymes, a closed loop of the small intestine was prepared according to the technique described.[8] Loops were made in 6 rats, 0.5 μg of $^{14}CH_3$-B_{12} and CH_3-B_{12}-^{57}Co was injected

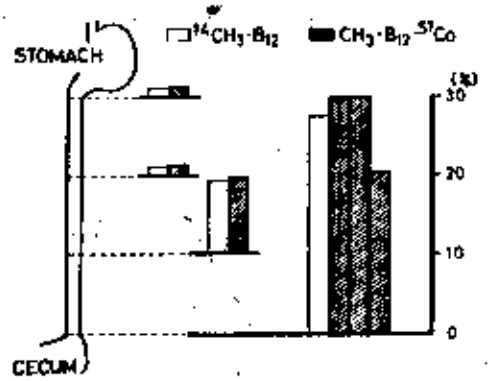

Fig. 1. Distribution of ^{14}C in the upper gastrointestinal tract 1 hour after oral administration of 2 μg each of $^{14}CH_3$-B_{12} and CH_3-B_{12}-^{57}Co (averages of 2 rats).

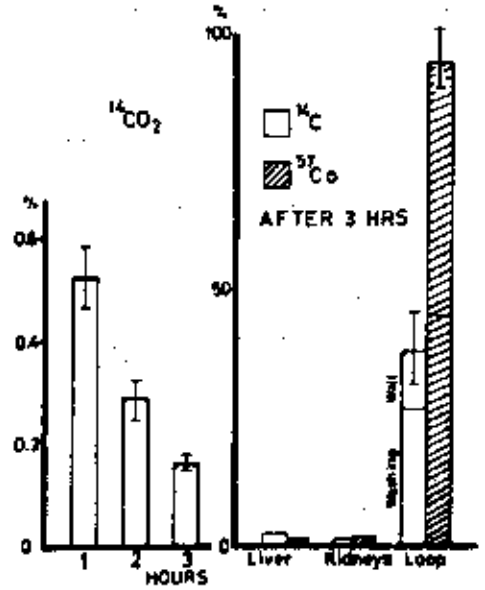

Fig. 2. $^{14}CO_2$ expiration following instillation of 0.5 μg of $^{14}CH_3$-B_{12} in a rat intestinal loop (left), and recovery of ^{14}C from the loop and major organs in comparison with that of ^{57}Co after instillation of the same dose of CH_3-B_{12}-^{57}Co (right). Figures are averages of 3 rats.

into 3 rats each, animals receiving the former were kept in the CO_2-trapping apparatuses, and they were killed after 3 hours. The amounts of $^{14}CO_2$ evolved and the radioactivities recovered from organs, the loop wall, and in its washing are shown in Fig. 2. Expiration of $^{14}CO_2$ in the first hour was 0.5 per cent to decline thereafter, and about 60 per cent of ^{14}C disappeared from the loop in 3 hours. In contrast, less than 10 per cent of ^{57}Co was absorbed in the same period of time. It was thus suggested that the loss of CH_3 occurs in the small intestine without the influences of gastric and pancreatic juices.

In another set of rats, a mixture of $^{14}CH_3$-B_{12} and CH_3-B_{12}-^{57}Co was administered orally and the distribution and ratio of the two labels were studied.

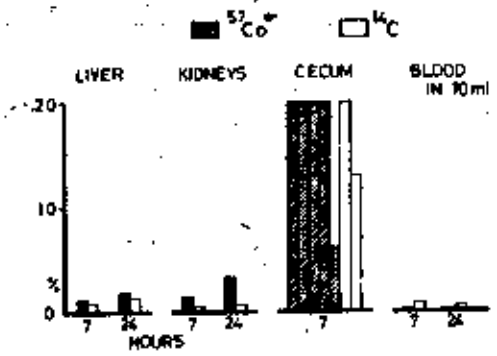

Fig. 3. Tissue distribution of ^{14}C and ^{57}Co after oral administration of 1.7 μg of $^{14}CH_3$-B_{12}-^{57}Co expressed as per cent of the dose of each label (averages of 3 rats).

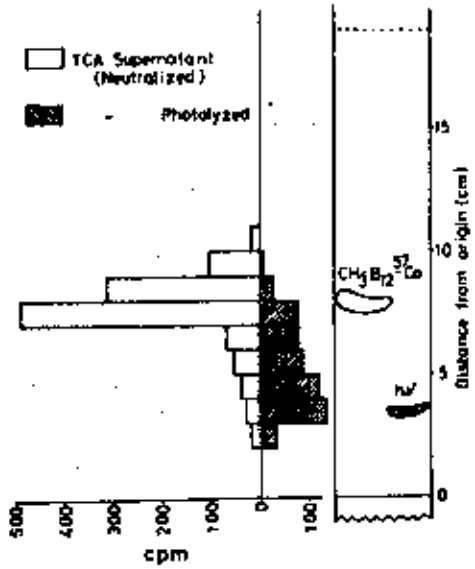

Fig. 4. Paper chromatography of digested ileal mucosa, taken 1.5 hours after oral administration of 150 ng. of CH_3-B_{12}-^{57}Co. On the strip in the right are shown the location of authentic $^{14}CH_3$-B_{12} and hv-CH_3-B_{12}.

If the CH_3 group was stable, both labels should be parallel in distribution and in the amount relative to the original dose. The dose was prepared in such a way that it contained a total of 1.7 μg of B_{12} with minimal interference of ^{57}Co in ^{14}C counting. Fig. 3 represents the average of 3 rats killed after 7 hours and 24 hours. The unabsorbed portion recovered from the cecal content at 7 hours was 33 per cent for ^{14}C, in a distinct contrast to 80 per cent for ^{57}Co. The organ radioactivities were not in proportion to the net absorption, and the amount of ^{14}C in kidneys, in particular, was much smaller than ^{57}Co at 7 hours and 24 hours after administration.

Detection of CH_3-B_{12} in the mucosa. $^{14}CH_3$-B_{12}-^{57}Co in a dose of 150 ng. was given orally to a fasting rat, and the small intestine was removed after 1.5 hours. The mucosa of the distal half was washed thoroughly with warm saline,

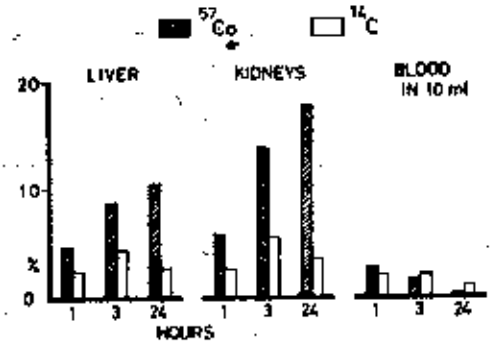

Fig. 5. Tissue distribution of ^{14}C and ^{57}Co after intramuscular injection of 1.7 μg of $^{14}CH_3$-B_{12}-^{57}Co expressed as per cent of the dose of each label.

Table V. Stability of $^{14}CH_3$-B_{12} in human blood

Tube	Blood* (ml.)	$^{14}CH_3$-B_{12}	Incubation (hrs.)	IFC (mg.)	Dialysis of plasma	c.p.m./ml.	%
Control 1	6	0.6 (μg)	—	1.5	-	2,170	100
Control 2	6	0.6 (μg)	—	1.5	+	2,074	96
Control 3	6	0.6 (hv)	3	1.5	+	48	2
Experimental	6	0.6 (hv)	3	1.5	+	2,262	104

*Contained 5 mg. of CN-B_{12} per milliliter to saturate B_{12} binding capacity.

scraped, and made into a 20 per cent homogenate with a physiologic saline. The homogenate was digested with Pronase, 2 mg. per milliliter, for 1 hour, and then centrifuged. The supernatant was precipitated with trichloroacetic acid to remove proteins, and the supernatant was immediately neutralized. One-half portion was exposed to light. Paper chromatography was carried out for the two kinds of supernatant, photolyzed and not photolyzed, and the Rf of ^{57}Co was measured after 20 hours. Concentrated CH_3-B_{12}-^{57}Co and its photolysis product were also applied and chromatographed simultaneously. The distribution of radioactivity measured in the cut strips of 1 cm. width, relative to the localization of authentic specimens grossly depicted are shown in Fig. 4. The bulk of radioactivity in the digested mucosa was located at 7 to 9 cm. from the origin, corresponding to the spot of CH_3-B_{12}. The radioactivity of the photolyzed supernatant was distributed in a wide area closer to the origin, and its peak at 3 to 4 cm. corresponded to that of authentic $he^{14}H_3$-B_{12}. Under the same conditions, DAB_{12} and CN-B_{12} are located between the two spots.

Parenteral administration of doubly labeled CH_3-B_{12}. The same mixture of CH_3-B_{12}-^{57}Co and $^{14}CH_3$-B_{12} as in the previous study was injected intramuscularly to 9 rats, and they were killed at 1, 3, and 24 hours. The amounts of ^{14}C in the liver and kidneys were always smaller than those of ^{57}Co, and the concentration of ^{57}Co in blood decreased after 3 hours to become almost immeasurable while considerable amounts of ^{14}C were still detected in blood after 24 hours (Fig. 5). $^{14}CO_2$ evolution following intramuscular injection of $^{14}CH_3$-B_{12} and $he^{14}CH_3$-B_{12} in a dose of 1.2 μg was measured in 3 rats each. About 43 per cent of the dose was expired in the first hour and a total of 61 per cent in

6 hours with hv$^{14}CH_3$-B_{12}, and the corresponding figure for $^{14}CH_3$-B_{12} were 2 and 13 per cent, respectively.

Stability of CH_3-B_{12} in blood. Six milliliters of heparinized human whole blood were mixed first with 30 ng. of CN-B_{12} which was to saturate the binding capacity of transcobalamins, and then with 0.6 μg of $^{14}CH_3$-B_{12} 30 minutes later. The mixture was incubated for 3 hours, and 1.5 mg. of hog IFC was added to render free cobamide nondialyzable. The mixture was centrifuged, plasma was divided and dialyzed in duplicate for 24 hours against saline with 3 changes. The radioactivity remaining in the tube was compared with that obtained in 3 sets of control as shown in Table V. In Control 1, which was not dialyzed, and in Control 2 which was not incubated but was immediately chilled in ice water and dialyzed, the radioactivities in plasma and in the dialyzed plasma were practically the same as that of the experimental. The third control in which hv$^{14}CH_3$-B_{12} was used with IFC, radioactivity was completely dialyzed away. These data clearly indicate that $^{14}CH_3$-B_{12} was stable in whole blood for at least 3 hours, because the label of $^{14}CH_3$ was rendered nondialyzable by IFC, which does not bind detached $^{14}CH_3$.

Discussion

It has been shown that free CH_3-B_{12} is not very stable in the gastrointestinal tract and considerable loss of the methyl group takes place. Almost 60 per cent of the label of large oral doses of $^{14}CH_3$-B_{12} disappeared from the bowels in 1 hour during which time only negligible absorption of the entire molecule of B_{12} takes place.[11] Because of the low specific activity of $^{14}CH_3$-B_{12}, supraphysiologic doses had to be used, and these data may not necessarily apply to the physiologic situation where small quantities of CH_3-B_{12} are gradually freed from food and are bound by IF to form a complex which resists proteolysis.[12]

The label of photolyzed $^{14}CH_3$-B_{12} was very quickly absorbed and expired as $^{14}CO_2$ in large quantities, whereas only small fractions of absorbed label were expired when $^{14}CH_3$-B_{12} was administered. It seems, therefore, that the mode of detachment in the intestine of the CH_3 group from the cobalt atom of corrin ring is different from that in vitro or by photolysis, although conversion of the cobalamin moiety to OH-B_{12} may be the same. Similar differences were also noted in the experiment in which $^{14}CH_3$-B_{12} and hv$^{14}CH_3$-B_{12} were injected intramuscularly; much less label was taken up by the kidney with the latter. In solution and when oxygen is available, the methyl group is converted mainly to ^{14}CHO, and probably also to CH_4, C_2H_6, and CH_3OH in minute quantities,[9] but in vivo, the biochemical environment is different and the methyl group may be transferred to or exchanged by certain organic substances. There was also some difference in urinary excretion and organ uptake between the oral and the intramuscular route of administration of $^{14}CH_3$-B_{12}.

Paper chromatography of the ileal mucosal extract prepared in the absorptive phase after oral administration of a relatively small dose of CH_3-B_{12}-^{57}Co, demonstrated that the Rf of the bulk of radioactivity corresponded to that of CH_3-B_{12}. It had been thought that more of the B_{12} in mucosa was OH-B_{12} as a result of the loss of CH_3. The radioactivity in the mucosal extract after photolysis did not localize itself at the spot for hvCH_3-B_{12} (OH-B_{12}), but was also spread in

the area between the positions for CH_3-B_{12} and OH-B_{12} where DAB$_{12}$ and CN-B_{12} are to be located. It may be that some of the absorbed cobamide had already been converted to other forms of cobamide that did not yield OH-B_{12} upon photolysis. It is also possible that IF-bound CH_3-B_{12} was protected from chemical change and entered the mucosa as such, while the unprotected and unaided molecule became OH-B_{12} and remained largely unabsorbed. Despite its stability in blood in vitro, it is not unexpected that CH_3-B_{12} quickly loses its methyl moiety in tissues where a multitude of reactions are going on. CH_3-B_{12} constitutes a large portion of plasma- or transcobalamin-bound B_{12},[3, 13] and a cycle of regeneration and degradation of CH_3-B_{12} synchronous with its movement in and out of plasma may be conceived. Our previous study on DAB$_{12}$ in rats demonstrated a much greater stability in the gastrointestinal tract and slower changes in tissue. The differences may be due to a greater stability of the 5'-deoxyadenosyl moiety which attaches itself to IF and probably to other binders as shown by its non dialyzability after photolysis.[10]

The binding of CH_3-B_{12} to IFC and plasma was the same as that of CN-B_{12}, whereas the behavior of CH_3-B_{12} or OH-B_{12} was quite different and the greater its amount relative to binders, the more was bound to them.[11] The greater hepatic uptake that became evident after 8 hours following oral administration of CH_3-B_{12}-^{57}Co compared with CN-B_{12}-^{57}Co, indicated a slow conversion of CH_3-B_{12} to free OH-B_{12} and cobamide coenzyme that have a greater affinity for liver.[15, 16] It is to be noted that in this absorption study, a physiologic dose was used, and the differences from experiments employing large doses of CH_3-B_{12} may be accounted for by IF which is operative in the former situation. The result of the Schilling test also indicates conversion of the dose to other natural cobamides which are excreted in urine to a lesser extent than CN-B_{12}.[16, 17]

The biochemical steps of B_{12} absorption involving IF have been the subject of extensive studies.[18, 19] However, very little information is available as to the chemical changes as might occur to B_{12} molecule during absorption. Peters and associates[20, 21] recently demonstrated in guinea pigs that uptake of CH_3-B_{12}-^{57}Co by the enterocyte was similar to that of CN-B_{12}-^{57}Co, and that these cobamides were localized in the mitochondria during an early phase of absorption. They also showed the conversion in the ileal mucosa of CN-B_{12} to DAB$_{12}$ and other unidentified cobamides, but not to CH_3-B_{12}.[22] The liver is not the only organ where interconversion of cobamides takes place, and whatever form of cobamide is released from food, it will be absorbed by the enterocyte with the aid of, and under protection of, IF and enter the body pool of cobamides which are in equilibrium among tissues.

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REFERENCES

1. Smith EL, Mervyn L, Johnson AW, et al: Partial synthesis of vitamin B_{12}-coenzyme and analogues. Nature 194: 1175, 1962.
2. Guest JR, Friedman S, and Woods DD: A methyl analogue of cobamide coenzyme in relation to methionine synthesis by bacteria. Nature 194: 340-342, 1962.
3. Lindstrand K and Ståhlberg KG: On vitamin B_{12} forms in human plasma. Acta Med Scand 174: 665-669, 1963.

4. Lindstrand K: Isolation of methylcobalamin from natural source material. Nature **204**: 188-189, 1964.
5. Toohey JL and Barker HA: Isolation of coenzyme B_{12} from liver. J Biol Chem **236**: 560-563, 1961.
6. Hogenkamp HPC: The photolysis of methylcobalamin. Biochemistry **5**: 417-422, 1966.
7. Dolphin D, Johnson AW, and Rodrigo R: Some reactions of the vitamin B_{12} coenzyme and its alkyl analogues. Ann NY Acad Sci **112**: 500-600, 1964.
8. Okuda K: Vitamin B_{12} absorption in rats, studied by a "loop" technique. Am J Physiol **199**: 84-90, 1960.
9. Okuda K: Binding of vitamin B_{12} by intrinsic factor and intestinal absorption. Clin Chim Acta **7**: 778-787, 1962.
10. Okuda K, Yoshina T, and Takamatsu M: Intestinal absorption of colamide coenzyme in relation to structural integrity and intrinsic factor. J Lab Clin Med **74**: 218-228, 1969.
11. Okuda K, Kitazaki T, and Takamatsu M: Inactivation of vitamin B_{12} by a binder in rat intestine and the role of intrinsic factor. Digestion **4**: 35-48, 1971.
12. Grisolock R: Intrinsic factor and the other vitamin B_{12} transport proteins. Progr Hematol **6**: 233-260, 1969.
13. Hall CA: Transport of vitamin B_{12} in man. Br J Haematol **16**: 429-433, 1969.
14. Tsutsumi C: Fundamental studies on the blood concentration of injected hydroxocobalamin. Vitamins **33**: 381-397, 1966.
15. Glass GBJ, Lee DH, and Hardy WW: Hydroxocobalamin. II. Absorption from site of injection and uptake by liver and calf muscle in man. Blood **18**: 522-526, 1961.
16. Okuda K and Tantengco V: Intestinal absorption of 5,6-dimethyl-benzimidazolyl colamide coenzyme in the rat. Proc Soc Exp Biol Med **110**: 396-400, 1962.
17. Killander A and Schilling RF: Studies on hydroxocobalamin I. Excretion and retention of massive doses in control subjects. J Lab Clin Med **57**: 553-561, 1961.
18. Glass GBJ: Gastric intrinsic factor and its function in the metabolism of vitamin B_{12}. Physiol Rev **43**: 529-549, 1963.
19. Mackenzie IL and Donaldson RM: Vitamin B_{12} absorption and intestinal cell surface. Fed Proc **28**: 41-45, 1969.
20. Peters TJ and Hoffbrand AV: Absorption of vitamin B_{12} by the guinea pig. I. Subcellular localization of vitamin B_{12} in the ileal enterocyte during absorption. Br J Haematol **19**: 369-382, 1970.
21. Peters TJ, Quinlan A, and Hoffbrand AV: II. The subcellular localization of cyano-, methyl-, and adenosyl-cobalamins, and the effect of fluoroacetate on cyanocobalamin absorption. Br J Haematol **20**: 123-129, 1971.
22. Peters TJ, Linnell JC, Matthews DM, et al: III. The forms of vitamin B_{12} in ileal mucosa and portal plasma in the fasting state and during absorption of cyanocobalamin. Br J Haematol **20**: 299-305, 1971.

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Effects of Radiation and Diarrhea on Intestinal Absorption of Vitamin B_{12}

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Physiological absorption of B_{12} involves gastric intrinsic factor (IF) and divalent cations. It is generally agreed that an IF-B_{12} complex is formed in the gastrointestinal tract and it is absorbed into the mucosa[1], but the subsequent steps have not been elucidated. One of the possible and recently proposed mechanisms is that this complex enters the mucosal cell intact probably by pinocytosis[2]. The present study was undertaken to produce by means of irradiation, a mucosal damage of the intestine sufficiently severe so that one would expect reduction of pinocytic activities, and to see whether IF-mediated absorption of B_{12} would be altered. Since diarrhea inevitably develops following large doses of radiation, effects of diarrhea itself were investigated.

Adult rats were exposed to whole body X-ray irradiation at the dose range of 1,000–1,500γ, and absorption of ^{57}Co-cyanocobalamine was measured from fecal recovery of unabsorbed portions and organ uptakes following intraduodenal administration. This route, executed by laparotomy and trans-serosal injection of the dose, was used because of the delayed emptying time of the stomach after irradiation.

For the study of the effect of IF on B_{12} absorption under the influence of radiation, an *in vivo* system of the rat intestinal loop[3] was employed in which small intestine was washed free of endogenous IF, disconnected from the stomach, made into a closed loop, to which ^{57}Co-B_{12} with or without IF was applied, and absorption was measured from the decrease of radioactivity from the loop. To vary the speed of dose descent from the stomach and in the bowel for the study of the effect of diarrhea on absorption, castor oil, 30% $MgSO_4$, and Tween 80 which forms a gel in the stomach with an aqueous dose of B_{12}, were employed. The descent of the dose was estimated from the co-administered Indian ink and the distribution

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of radioactivity.

It was found that oral doses after irradiation were retained in the stomach very long but the intraduodenally instilled doses descended at almost the same speed as in the non-irradiated control during the first 24 hours. Absorption from the intestinal loop of physiological doses of B_{12} with the aid of homologous IF as well as of supraphysiological doses of B_{12} without IF, was found not to be

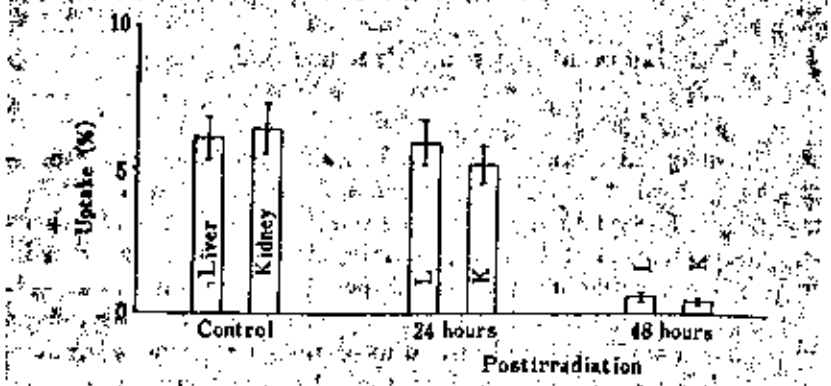

Fig. 1 Effect of radiation on B_{12} absorption. Rats received 1,000 γ 24 and 48 hours prior to test, 1 mμg ^{57}Co-B_{12} was placed in the duodenum and absorption estimated at 3 hours from organ uptakes.

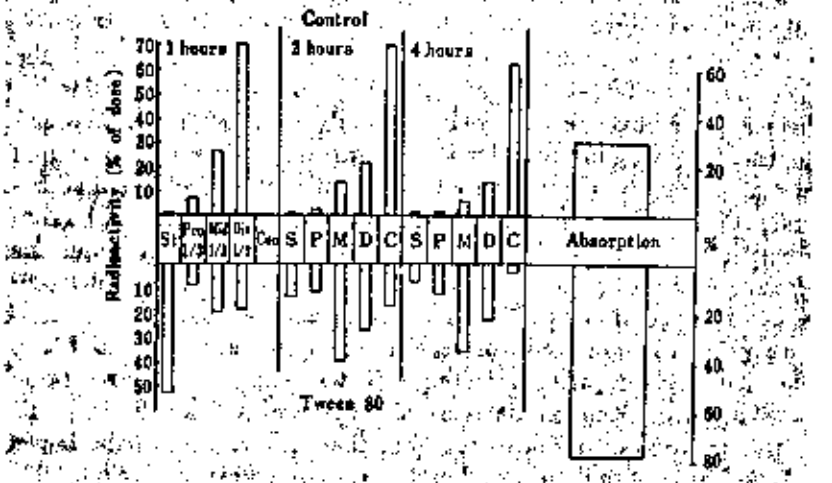

Fig. 2 Relationship between speed of dose descent and absorption of B_{12}. 50 mμg ^{57}Co-B_{12} was fed with or without Tween 80 to be mixed in the stomach. Intestinal distribution at short intervals and absorption by fecal test after 3 days were determined.

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reduced shortly after irradiation. Mucosal absorption of IF-B_{12} complex, which is measured by washing the removed loop throughly with saline and determining radioactivity adhering to the loop wall, is normally complete within 45 minutes of application of the dose. After irradiation, this process and subsequent absorption were almost normal within 24 hours, but they became increasingly impaired as time elapsed thereafter (Fig. 1). Absorption of B_{12} was inversely correlated with the speed of dose descent in the small intestine (Fig. 2). Diarrhea caused by $MgSO_4$ was exception, and absorption of both physiological and supraphysiological doses was increased when co-administered with this salt. It is probably related to the amount of divalent ions in the intestinal lumen.

These data by and large, suggest that absorption of B_{12} from the small intestine is little affected by lethal doses of radiation for 24 hours postirradiation, after which absorption is impaired due to histoanatomical damage to the mucosa. Although no support to the pinocytic theory for the absorption of IF-B_{12} complex has been obtained by this study, interpretation of the results depends on the effect of radiation on pinocytic activities of the mucosal cell which has as yet to be determined.

References

1. Okuda, K.: Mucosal adsorption and absorption of vit. B_{12} in the intestine of the rat. Proc. Soc. Exp. Biol. Med. *111*: 320–323, 1962.
2. Wilson, T. H.: Intestinal absorption of vit. B_{12}. Physiologist 6: 11–26, 1963.
3. Okuda, K.: Vit. B_{12} absorption in rats, studied by a 'loop' technique. Amer. J. Physiol. *199*: 84–90, 1960.

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Antibody to Transcobalamin II in Patients Treated with Long Acting Vitamin B_{12} Preparations

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^{57}Co-labelled vitamin B_{12} bound to transcobalamin I, transcobalamin II or non-protein bound, has been mixed with serum from patients with pernicious anaemia treated with long acting vitmain B_{12} preparations. By paper electrophoresis, immunoelectrophoresis and Sephadex G-200 gel filtration, an antibody of the γG-globulin type reacting with transcobalamin II was demonstrated. By immunoelectrophoresis the antibody was demonstrated in 8 of 19 patients. Gel filtration experiments showed that the affinity of the antibody to TC II is very sensitive to changes in pH, ionic strength and temperature.

Treatment of patients with pernicious anaemia (P.A.) with long acting vitamin B_{12} preparations (Betolvex®** or hydroxocobalamin) every second to third month has been able, in all cases, to give total remission or sustain the remission already obtained with other preparations (Bastrup-Madsen et al. 1966). In some patients the serum vitamin B_{12} concentration is not only normalized, but levels of over 1000 pg./ml. are attained for long periods of time. The total vitamin B_{12} binding capacity of these sera is also increased.

The reason for this is the presence of an antibody of the γG-globulin type, as has been shown by Schwartz & Bastrup-Madsen (1968). On adding ^{57}Co-labelled vitamin B_{12} to serum, autoradiography of a paper electrophoresis showed the activity to be localized in a rather narrow band in the

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* U. S. Public Health Service Postdoctoral Research Fellow (1 F2 AM 28,845 01A1) of the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health 1966–67.

** Cyanocobalamin-tannate suspended in aluminium-monostearate-sesame oil.

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fast γ-globulin region. This is in contrast to the vitamin B_{12} binders in normal human serum, transcobalamin I and II, that are localized in the α_1-globulin and β-globulin regions, respectively.

In the present work, the purpose has been to extend these studies, to classify what the antibody is combining with, to obtain an impression of the frequency of the antibody and to study, by gel filtration, the influence of different experimental conditions on the antigen-antibody reaction.

PATIENTS, MATERIALS AND METHODS

Patients. Serum from 15 patients treated with Betolvex®, 4 patients treated with hydroxocobalamin (Vibeden®), and 1 normal person was studied. In the treated patients the diagnosis of pernicious anaemia was based upon the results of conventional haematologic investigations including Schilling tests. All serum samples were obtained two months after the last vitamin B_{12} injection.

Serum vitamin B_{12} concentrations were determined by a modification (Raven et al. 1966) of the method of Lau et al. (1963).

Serum unsaturated vitamin B_{12} binding capacity was determined by the method of Gottlieb et al. (1965).

Paper electrophoresis was performed in veronal buffer (Laurell et al. 1956). *Autoradiography* was made with the dried strips before washing and staining to avoid elution of the labelled vitamin B_{12}.

Immunoelectrophoresis was performed by a modification of the micromethod of Scheidegger (1955). After the immunodiffusion, the plates were washed in deionized water for one hour, dried and exposed to X-ray film for 5 weeks. Antitotal human serum, anti-γG-, γA- and γM-globulin, and anti-kappa and anti-lambda antibodies were produced in rabbits in this laboratory.

Sephadex G-200 gel filtration was performed in columns of 100 x 2.3 cm. For buffers see Table I. Fractions of 3 ml. were collected by an automatic fraction collector.

Molecular sizes were estimated from the gel filtration data by use of the correlation between the logarithm of the molecular weight (mol. wt.) for γG-globulin (protein peak II), albumin (protein peak III), the unknown substance and their respective elution volumes by the method of Andrews (1964).

Diethylaminoethyl (DEAE)-cellulose chromatography was performed with gradient elution using a cone sphere arrangement. The starting buffer was 0.01 M sodium phosphate, pH 8.0 and the limit buffer was 0.3 M sodium phosphate pH 4.5 (Fahey et al. 1958).

γG-globulin from patient 1-D.S. (see Table II) was the breakthrough fraction from a DEAE-cellulose chromatography.

Transcobalamin I and II were separated by Sephadex G-200 gel filtration and labelled with ^{57}Co-labelled vitamin B_{12} as described by Hom & Olesen (1967). These preparations will be designated ^{57}TC I and ^{57}TC II in the following.

^{57}Co-labelled vitamin B_{12} ($^{57}B_{12}$) with a specific activity of 250 μc. per μg. cyanocobalamin was obtained from Philips Duphar, Amsterdam, Holland. The activity was measured in a well-type scintillation counter.

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TABLE I

Sephadex G-200 gel filtration of serum I-D.S. under different conditions

Exp. no.	I-D.S. serum µl.	*B_{12} added	Buffer	pH	Molarity	NaCl mole	Temp. °C.	Per cent labelled B_{12} bound to type of binders			
								Complex mol. wt. 240,000	*TC I mol. wt. 121,000	*TC II mol. wt. 38,000	
1	500	–	Tris	8.0	0.1	1	20	93.6[1]		6.4[1]	
2	500	*B_{12}	Tris	8.0	0.1	1	20	48.9		51.1	Trailing
3	500	*TC II	Tris	8.0	0.1	1	20	25.8		74.2	Trailing
4	500	*TC I	Tris	8.0	0.1	1	20		100		
5	300	*B_{12}	Naph	7.5	0.04	–	4	97.5		2.5	
6	150	*TC II	Naph	7.5	0.04	–	4	>98			
7	150	*TC II	Naph	4.5	0.3	1	20			>95	
8	500	*TC II	Serum	7.4		0.15	37	>99			
9	0	*TC II	Serum	7.4		0.15	37	>95			
10	450 NS[2]	*TC II	Naph	7.5	0.04	–	4	>95			Trailing
11	500 NS	*B_{12}	Tris	8.0	0.1	1	20	0.2	38.2	61.6	

1. Endogenous vitamin B_{12}.
2. Normal serum.

RESULTS

A. *Studies on serum from patient I-D.S.*

The patient is a 52 year-old female diagnosed as having P.A. in 1955 based upon the results of conventional haematological examinations including bone marrow puncture. The patient was treated with a variety of oral vitamin B_{12} and intrinsic factor preparations until 1962, when refractoriness to hog intrinsic factor developed. From March 1962, the patient has received bi-

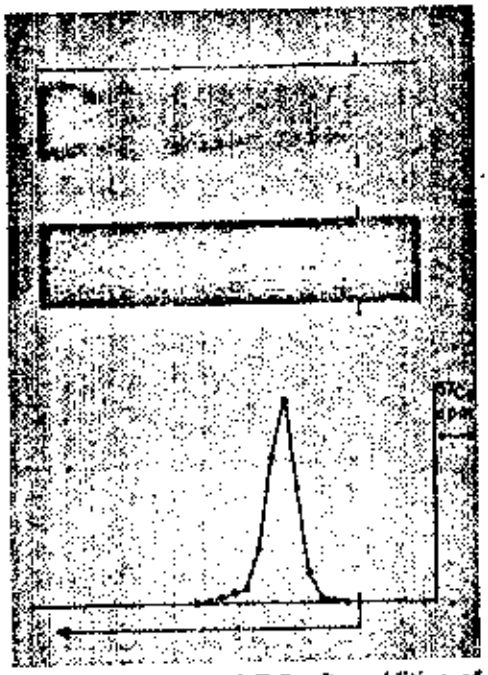

Figure 1. Paper electrophoresis of serum I-D.S. after addition of ^{57}Co-B_{12}-TC II. The radioactivity was localized in a narrow band in the fast γ-globulin region. In normal serum labelled TC II migrates with the β-globulins. The anode is to the left.

monthly intramuscular injections of Betolvex®. Within 9 months, there was about a ten-fold increase in the serum vitamin B_{12} level to 4450 pg./ml. Maximum values of about 100,000 pg./ml. were obtained in 1964 and 1965. During this period, the serum unsaturated vitamin B_{12} binding capacity was also markedly elevated with values between 51,000 and 125,000 pg./ml. The patient has remained in clinical and haematological remission throughout.

a. *Paper electrophoresis* (Figure 1). When $^{*}B_{12}$ or *TC II was added to serum I-D.S. the activity was localized to the fast γ-globulin region. In normal serum, *TC II migrates with the β-globulins and *TC I with the α_1-globulins.

TABLE II

Studies on sera from pernicious anaemia patients treated with Betolvex® or hydroxocobalamin

Patient	P.A. Duration years	Treatment		Serum		Autoradiography of immunoelectrophoresis						
		Type	Duration months	B_{12}	$TB_{12}BC$[1]	*B_{12} added	Type of rabbit antibody					
				pg./ml.			Total	γG	γA	γM	kappa	lambda
1-D.S.	12	B[2]	54	28,000	60,500	*B_{12}	γG	γG	–	γM	γG	γG
						*TC II	γG	γG	–	γM	γG	γG
						*TC II[3]	–	–	–	–	–	–
2-O.A.P.	12	B	66	34,800	62,300	*B_{12}	γG	γG	–	–	γG	–
						*TC II	γG	γG	–	–	γG	–
3-B.M.	3	B	38	4,360	10,290	*TC II	γG	γG	–	–	γG	–
4-A.J.	5	B	60	590	8,510	*TC II	γG	γG	–	–	γG	–
5-E.L.	14	B	66	4,200	6,420	*TC II	γG	γG	–	–	–	–
6-K.P.	6	B	68	2,310	4,480	*TC II	γG	γG	–	–	γG	–
7-K.L.	8	B	64	2,080	3,370	*TC II	γG	γG	–	–	γG	–
8-M.N.	3	B	36	764	3,084	*TC II	–	–	–	–	–	–
9-H.N.	14	B	66	1,086	2,526	*TC II	–	–	–	–	–	–
10-E.J.	14	B	66	748	2,378	*TC II	–	–	–	–	–	–
11-E.H.	4	B	38	646	2,066	*TC II	–	–	–	–	–	–
12-B.C.	18	B	66	568	1,808	*TC II	–	–	–	–	–	–
13-V.C.	16	B	66	400	1,720	*TC II	–	–	–	–	–	–
14-E.H.	13	B	68	476	1,631	*TC II	–	–	–	–	–	–
15-M.N.	13	B	66	504	1,544	*TC II	–	–	–	–	–	–
16-A.R.	1.5	V[4]	18	8,940	9,857	*TC II	–	–	–	–	–	–
17-E.J.	2	V	21	4,700	7,770	*TC II	γG	γG	–	–	γG	γG
18-K.S.	1	V	11	2,572	3,397	*TC II	–	–	–	–	–	–
19-I.C.	1.5	V	17	1,474	2,302	*TC II	–	–	–	–	–	–
20 normal serum						*TC II	–	–	–	–	–	–
21 no serum added						*TC II	–	–	–	–	–	–

1. Total vitamin B_{12} binding capacity (normal range: 972–2332 pg./ml.).
2. Betolvex®.
3. Added to γG-globulin from serum 1-D.S. purified by DEAE-cellulose chromatography.
4. Vibeden® (hydroxocobalamin).

b. *Immunoelectrophoresis* (Figure 2 and Table II). Autoradiography of the immunoelectrophoresis of the mixture of $^{57}B_{12}$ or ^{57}TC II and serum 1-D.S. showed that the ^{57}Co-activity was localized in the anodal part of the γG-globulin precipitation line, and antibodies of both the kappa and the lambda type reacted. A very weak line of the γM-globulin type was also formed.

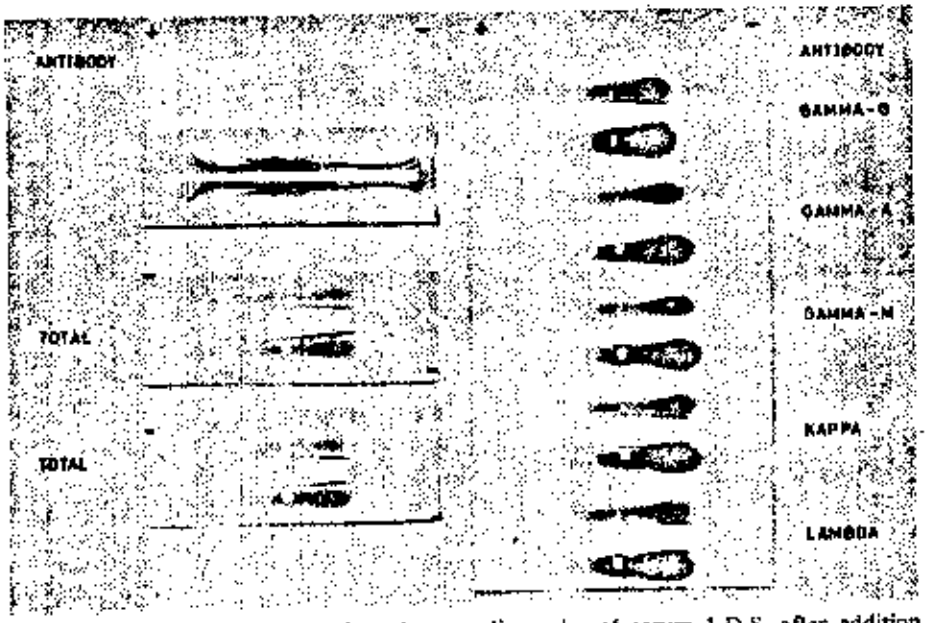

Figure 2. Immunoelectrophoresis and autoradiography of serum 1-D.S. after addition of ^{57}Co-B_{12}-^{57}TC II. The radioactivity was localized in the anodal part of the γG-globulin precipitation line. This is best seen in the two photographs on the lower left where serum was run against anti-total human serum. The stained slide in the upper left shows the length of the normal γG-globulin precipitation line. The photograph of the autoradiography on the right after the use of specific antisera, demonstrates that the antibody was type γG and, further, that antibodies of both the kappa and the lambda type reacted.

c. *DEAE-cellulose chromatography* (Figure 3). When serum 1-D.S. without added vitamin B_{12} was subjected to DEAE-cellulose chromatography both the endogenous vitamin B_{12} and the unsaturated binding capacity were eluted prealbumin. None appeared in the breakthrough fraction.

The breakthrough fraction containing γG-globulin was concentrated, dialysed versus isotonic saline and mixed with ^{57}TC II. By autoradiography of the immunoelectrophoresis, no blackening corresponding to the γG-globulin line was obtained (Table II), indicating that this fraction of the γG-globulin was not able to combine with ^{57}TC II.

d. *Sephadex G-200 gel filtration* (Table I). For comparison with the following experiments, the result obtained when $^{*}B_{12}$ in excess of the binding capacity is added to normal human serum and filtered through Sephadex G-200 is shown in Figure 4 (Exp. 2). Three major activity peaks are seen, one for TC I, one for TC II and a third for free vitamin B_{12}.

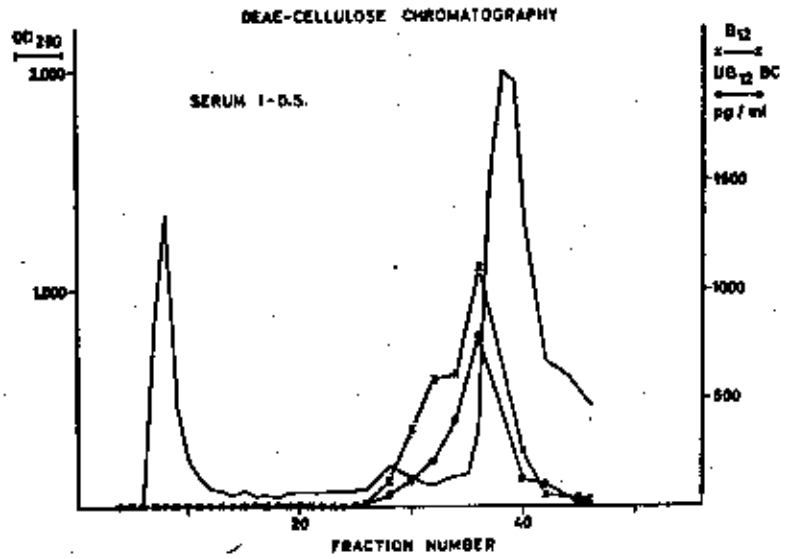

Figure 3. DEAE-cellulose chromatography of 1 ml. of serum 1-D.S. No vitamin B_{12} was added. Both the endogenous B_{12} and the unsaturated B_{12} binding capacity were eluted prealbumin. None appeared in the breakthrough (unbound) fraction which comprises about 50 % of the serum γG-globulin.

With serum 1-D.S. without added vitamin B_{12} (Figure 4, Exp. 1) the major part of the endogenous vitamin B_{12} appeared in a well defined peak between protein peaks I and II corresponding to a molecular size of about 240,000, while only 6.4 per cent was eluted postalbumin. The unsaturated B_{12} binding capacity however was rather evenly distributed at these two elution sites for endogenous vitamin B_{12}. Only a poor separation was obtained between the two peaks in the buffer used.

When $^{*}B_{12}$ was added to the serum (Figure 5, Exp. 2) two distinct peaks of about equal size were obtained at the same sites as for the endogenous vitamin B_{12} in the gel filtration Exp. I, confirming the results obtained by determination of the unsaturated B_{12} binding capacity performed on individual effluent fractions.

A similar result was obtained when *TC II (Exp. 3) was added to serum 1-D.S. (Figure 5). However, with *TC I and 1-D.S. serum (Figure 5) the

^{57}Co-activity appeared between protein peaks II and III at the site where TC I normally appears (see Figure 4).

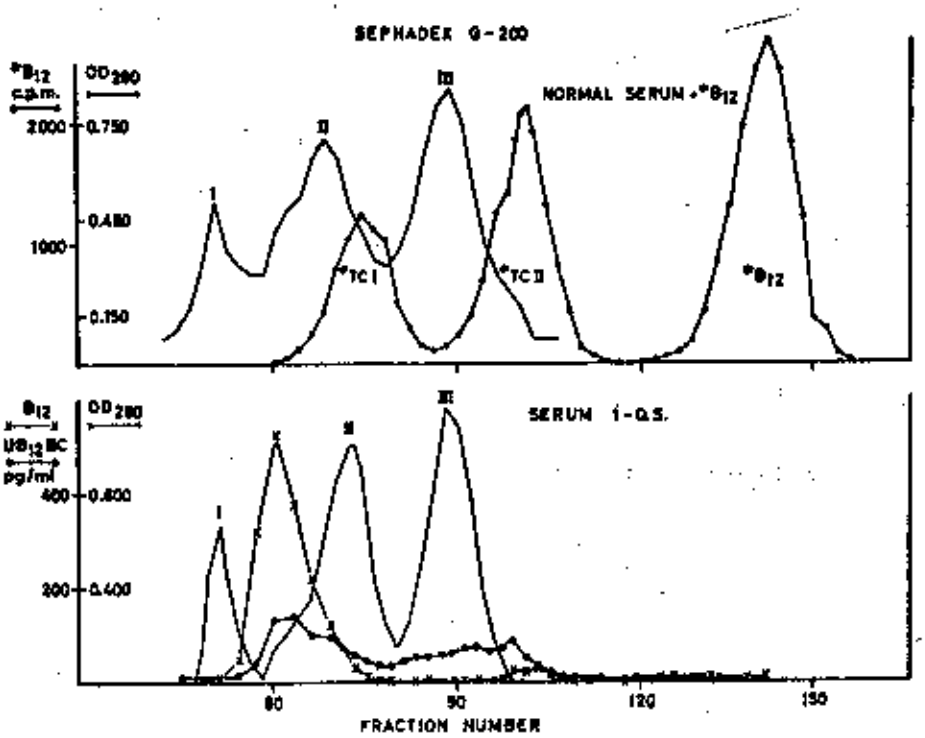

Figure 4. Sephadex G-200 gel filtration. *Upper diagram*, normal human serum after addition of an excess amount of radioactive B_{12}. *TC I appears between protein peak II (primarily γG-globulins) and protein peak III (albumin), while *TC II appears after protein peak III. The non-protein bound $^*B_{12}$ appears last. *Lower diagram*, serum 1-D.S. with no added B_{12}. The major part of the endogenous B_{12} appears in a well-defined peak between protein peaks I and II indicating a mol. wt. of about 240,000. A small fraction (6.4 %) of the endogenous B_{12} appears after the albumin peak. The unsaturated B_{12} binding capacity was rather evenly distributed between these two elution sites. No discrete binding by TC I could be detected.

e. *Sephadex G-200 gel filtration at different pH, ionic strength and temperature* (Table I). At the pH of normal serum, a low ionic strength and a low temperature nearly all of $^*B_{12}$ or *TC II added to 1-D.S. serum appeared presumably bound to the TC II antibody (Exp. 5 and 6), while an increase of temperature and molarity and a decrease in pH to 4.5 resulted in nearly quantitative elution of *TC II after the albumins (Figure 5, Exp. 7), indicating that the complex has dissociated.

When *TC II was added to normal human serum (Exp. 10) and run at low

ionic strength, low temperature and pH 7.5 the activity was eluted between protein peaks I and II, that is, in a high molecular weight form.

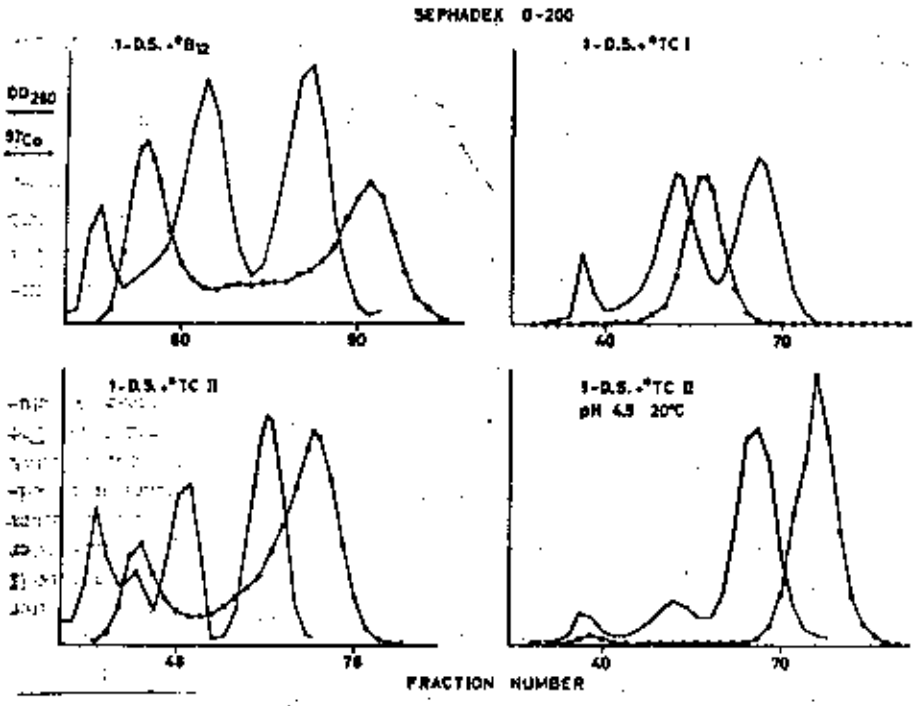

Figure 5. Sephadex G-200 gel filtration of serum 1-D.S. *Left diagrams*, after addition of either $^{*}B_{12}$ or *TC II, the radioactivity appears as a high molecular weight complex between protein peaks I and II and after protein peak III where TC II normally appears. There is considerable trailing between the two radioactive peaks. *Upper right*, after addition of *TC I, the radioactivity appears between protein peaks II and III at the site where TC I normally elutes. *Lower right*, after addition of *TC II and gel filtration at a higher ionic strength, higher temperature and a lower pH, the radioactivity appears after protein peak III, demonstrating that the high molecular weight complex had dissociated.

When a mixture of *TC II and ^{131}I-labelled γG-globulin was filtered through a Sephadex G-200 column equilibrated with normal human serum and run at 37° C., pH 7.4, the *TC II was eluted before the ^{131}I-labelled γG-globulin, that is, in a high molecular weight form (Exp. 8).

B. *Studies on sera from Betolvex® or hydroxocobalamin treated patients*

a. *Serum vitamin B_{12} and binding capactiy* (Table II). The levels of vitamin B_{12} and the total vitamin B_{12} capacities including transcobalamin I, trans-

cobalamin II, and the complex form is given in Table II. In 11 cases the vitamin B_{12} was over 800 pg./ml. and in 12 cases the binding capacity was over 2,500 pg./ml.

b. *Autoradiography of immunoelectrophoresis* (Figure 2 and Table II). TC II was mixed with the patients sera and subjected to immunoelectrophoresis and autoradiography. The results obtained with the different antisera are given in Table II. In 7 of the 15 Betolvex® treated patients and 1 of the 4 patients treated with hydroxocobalamin, ^{57}Co-activity localized to the anodal part of the γG-globulin precipitation lines. In two patients γG-globulin molecules of both kappa and lambda light chain type reacted. In the remaining 6 cases only kappa type molecular reacted. In patient 1-D.S. a faint line of the γM-globulin type was also seen. The light chain type of these molecules was not studied.

Antibody to TC II was found in cases where the total vitamin B_{12} binding capacity was higher than 3,200 pg./ml.

DISCUSSION

The results obtained with serum 1-D.S. show that this serum contains an antibody of the γG-globulin type, specifically reacting with transcobalamin II. It did not react with transcobalamin I, and the reaction with free vitamin B_{12} is caused by the binding of TC II to the antibody. The molecular size of the vitamin B_{12}-TC II-γG-globulin complex correspond to a molecular weight of about 240,000. As the number of combining sites on γG-globulin molecules is two (Eisen et al. 1949) it seems reasonable to assume that the complex consist of one γG-globulin molecule (mol.wt. 150,000), two TC II molecules (mol. wt. 38,000) (Hom et al. 1967) and possibly two vitamin B_{12} molecules.

The combination of B_{12}-TC II and antibody was very sensitive to changes in pH, ionic strength and temperature. The strongest binding occurred at low temperature, low ionic strength and pH 7.5, while a nearly quantitative dissociation was seen at high temperature, high ionic strength and a shift of pH to 4.5, as might be expected (Hughes-Jones 1963).

From this information the binding energy *in vivo* at 37° C. should be relatively small. In spite of this a high percentage of TC II in the complex form occurs *in vivo* presumably because of a higher concentration of antibody than of TC II.

In order to approximate *in vivo* conditions as much as possible, the experiment with human serum as buffer was performed. As an equilibration of a Sephadex G-200 column with serum 1-D.S. was not possible, because of lack of adequate serum, pooled normal human serum was used. When

gel filtration was performed at 37° C. it was expected that ^{57}TC II alone appeared before the ^{131}I-γG-globulin, suggesting that normal human serum also contains a protein being able to complex with TC II, although this could not be demonstrated by paper electrophoresis, immunoelectrophoresis or Sephadex gel filtration at high ionic strength.

This was further substantiated by the finding that ^{57}TC II complex formation was performed at pH 7.5, 4° C. at low ionic strength (Table I, Exp. X). The nature of this complex is unknown, but it cannot be excluded that weak antibodies to TC II are present in normal human serum.

Whether the increase of transcobalamin II seen in patient 1-D.S. is due to an increase in the rate of production or to a decrease in the degradation rate is being studied (Hom et al., in preparation).

Of the methods used to detect the antibody to TC II autoradiography of the immunoelectrophoresis proved most specific and most sensitive. However in two patients with total vitamin B_{12} binding capacities of more than 3,200 pg./ml., antibody to TC II could not be demonstrated. The reason for this is unknown; both sera were from hydroxocobalamin treated patients.

The fast electrophoretic mobility of the $^{57}B_{12}$-TC II-γG-globulin complex found in all sera with TC II antibody might be caused either by the β-mobility of TC II or by the antibody *per se* having a high mobility. By DEAE-cellulose chromatography, γG-globulins are eluted in the order of increasing electrophoretic mobility, the γG-globulins in the breakthrough fraction having the lowest mobility (Fahey et al. 1958). Since the breakthrough fraction obtained from serum 1-D.S. did not combine with $^{57}B_{12}$-TC II, it is concluded that the γG-globulin being able to combine with $^{57}B_{12}$-TC II must have been eluted later, and hence have a higher mobility.

From the gel filtration experiment no. I showing that more B_{12}-TC II was in the complex form than TC II without bound B_{12}, it seems that the antibody is primarily directed against B_{12}-TC II. Normally B_{12} cannot be demonstrated bound to TC II (Mendelsohn et al. 1958, Gabuzda et al. 1965) and thus the reason for antibody formation might be that long acting preparations result in the *persisting* occurrence of B_{12}-TC II in plasma.

REFERENCES

Andrews, P. (1964) 'Estimation of the molecular weights of proteins by Sephadex gel-filtration.' *Biochem. J.* 91, 222–33.

Bastrup-Madsen, P., Nørregaard, S., Schwartz, M., Hansen, T. & Meulengracht, E. (1966) 'Serum-vitamin B_{12} during maintenance therapy in pernicious anæmia with a depot preparation of vitamin B_{12}.' *Lancet* I, 739–41.

Eisen, N. H. & Karush, F. (1949) 'The interaction of purified antibody with homologous hapten. Antibody valence and binding constant.' *J. Amer. chem. Soc.* 71, 363–64.

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Fahey, J. L., McCoy, P. F. & Goulian, M. (1958) 'Chromatography of serum proteins in normal and pathological sera: The distribution of protein-bound carbohydrate and cholesterol, siderophilin, thyroxin-binding protein, B_{12}-binding protein, alkaline and acid phosphatases, radio-iodinated albumin and myeloma proteins.' *J. clin. Invest.* **37**, 272–84.

Gabuzda, T. G., Worm-Petersen, J. & Lous, P. (1965) 'The binding of vitamin B_{12} added in vitro and in vivo to normal human serum proteins separated in ion exchange cellulose.' *Scand. J. Haemat.* **2**, 61–66.

Gottlieb, C., Lau, K.-S., Wasserman, L. R. & Herbert, V. (1965) 'Rapid charcoal assay for intrinsic factor (I. F.), gastric juice unsaturated B_{12} binding capacity, antibody to IF, and serum unsaturated B_{12} binding capacity.' *Blood* **25**, 875–84.

Hom, B. & Olesen, H. (1967) 'Molecular weights of vitamin B_{12} binding proteins in human serum determined by Sephadex G-200 gel filtration.' *Scand. J. clin. Lab. Invest.* **19**, 269–73.

Hughes-Jones, N. C. (1963) 'Nature of the reaction between antigen antibody.' *Brit. med. Bull.* **19**, 171–77.

Lau, K.-S., Gottlieb, C., Wasserman, L. R. & Herbert, V. (1965) 'Measurement of serum vitamin B_{12} level using radio-isotope dilution and coated charcoal.' *Blood* **26**, 202–14.

Laurell, C.-B., Laurell, S. & Skoog, N. (1956) 'Buffer composition in paper electrophoresis.' *Clin. Chem.* **2**, 99–111.

Mendelsohn, R. S., Watkin, D. M., Horbett, A. P. & Fahey, J. L. (1958) 'Identification of the vitamin B_{12}-binding protein in the serum of normals and of patients with chronic myelotic leukemia.' *Blood* **13**, 740–47.

Raven, J. L., Walker, P. L. & Barkhan, P. (1966) 'Comparison of the radioisotope dilution-coated charcoal method and a microbiological method (L. Leichmannii) for measuring vitamin B_{12} in serum.' *J. clin. Path.* **19**, 610–13.

Scheidegger, J. (1955) 'Une microméthode de l'immunoélectrophorese.' *Int. Arch. Allergy* **7**, 103–10.

Schwartz, M. & Bastrup-Madsen, P. (1968) 'A new vitamin B_{12}-binding protein in serum causing excessively high serum vitamin B_{12} values.' *Scand. J. Haemat.* **5**, 35–40.

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Erythrocyte Vitamin B_{12} Activity in Health, Polycythemia, and in Deficiency of Vitamin B_{12} and Folate

By A. Omer, N. D. C. Finlayson, D. J. C. Shearman, R. R. Samson and R. H. Girdwood

THERE HAS BEEN RELATIVELY LITTLE WORK on the estimation of erythrocyte vitamin B_{12} activity in man, and consequently there are few reports of its activity in disease states. Sobotka et al.[1] reported low levels in four cases of pernicious anemia (P. A.), using *E. gracilis* and *O. malhamensis*. This was confirmed by Biggs et al.,[2] who also reported the levels found in treated cases of polycythemia vera and in various leukemic states using *L. leichmannii*.

Recently, we have developed a method for measuring vitamin B_{12} activity in erythrocytes using *E. gracilis*. This paper reports the levels found in control subjects, in primary and secondary polycythemia, and in vitamin B_{12} and folate deficiency states.

Materials and Methods

Methods

Blood samples were taken for routine hematological estimations using sequestrene as the anticoagulant. Heparinized blood samples were used for plasma vitamin B_{12} and folate determinations, and for erythrocyte vitamin B_{12} estimations.

Routine hematological methods, including red-blood-cell volume measurements, as described by Dacie and Lewis,[3] were used. The method of Anderson[4] for the measurement of serum vitamin B_{12} modified by using Difco medium was used to measure plasma vitamin B_{12}, and the method of Waters et al.[5] for the measurement of serum folate, modified by the use of 10 mg. ascorbate/ml. plasma, and a Difco *L. casei* assay medium, was used for plasma folate assay. In our laboratory, using these methods, similar results for folate and for vitamin B_{12} levels were obtained whether serum or plasma was assayed. The normal range for plasma folate is 2.2–18 ng./ml.,[6] and for serum vitamin B_{12} 150–1000 pg./ml.*

Erythrocyte vitamin B_{12} activity was measured using *E. gracilis*. Heparinized blood was centrifuged at 1500 g. for 30 minutes and the plasma removed for vitamin B_{12} and folate

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assay. The erythrocytes collected in such a way that contamination with leucocytes was avoided, were washed once with isotonic saline, recentrifuged as before, and 2 ml. of packed cells washed into 2 ml. of acetate buffer (0.4 M and pH 5.3) and 16 ml. of distilled water, giving a 1/10 dilution. In a few cases the initial saline washings were assayed and negligible amounts of vitamin B_{12} were found. The proteins were then precipitated by autoclaving at 10 lbs. pressure for five minutes and removed, the supernatant being used for assay. Biochemical analysis of the supernatant by a modification of the method of Meulemans[7] showed that less than 5-10 mg. protein/100 ml. of filtrate were present and this is insignificant. At this dilution (1/10) the supernatant was found to inhibit the growth of *E. gracilis*, an effect due to the acetate buffer which was eliminated by a final assay dilution of 1/60 (Fig. 1). Subsequent investigation has shown that the in-

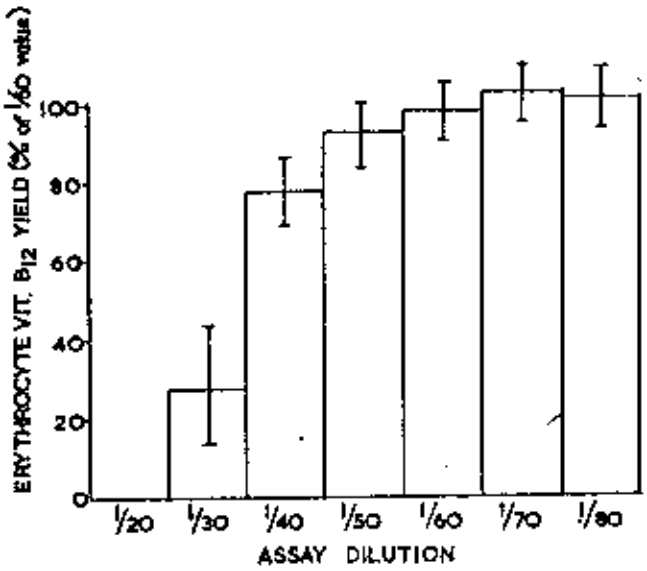

Fig. 1.—Erythrocyte vitamin B_{12} yield at increasing assay dilutions.

hibiting effect of acetate buffer could be eliminated by substituting citrate–phosphate buffer (0.1 M of citrate and 0.2 M of phosphate at pH 5.3) which had no inhibiting effect. Results using this buffer are similar to those obtained at a 1/60 dilution using acetate buffer. Aqueous standard tubes containing 0–12 pg./ml./tube were used, and all samples were assayed in duplicate. Vitamin B_{12} recovery studies were carried out on erythrocytes from three normal persons using the citrate–phosphate buffer. Addition of 100 and 200 pg./ml. of vitamin B_{12} in each case gave a recovery range of 97 per cent–128 per cent. Individual values are given in Table 1.

Table 1.—Recovery of Vitamin B_{12} from Erythrocytes (three normal subjects)

Subject Number	Vitamin B_{12} Recovery (pg./ml.)		
	Basal Level	100 pg./ml. Added	200 pg./ml. Added
1	120	261	411
2	140	233	344
3	135	255	368

Subjects

Measurements were carried out in the following groups:

(1) *Normal Subjects* (29). These were healthy blood donors and laboratory staff with normal peripheral blood counts.

(2) *Deficiency of Vitamin B_{12} or Folate* (27). Twenty-four patients who had been

Table 2.—Folate Deficient Patients (Hematological Investigations and Diagnoses)

No.	Vitamin B_{12} R.B.C. (pg./ml.)	Vitamin B_{12} Plasma (pg./ml.)	Cyanocobalamin Absorption Schilling Test	Cyanocobalamin Absorption Whole Body Count	Folate Plasma (ng./ml.)	Marrow	Diagnosis
1	24	208	16 per cent	–	0.8	Megaloblastic	Primary Malabsorption
2	21	132	5.7 per cent (+IF*—6 per cent)	–	2.0	Megaloblastic	Primary Malabsorption
3	93	216	–	–	2.0	Megaloblastic	Epileptic on Phenytoin Treatment
4	87	132	–	59 per cent	2.2	Megaloblastic	Alcoholic
5	87	168	–	31 per cent:59 per cent	1.6	Megaloblastic	Idiopathic Folate Deficiency
6	120	124	–	–	1.6	–	Crohn's Disease
7	132	292	11.1 per cent	–	1.5	Megaloblastic	Rectal Carcinoma
8	195	621	–		1.6	–	Rheumatoid Arthritis

* I.F. = Intrinsic Factor.

Table 3.—Primary Polycythemia Patients (Hematological Investigations)

No.	Sex	Hb (G per cent)	Peripheral Blood R.B.C. (M/cu. mm.)	Peripheral Blood W.B.C. (cu. mm.)	Peripheral Blood Platelets (1000s/cu. mm.)	Blood Volume R.B.C. (ml./Kg.)	Blood Volume Plasma (ml./Kg.)	Vitamin B_{12} R.B.C. (pg./ml.)	Vitamin B_{12} Plasma (pg./ml.)	Folate Plasma (ng./ml.)
1	F	19.0	6.5	9000	736	–	–	198	688	5.0
2	F	20.4	7.7	10400	580	63.0	26.4	111	1120	4.8
3	F	18.6	5.7	9800	360	51.8	31.0	120	816	5.5
4	F	17.4	6.5	5700	635	34.7	38.6	171	452	5.0
5	M	17.9	6.0	11300	442	43.5	22.0	198	1088	11.6
6	F	17.2	8.1	13700	320	55.0	37.0	114	544	5.0
7	F	18.2	6.0	18000	550	45.4	31.0	159	836	4.2
8	M	20.0	7.5	8000	250	46.4	25.0	165	1384	3.6
9	M	17.0	6.2	13400	650	42.0	44.4	152	336	4.3
10	M	19.0	8.1	19100	330	63.0	42.0	51	620	7.8
11	F	20.4	9.1	19700	205	68.8	31.7	54	960	4.4
12	M	18.0	7.2	21200	305	60.0	38.0	90	552	3.0
13	F	14.8	5.0	46000	670	35.7	44.0	60	452	2.9
14	F	19.1	7.4	12600	200	48.0	37.0	66	723	4.1

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found to have a megaloblastic marrow picture were studied. Eighteen of these cases were then classified as vitamin B_{12} deficient (plasma vitamin B_{12} < 100 pg./ml.), and six cases as folate deficient (plasma folate < 2.4 ng./ml.).

In three cases marrow examination was not carried out. One such patient was vitamin B_{12} deficient as shown by a plasma vitamin B_{12} level of 48 pg./ml. and a plasma folate value of 20.1 ng./ml. In two cases folate deficiency was diagnosed from plasma folate levels of 1.6 and 1.6 ng./ml. with plasma vitamin B_{12} levels of 621 and 124 pg./ml., respectively.

All the patients suffering from vitamin B_{12} deficiency had pernicious anemia. The diagnosis and information regarding B_{12} absorption in the folate deficient group is shown in Table 2.

(3) *Polycythemia* (22). In 17 cases the red blood cell volume was above 35 ml./Kg. in males and above 30 ml./Kg. in females. In five cases volume studies were not considered justifiable. Four were children with congenital heart disease who had all been cyanosed from birth, were still so when blood was collected, and who had PCV values above 55 per cent. One was a pregnant woman with the clinical features of polycythemia vera including splenomegaly, Hb. 19 Gm./100 ml. PCV 60 per cent, WBC 9000/cu. mm., platelets 736,000 cu.mm., leucocyte alkaline phosphatase 163 counts per 100 consecutive neutrophils (control 64 counts), and a marrow picture showing hyperplasia of the erythroid, myeloid, and megakaryocytic series.

These cases were then classified into those in whom no cause for polycythemia could be found (polycythemia vera), and those in whom polycythemia was secondary to a recognized lesion. The latter group included four cases of congenital cyanotic heart disease, two cases of chronic bronchitis and cor pulmonale without clinical signs of cardiac failure, and two cases of erythrocytosis, accompanied in one by a large bronchial carcinoma and in the other by a calcified intracerebral lesion of unknown type. In no case had treatment for the polycythemia been given.

In the statistical analysis of results, the Behrens–Fisher test was used, and all significant differences were at the 5 per cent level.

Results

Polycythemia

Figure 2 shows the values obtained for erythrocyte and plasma vitamin B_{12} activity in control subjects, and in primary and secondary polycythemia. The mean values in controls were, for erythrocytes, 158 pg./ml. of packed cells (S.D. 31 pg.), and for plasma 349 pg./ml. (S.D. 133 pg.); in primary polycythemia, for erythrocytes, 122 pg./ml. of packed cells (S.D. 53 pg.), and for plasma 755 pg./ml. (S.D. 299 pg.); in secondary polycythemia, for erythrocytes, 170 pg./ml. (S.D. 92 pg.), and for plasma, 692 pg./ml. (S. D.±234 pg.).

The difference between the erythrocyte vitamin B_{12} level in controls and in primary polycythemia was significant, but that between controls and secondary polycythemia was not. The differences between the controls and the two polycythemic groups for plasma vitamin B_{12} was significant.

Table 3 shows hematological data for patients with primary polycythemia. The final five patients are those with abnormally low erythrocyte vitamin B_{12}. These patients do not show any feature which distinguishes them from those with a normal erythrocyte vitamin B_{12} level, and none of them had an excessively low plasma folate.

Figure 3 shows the yield of vitamin B_{12} from erythrocytes as a percentage of the 1/60 dilution value in normals and four cases of polycythemia, three of whom had low red cell vitamin B_{12} levels, at varying assay dilution. No in-

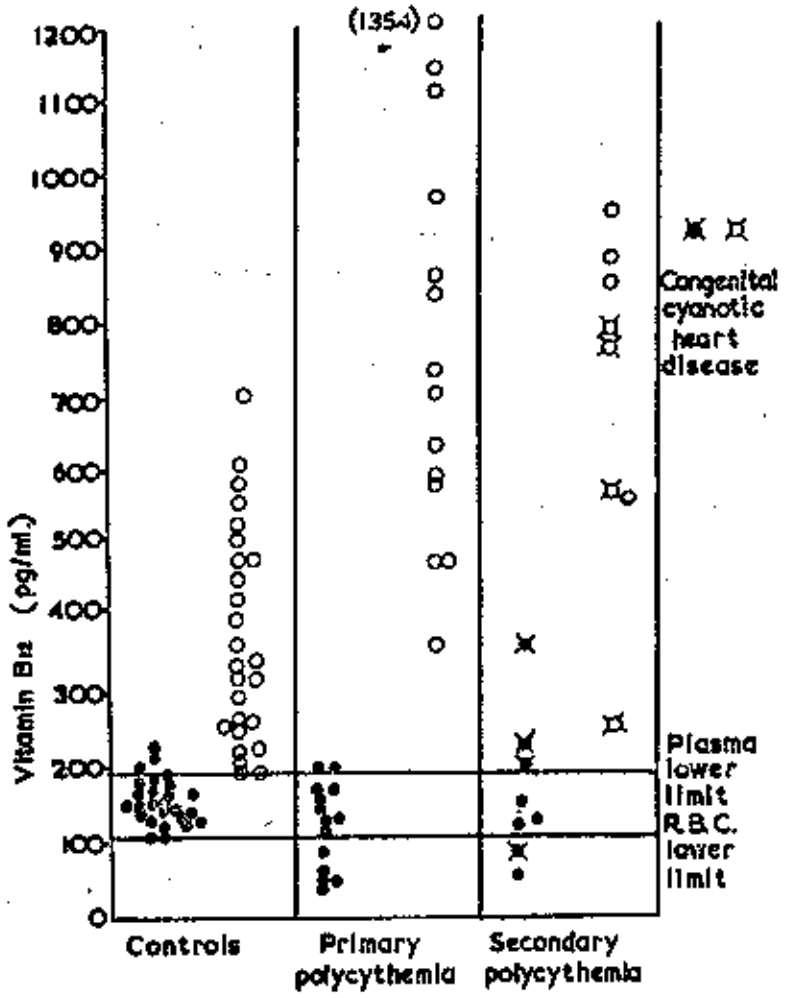

Fig. 2.—Erythrocyte (filled circles) and (plasma open circles) vitamin B_{12} levels in controls and polycythemia.

hibitor peculiar to the patients with primary polycythemia was demonstrated.

Table 4 shows the hematological data and diagnosis in those patients with secondary polycythemia.

B_{12} and Folate Deficiency

Figure 4 shows the values obtained in vitamin B_{12} and folate deficiency as compared to the same controls. The mean values for the vitamin B_{12} deficient patients were, for erythrocytes, 53 pg./ml. of packed cells (S.D. 32 pg.), and for plasma, 32 pg./ml. (S.D. 28 pg.), and for the folic acid deficient group, for erythrocytes, 95 pg./ml. of packed cells (S.D. 57 pg.), and for plasma, 237 pg./ml. (S.D. 165 pg.).

The difference between the erythrocyte vitamin B_{12} level in controls and in vitamin B_{12} deficiency was significant, as was the difference between controls and patients suffering from folate deficiency.

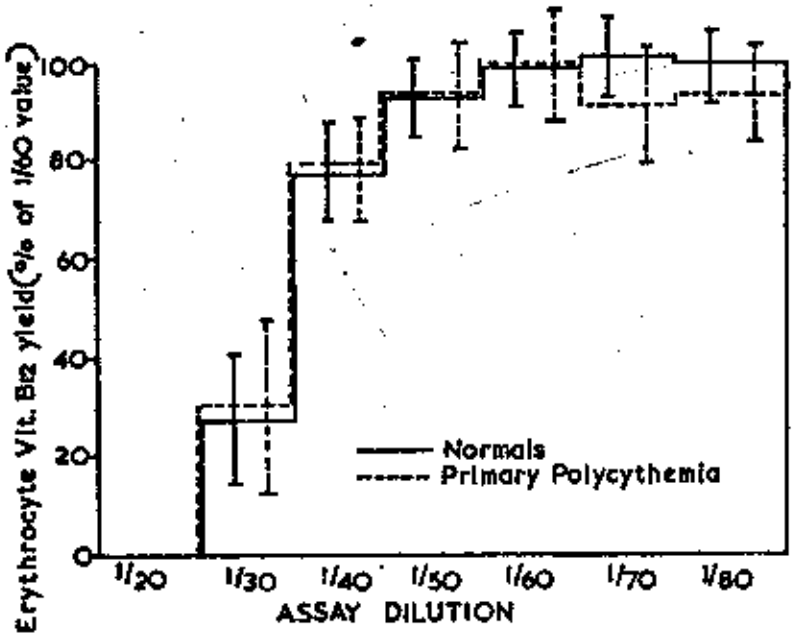

Fig. 3.—Erythrocyte vitamin B_{12} yield at increasing assay dilution in normals and primary polycythemia (four cases).

There was no significant difference between the erythrocyte vitamin B_{12} levels in vitamin B_{12} and folate deficiency. The differences between the controls, and both the vitamin B_{12} and folate deficient groups with regard to plasma vitamin B_{12} were significant, the level in the vitamin B_{12} deficient group being significantly lower than in the folate deficient group.

Discussion

The results obtained in this study for the erythrocyte vitamin B_{12} activity in normal persons agrees with those obtained by other workers.[1,2,3] Regarding patients with primary polycythemia (polycythemia vera), it has already been shown[2] that, in a treated group of patients, the erythrocyte B_{12} level is significantly below normal. This study confirms these findings in untreated patients, but it is noted that only five of the 14 patients had levels below the normal range (lower level 110 pg./ml.). These five patients were not clinically different from others, and in the group as a whole there was no relationship between the erythrocyte vitamin B_{12} level and the hemoglobin, white cell count, platelet count, red cell mass, plasma folate, or plasma vitamin B_{12} level. As shown in Fig. 3, no effective erythrocyte inhibitor could be found in the patients studied. No reason could be found for the reduced level of vitamin B_{12} in the erythrocytes. In untreated secondary polycythemia, the erythrocyte vitamin B_{12} level of the group as a whole was not significantly different from that of the normals, but there were two patients in the group with levels below the normal range, one with congenital cyanotic heart disease (P.C.V. 75 per cent), and one with cor pulmonale due to chronic bronchitis (red cell volume

Table 4.—Secondary Polycythemia Patients (Hematological Investigations and Diagnoses)

No.	Sex	P.C.V. per cent	Blood Volume R.B.C. (ml./Kg.)	Blood Volume Plasma (ml./Kg.)	Vitamin B_{12} R.B.C. (pg./ml.)	Vitamin B_{12} Plasma (pg./ml.)	Folate Plasma (ng./ml.)	Diagnosis
1	M	57	40.6	37.5	128	880	2.3	Bronchial Carcinoma
2	F	62	47.4	33.0	126	948	6.0	Calcified Cerebral Lesion
3	M	65	68.0	31.0	153	524	4.4	Chronic Bronchitis Cor Pulmonale
4	M	58	61.0	39.0	75	852	2.8	"
5	F	55	–	–	222	520	6.3	Tetralogy of Fallot
6	F	57	–	–	201	784	3.4	"
7	F	70	–	–	360	764	6.2	"
8	M	75	–	–	93	260	4.0	"

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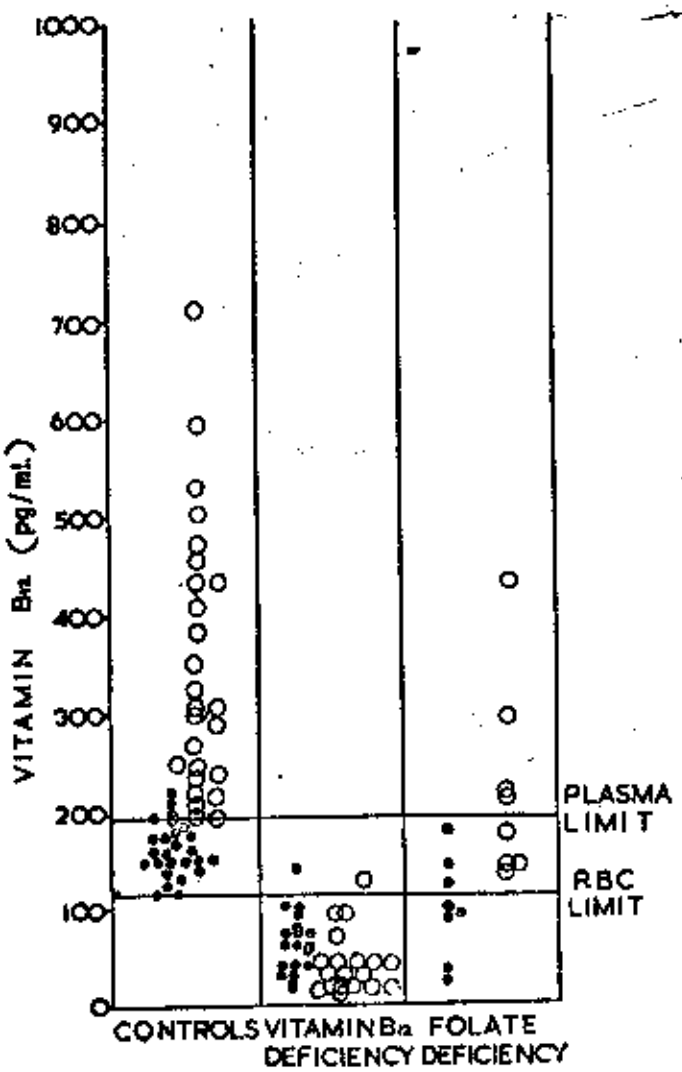

Fig. 4.—Erythrocyte (filled circles) and plasma (open circles) vitamin B_{12} levels in controls, and in vitamin B_{12} and folate deficiency.

61 ml./Kg.). No explanation was found for this, but it presents the possibility that a low erythrocyte vitamin B_{12} level may be related to polycythemic states in general, rather than to the primary form alone. It is known that the plasma vitamin B_{12} may be high in primary polycythemia,[8] and this finding is confirmed here In addition, five of the eight secondary cases had levels above the normal range, though two of these were the patients with isolated erythrocytosis who might in reality be primary cases.

It has already been shown in small numbers of cases that the erythrocyte vitamin B_{12} level is low in pernicious anemia.[1,2,3] This reduction is less than that of the plasma so that the erythrocyte concentration exceeds that of the plasma, and this is the reverse of the normal finding. This was confirmed, but it was found that the level of vitamin B_{12} in erythrocytes is not always greater than in plasma. In five of the 19 patients, the plasma vitamin

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B_{12} level exceeded the erythrocyte level, and in one they were the same. Two patients deserve special mention: in one with frank pernicious anemia (Hb 7.8 Gm./100 ml. : marrow megaloblastic) the level of vitamin B_{12} in the plasma was virtually unrecordable at 8 pg./ml., yet the erythrocyte level was normal at 144 pg./ml., while in the other, a patient found to have pernicious anemia during infertility investigations (Hb 12.2 Gm./100 ml.: marrow megaloblastic), the plasma vitamin B_{12} reading was 120 pg./ml. and that of the erythrocyte 67 pg./ml. In both patients the plasma folate level was normal. Clearly the relationship of the plasma to the erythrocyte vitamin B_{12} is not a simple one. There was no relationship between either the erythrocyte or plasma vitamin B_{12}, and the hemoglobin or red cell count in these patients.

We have found no previous reports of erythrocyte vitamin B_{12} levels in folate deficiency. Five of the eight cases studied had levels below the normal range, and in all of these the plasma vitamin B_{12} was normal. In one, a case of primary malabsorption, cyanocobalamin absorption was poor (Schilling test 6 per cent), and in two it was normal. Thus, in folate deficiency, a low erythrocyte vitamin B_{12} level is not related to malabsorption of cyanocobalamin. As has been reported by other workers, the plasma vitamin B_{12} was found to be low in this group, but no relationship between the plasma and erythrocyte vitamin B_{12} was found.

Summary

A method for the estimation of vitamin B_{12} in erythrocytes using *E. gracilis* is described. In 29 normal subjects the mean value was 158 pg./ml. (S.D. 31 pg.) packed cells.

Five of 14 patients with untreated primary polycythemia had very low erythrocyte vitamin B_{12} levels, and in the group as a whole the value was significantly less than normal. In eight patients with secondary polycythemia the erythrocyte vitamin B_{12} did not differ from normal, though two patients had values below the normal range.

In 19 cases of pernicious anemia the mean erythrocyte vitamin B_{12} level was 53 pg./ml. (S.D. 32 pg.) packed cells which was significantly reduced. There was no constant relation between the erythrocyte and plasma levels. In eight cases of folate deficiency the erythrocyte vitamin B_{12} level was significantly reduced, with a mean value of 95 pg./ml. (S.D. 57 pg.) packed cells.

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REFERENCES

1. Sobotka, H., Baker, H., and Ziffer, H.: Distribution of vitamin B_{12} between plasma and red cells. Amer. J. Clin. Nutr. 8:283, 1960.

2. Biggs, J. C., Mason, S. L. A., and Spray, G. H.: Vitamin B_{12} activity in red cells. Brit. J. Haemat. 10:36, 1964.

3. Dacie, J. V., and Lewis, S. M.: Practical Haematology: London, J. & A. Churchill Ltd., 1963.

4. Anderson, B. B.: Investigations into the euglena method for the assay of the vitamin B_{12} in serum. J. Clin. Path. 17:14, 1964.

5. Waters, A. H., and Mollin, D. L.:

1957

Observations on the metabolism of folic acid in pernicious anaemia. Brit. J. Haemat. 9: 319, 1963.

6. Girdwood, R. H., Thomson, A. D., and Williamson, J.: Folate status in the elderly. Brit. Med. J. 2:670, 1967.

7. Meulemans, O.: Determination of total protein in spinal fluid with sulphosalicylic acid and trichloroacetic acid. Clin. Chim. Acta 5:757, 1960.

8. Kelly, A., and Herbert, V.: Coated charcoal assay of erythrocyte vitamin B_{12} levels. Blood 29:139, 1967.

9. Delamore, I. W.: Vitamin B_{12} metabolism in polycythaemia vera. Clin. Sci. 20: 177, 1961.

2077

Osol, A., R. Pratt and M.D. Altschule (ed.) 1967

The United States Dispensatory and Physicians Pharmacology, XXI

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404-413

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Controlled trial of hydroxocobalamin and riboflavine in Nigerian ataxic neuropathy

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SUMMARY The results are presented of a double-blind therapeutic trial of hydroxocobalamin and riboflavine in Nigerian patients suffering from a degenerative neuropathy. Although no benefit from either vitamin was demonstrated, this may reflect the inadequacy of the dosages used. The results are discussed in relation to the hypothesis that dietary cyanide and cyanogens are responsible, at least in part, for the occurrence of this disease in a malnourished population.

There is increasing evidence that a degenerative neurological disease known as 'tropical ataxia' or 'tropical ataxic neuropathy' in Nigeria may be caused, at least partly, by the consumption of cassava, the staple diet of large numbers of people living in tropical and sub-tropical areas.

A recent epidemiological study of the disease in Western Nigeria has shown that the prevalence of the syndrome varies with the amount of cassava eaten (Osuntokun, Monekosso, and Wilson, 1969) but it is also recognized that there is co-existing malnourishment in other respects, notably in the intake of the sulphur-containing amino acids, and perhaps of riboflavine.

It is well known that the tuber of cassava (*Manioc utilissima*) contains large amounts of the cyanogenetic glycoside linamarin in its outer integument and, although the indigenous methods of food preparation eliminate most of this compound from the edible product, significant quantities of cyanogenetic material, and perhaps free cyanide, persist and are ingested.

It has, therefore, been suggested that this degenerative neurological disease may represent a form of chronic cyanide toxicity which may be related to Leber's disease (Wilson, 1963, 1965), subacute combined degeneration of the cord (Wilson and Langman, 1966), and tobacco amblyopia (Smith, 1961).

Significantly higher concentrations of thiocyanate, an important detoxication product of cyanide, have been found in the serum of patients with tropical ataxia compared with that of control individuals (Monekosso and Wilson, 1966; Osuntokun, 1968; Osuntokun, Monekosso, and Wilson, 1969). Changes in the distribution of cyanide-displaceable protein-bound vitamin B_{12} in plasma have also been demonstrated (Monekosso and Wilson, 1966; Osuntokun, 1968; Osuntokun *et al.*, 1969), and the findings are consistent with the suggestion that cyanide or cyanogen ingestion in patients is excessive.

Since hydroxocobalamin has a strong affinity for cyanide *in vitro*, and has a demonstrable protective effect in acute cyanide poisoning *in vivo*, it was decided to conduct a therapeutic trial of this agent in a group of Nigerian patients suffering from the disease. Riboflavine was also included in the trial in view of the suggestion that deficiency of this vitamin may be a contributory factor.

PATIENTS

The 80 patients treated all showed the clinical features of the ataxic neuropathy as previously described (Osuntokun, 1968, 1969). They were newly diagnosed and, as far as could be ascertained, had not been treated (conventionally) previously. Those whose disease was so advanced that they were bedridden were not included in the trial because it was considered that irreversible neuropathological damage was established, and was not amenable to treatment. The patients were all seen at a District Research Centre maintained by the Wellcome Trust

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at Epe, a large town 70 miles south-east of Ibadan in an area where tropical neuropathy is common. Fifteen of the patients were also investigated in the Neurological Unit at University College Hospital Ibadan, for periods not exceeding one week.

METHODS

Identically-appearing ampoules containing, respectively, 1,000 μg hydroxocobalamin, 5 mg riboflavine dyed with amaranth, and 1 μg cyanocobalamin (as control) were prescribed by reference to a pre-arranged schedule prepared by one of us (M.J.S.L.). Treatment was randomized with the restriction that each group of six patients was divided equally between the three treatments. The patients themselves and their examiner (B.O.O.) were unaware of any differences of treatment, and we conclude that the trial was effectively double-blind. The ampoules were flown to Nigeria in a cold-storage compartment and stored throughout the trial at 4°C. Injections were given at weekly intervals for the first 12 weeks of treatment, and thereafter, because of the practical difficulties of giving weekly injections, at two-weekly intervals for a further 24 weeks.

Clinical assessment was completed before and after the trial according to a scheme modified from Alexander (1951) in which neurological signs and symptoms were scored numerically. Improvement or deterioration during the trial was represented by a reduction or an increase in the score respectively. The patients' practical disabilities were also assessed at the beginning and at the end of the trial by the method suggested by Miller, Newell, and Ridley (1961).

Plasma thiocyanate and cyanide concentrations were estimated by the method of Aldridge (1945), cyanide being separated from thiocyanate by an aeration technique described by Boxer and Rickards (1951). Serum vitamin B_{12} concentrations were estimated in duplicate, on aliquots of sera with and without the addition of cyanide to the buffer used in extracting vitamin B_{12} bound to serum proteins, as described by Smith (1961) and Wilson and Matthews (1966). It has been suggested that differences in 'extractability' thus demonstrated are the consequence of cyanide exposure.

RESULTS

Eighty patients were seen initially and treatment started, and all, except one treated with riboflavine, were examined at the end of the trial. Of the 79 patients assessed at the beginning and at the end of the trial 26 received vitamin B_{12}, 26 were given riboflavine, and 27 received the control preparation. But 12 (six treated with riboflavine, one with hydroxocobalamin, and five with the control preparation) failed to attend the clinic regularly. Details of clinical comparability of all these patients are given in Table 1 and show that there were no substantial differences in respect of age, the proportions of men and women, or their initial neurological score. The patients' ages proved hard to determine, since few knew their precise dates of birth; as a consequence 31 of the 80 patients' dates of birth were recorded simply in multiples of five. In Table 2 the mean results of serum vitamin B_{12} measurements with and without cyanide extraction are given. None of the patients showed evidence of deficiency of the vitamin. The high mean concentrations found were similar to those found previously in patients with the disease.

TABLE 1

COMPARABILITY OF PATIENTS GIVEN THE THREE TREATMENTS

	Treatment group		
	Hydroxocobalamine	*Riboflavine*	*Control*
Men	13	16	15
Women	11	10	12
Mean age (yr)	45·1	46·7	47·1
Mean initial neurological score	123·1	126·3	134·4

TABLE 2

MEAN SERUM VITAMIN B_{12} CONCENTRATIONS IN VARIOUS TREATMENT GROUPS AT BEGINNING OF TRIAL, WITH AND WITHOUT CYANIDE IN EXTRACTING BUFFER

Treatment group	*Mean B_{12} concentrations (pg/ml.) with S.E.M.*			
	No.	(A) *With cyanide*	(B) *Without cyanide*	(B)/(A) × 100
Hydroxocobalamin	23	1903 ± 204	1173 ± 154	58·5
Riboflavine	22	1927 ± 260	1223 ± 194	59·6
Placebo	22	1604 ± 110	941 ± 141	57·9

Assessment by disability scale added no useful information to that obtained from the neurological examination scores, and therefore in Table 3 the changes in these alone have been given. Comparisons between initial and final results were made by cal-

TABLE 3

OVERALL RESULTS OF NEUROLOGICAL ASSESSMENT SCORES

Treatment	*Mean percentage change in neurological scores*
Hydroxocobalamin	+9·6*
Riboflavine	−8·1
Placebo	−5·2

Individual final scores were expressed as percentages of initial scores and the means of these individual figures were calculated.

Positive changes indicate deterioration. Negative changes represent improvement.

*The omission of one figure of +210·0% reduces this figure to +1·6%.

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relating the percentage change in total neurological score for each patient—(for example, initial score = 50, final score = 60, therefore percentage change = 20% in deterioration). Table 3 shows the mean percentage change in neurological scores, but in making statistical comparisons a non-parametric ranking test (Mann Whitney U test) was applied: the differences found between hydroxocobalamin and control, and riboflavine and control were statistically insignificant ($P = 0{\cdot}1$ and $P = 0{\cdot}4$ respectively). To ensure that minor improvements in individuals with less severe neurological disease were not being masked by poor results in those with severe disease, the findings were considered separately for those with less severe disease (initial score less than 100) and severe disease (initial score 100 or more). The results, which are given in Table 4, show no evidence of such a masking effect. Separate consideration of the results obtained in the irregular attenders at the Clinic did not suggest that their neurological changes differed in any important respect from those found in the remainder. At the end of the trial patients were asked whether they considered that they had benefited from the treatment and Table 5 shows the results of these subjective assessments.

TABLE 4

MEAN NUMERICAL ASSESSMENT SCORES IN PATIENTS SUBDIVIDED ACCORDING TO SEVERITY OF INITIAL INVOLVEMENT

Treatment	*Initial*	*Final*	*Change*	*Number treated*
a. Initial score less than 100				
Hydroxocobalamin	63·3	79·6	+16·3	12
Riboflavine	59·5	52·3	−7·2	11
Placebo	67·2	64·1	−3·1	11
b. Initial score 100 or more				
Hydroxocobalamin	174·3	177·9	+3·6	14
Riboflavine	179·3	167·0	−0·3	15
Placebo	180·6	162·2	−18·4	16

TABLE 5

SUBJECTIVE CHANGES IN THERAPEUTIC TRIAL OF HYDROXOCOBALAMIN AND RIBOFLAVINE

Treatment	*Better*	*No change*	*Worse*	*Total*
Hydroxocobalamin	13	9	4	26
Riboflavine	8	16	1	25*
Placebo	9	13	5	27
	30	38	10	78

*In one individual no assessment was made of subjective change, and one further individual defaulted from the trial.

A greater proportion of individuals treated with hydroxocobalamin thought that they had benefited from treatment than did those given riboflavine or the control preparation but the difference was not statistically significant ($\chi^2 = 2{\cdot}20$ $P = 0{\cdot}34$) in comparing all improved with all 'no change' or 'worse'.

Plasma thiocyanate concentrations were determined at the start of the trial and at the mid-point and are shown in Table 6 for patients in each of the three treatment groups. The concentrations tended to fall irrespective of the treatment given and there were no significant differences between them. Plasma cyanide concentrations could be determined in only 28 of the patients at the start of the trial because of the problems posed by making the estimations in the District Centre. There was a correlation between plasma thiocyanate and cyanide concentration ($r = 0{\cdot}60$ $P < 0{\cdot}001$) with a tendency for increasing variability as both values rose.

TABLE 6

MEAN THIOCYANATE CONCENTRATIONS BEFORE AND DURING TRIAL IN INDIVIDUALS IN WHOM PAIRED MEASUREMENTS WERE MADE

Treatment group	*Mean concentrations (μ-mole/100 ml.) with S.E.M.*		
	Before	*During*	*Number*
Hydroxocobalamin	10·7 ± 0·9	9·0 ± 0·7	26
Riboflavine	12·0 ± 1·0	10·6 ± 0·9	21
Placebo	9·9 ± 0·6	7·9 ± 0·7	25

DISCUSSION

The present study leaves no doubt that, under the conditions of the trial, there was no significant objective benefit attributable to treatment with either hydroxocobalamin or riboflavine, although, as judged subjectively, there was some evidence favouring hydroxocobalamin.

The organization of such a trial as this presented formidable practical difficulties in a rural Nigerian setting, but we are satisfied that the double-blind scheme adopted for the trial was effective and that the patients allocated the three different treatments were comparable with each other.

The biochemical features of the patients were similar to those seen in other series (Monekosso and Wilson, 1966; Osuntokun, 1968) and almost certainly reflect the high cyanide or cyanogen exposure experienced by this population, which consumes large quantities of cassava. As in other series, the high serum levels of vitamin B_{12} remain unexplained. The slight fall in mean plasma thiocyanate concentrations in the three treatment groups seen during the trial may be a reflection of the longitudinal variation of cyanide or cyanogen intake according

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to the season rather than of any non-specific treatment effects.

Previous therapeutic trials using B vitamin preparations have yielded conflicting results. Spillane (1947) was disappointed with the results of thiamine administration in wartime cases of tropical neuropathy that he studied, and Pallister (1940) and McLaren (1960) noted the absence of improvement with the administration of vitamin B complex in their cases. Others (Wright, 1928; Nicholls, 1935) claimed some improvement with yeast or Marmite, and assumed that this was due to the content of B vitamins. However, other nutritional factors present—for instance, cystine—may have been effective. The same may be true of the trial reported by Monekosso, Annan, and Ashby (1964) in which controls as well as patients treated with thiamine improved, for both groups were hospital in-patients and therefore probably benefited from non-specific dietary improvements.

Clark (1936) claimed that Oluwole successfully treated Nigerian children with tropical amblyopia by reducing the amount of cassava in the diet. These observations have not been deliberately reproduced subsequently, but Moore (1937) treated a small group of Nigerians suffering from the amblyopic syndrome with intravenous thiosulphate solution, as an antidote to cyanogen derived from cassava, without apparent benefit.

The present trial was designed to test the hypothesis of cyanogen intoxication by including further hydroxocobalamin as a potent antagonist of cyanide. The lack of therapeutic success does not necessarily invalidate the hypothesis, for the absence of clear-cut improvement could be due among other things to inadequate dosage, both of hydroxocobalamin and of riboflavine.

While hydroxocobalamin binds cyanide very quickly, weight for weight it is relatively inefficient, a reflection of its very high molecular weight. In addition, it must be emphasized that the patients in this trial continued to subsist on their normal diet with its inevitably high cyanogen content. It is improbable that well-established dietary habits can be substantially modified in this community but a further trial using much higher doses of hydroxocobalamin, for comparison with large doses of vitamin B complex and cystine, as a further cyanide detoxicant, is now in progress because it is not considered that this initial negative therapeutic result invalidates the hypothesis of cyanide intoxication.

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REFERENCES

Aldridge, W. N. (1945). The estimation of micro quantities of cyanide and thiocyanate. *Analyst*, **70**, 474-475.

Alexander, L. (1951). New concept of critical steps in course of chronic debilitating neurologic disease in evaluation of therapeutic response; longitudinal study of multiple sclerosis by quantitative evaluation of neurologic involvement and disability. *Arch. Neurol. Psychiat. (Chic.)*, **66**, 253-271.

Boxer, G. E., and Rickards, J. C. (1950). Chemical determination of vitamin B_{12}. II. The quantitative isolation and colorimetric determination of milligrammicrogram quantities of cyanide. *Arch. Biochem.*, **30**, 372-381.

Clark, A. (1936). Report on the effects of certain poisons contained in food-plants of West Africa upon health of native races. *J. trop. Med. Hyg.*, **39**, 269-276 and 285-295.

McLaren, D. S. (1960). Malnutrition and eye disease in Tanganyika. *Proc. Nutr. Soc.*, **19**, 89-91.

Miller, H., Newell, D. J., and Ridley, A. (1961). Multiple sclerosis. Treatment of acute exacerbations with corticotrophin (A.C.T.H.). *Lancet*, **1**, 1120-1122.

Monekosso, G. L., Annan, W. C. T., and Ashby, F. H. (1964). Therapeutic effect of vitamin B complex on an ataxic syndrome in Western Nigeria. *Trans. roy. Soc. trop. Med. Hyg.*, **58**, 432-436.

Monekosso, G. L., and Wilson, J. (1966). Plasma thiocyanate and vitamin B_{12} in Nigerian patients with degenerative neurological disease. *Lancet*, **1**, 1062-1064.

Moore, D. G. F. (1937). Retrobulbar neuritis cum avitaminosis (followed by post partial optic atrophy, now shown to be of pellagrinous nature. Its serious incidence and its need for general investigation). *West Afr. med. J.*, **9**, 35-40.

Nicholls, L. (1935). Nerve and cord degeneration referable to vitamin-A deficiency. *Indian med. Gaz.*, **70**, 550-552.

Osuntokun, B. O. (1968). An ataxic neuropathy in Nigeria. A clinical, biochemical and electrophysiological study. *Brain*, **91**, 215-248.

Osuntokun, B. O. (1969). Chronic cyanide intoxication and a degenerative neuropathy in Nigeria. Ph.D. Thesis, *University of Ibadan*.

Osuntokun, B. O., Monekosso, G. L., and Wilson, J. (1969). Relationship of a degenerative tropical neuropathy to diet. Report of a field survey. *Brit. med. J.*, **1**, 547-550.

Pallister, R. A. (1940). Ataxic paraplegia occurring amongst Chinese in Malaya. *Trans. roy. Soc. trop. Med. Hyg.*, **34**, 203-211.

Smith, A. D. M. (1961). Retrobulbar neuritis in Addisonian pernicious anaemia. *Lancet*, **1**, 1001-1002.

Spillane, J. D. (1947). *Nutritional Disorders of the Nervous System*. Livingstone: Edinburgh.

Wilson, J. (1963). Leber's hereditary optic atrophy—some clinical and aetiological considerations. *Brain*, **86**, 347-362.

Wilson, J. (1965). Leber's hereditary optic atrophy: a possible defect of cyanide metabolism. *Clin. Sci.*, **29**, 505-515.

Wilson, J., and Langman, M. J. S. (1966). Relation of subacute combined degeneration of the cord to vitamin B_{12} deficiency. *Nature (Lond.)*, **212**, 787-789.

Wilson, J., and Matthews, D. M. (1966). Metabolic interrelationships between cyanide, thiocyanate and vitamin B_{12} in smokers and non-smokers. *Clin. Sci.*, **31**, 1-7.

Wright, E. J. (1928). Disease due to A and B avitaminosis in Sierra Leone. *West Afr. med. J.*, **2**, 127-130.

1963

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SOME VARIATIONS IN THE AMOUNT OF VITAMIN B_{12} IN THE SERUM OF THE RHESUS MONKEY

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THE amount of vitamin B_{12} in the serum of many animals is usually greater than 150 μμg/ml. as measured by the growth response of the organism *Euglena gracilis*[1,2]. Exceptionally, values of the order of several thousand μμg occur in those species where mechanisms such as coprophagy allow the absorption of vitamin produced by the flora of the gut[3-5]. Values below 150 μμg/ml. of serum have been found in men suffering from pernicious anæmia[5] and in strict vegetarians[6].

Deficiency of vitamin B_{12} in experimental animals, leading to wide-ranging and gross pathological effects, is not easily produced and can often only be achieved by breeding from a generation which has itself been fed a diet deficient in the vitamin, by suppressing intestinal absorption of the vitamin or by increasing the requirements of the animal for the vitamin[7-9]. But the low levels of vitamin B_{12} in the serum in strict vegetarians occur readily and usually without overt signs of deficiency, although pernicious anæmia and sub-acute combined degeneration of the cord are somewhat commoner than in other people[6]. Similar low levels of vitamin B_{12} have been found in the serum of rhesus monkeys kept in captivity on a vegetarian diet for three months[10]. In these animals as in strict vegetarians there are no obvious signs of deficiency of vitamin B_{12}.

A number of recent reports have associated the metabolism of vitamin B_{12} with various facets of reproductive physiology. Thus, in the rabbit, the local concentration of the vitamin in the early pregnant uterus and the implanting blastocyst is high compared with that in the uterus of the non-pregnant or oestrogen-treated rabbit, while the concentration in the uterine secretion in the cow is greater than that in the Fallopian and follicular fluids[11]. Deficiency of the vitamin in the female has been associated with increased frequency of abortion and fewer and smaller litters in both the rat[12] and the pig[13]. It has also been suggested that there is a reduced amount of vitamin B_{12} in the semen in the majority of infertile men with abnormal

sperm[14]. Certainly the administration of vitamin B_{12} to the bull and its addition to bovine semen *in vitro* have been shown to improve the motility and viability of spermatozoa for artificial insemination[15]. Such possible effects on the reproductive processes could be manifested as the relative sterility that seems to occur in patients with pernicious anæmia and which appears to be relieved on treatment of the anæmia with vitamin B_{12} (refs. 16–18).

Apparently healthy animals in the Birmingham colony of rhesus monkeys have proved relatively infertile. Random routine investigations, such as for the presence of actively motile spermatozoa in the vagina after copulation, and for evidence at laparotomy of normal ovulation, have failed to reveal any obvious local conditions preventing conception. As a preliminary, therefore, to a long-term investigation—suggested by the foregoing reports—of the effects of supplements of vitamin B_{12} on fertility in rhesus monkeys, the amounts of the vitamin in the serum have been determined in relation to pregnancy and the fœtus, comparing: (*a*) recently captive animals and (*b*) animals maintained on the usual vegetarian diet in the Birmingham colony.

The amounts of vitamin B_{12} in the serum were estimated in 62 rhesus monkeys which had been in the Birmingham colony for from two to ten years, 28 monkeys which had been in the colony for up to 2 years and 81 monkeys examined directly on arrival in Britain from India. Serial determinations were obtained in seven monkeys during the early months of their captivity. Some of the estimations were on blood samples removed during pregnancy (in 18 of the recently captive monkeys and in 8 of the monkeys in the colony) and others were on samples removed after the termination of pregnancy (in 10 recently captive monkeys after hysterotomy near full term, and in 12 animals in the colony after normal parturition).

In addition, the amounts of the vitamin in the serum were determined in 10 full-term fœtuses of recently captive mothers, and in 8 new-born babies of mothers maintained in the colony. Serum levels were also determined in animals maintained in the colony: (*a*) in four non-pregnant mature females immediately before and approximately one month after injection with 1,000 μμg of cyanocobalamin, and (*b*) immediately before a similar injection in three pregnant animals, and again approximately one month later at parturition in mothers and infants. Estimations of vitamin B_{12} in the serum were also made in a number of other primates of which twelve had only recently entered captivity and seven had been in captivity for long periods of time (for details of species see Table 4).

The group of recently captive monkeys included six independent shipments at various times of the year. Their diet for 7–10 days after capture in India and for 4–9 days in England prior to the removal of blood samples was corn, fruit and vegetables and did not contain any supplements of vitamin B_{12} or any foods known to contain large amounts of the vitamin. The routine treatment (80 mg of terramycin daily) of newly arrived monkeys was withheld until the blood samples had been taken. In all cases samples of blood were withdrawn before the animals were used for any other purpose.

The diet fed to monkeys in the Birmingham colony consists of bread with a little dried milk, vegetables and fruit, and is supplemented with 'Bemax', ascorbic acid and halibut liver oil. Although some of these monkeys had received antibiotic drugs and had been used in experiments, blood samples were removed not earlier than one month following such treatments.

The amounts of vitamin B_{12} in the serum were estimated by a bioassay technique with *Euglena gracilis* as reported by Shinton[19]. The activity of the vitamin to the organism was distinguished as 'readily available', that is, the activity of untreated serum, and as 'total', that is, the activity of heat-treated serum[3].

Because of the well-known association in man of low levels of vitamin B_{12} in the serum and macrocytic anæmia, routine hæmatological examinations were carried out on 66 monkeys. These showed no evidence of macrocytosis and no significant anæmia (as judged by the amount of hæmoglobin in the blood—mean 12 g per cent).

The initial finding that recently captive rhesus monkeys have levels of vitamin B_{12} in the serum which are high compared with those of the animals maintained in the colony[16] has been confirmed—the mean level in 43 recently captive monkeys being 233 μμg/ml. (range 110–680), and in 34 animals kept in the colony, 35 μμg/ml. (range 20–70). But the observations already reported, that the levels of the vitamin in the serum fall rapidly within the first three months[16], have now been shown by a cross-sectional examination (Fig. 1) to be the first rapid phase of a process which continues for 18 months–2 years after captivity. A longitudinal investigation confirms that in each animal the level does indeed fall and that the changing rate of the fall is similar in the different individuals (Fig. 2). It is within this context that the levels in the serum in pregnant, puerperal and perinatal monkeys must be discussed.

It is known that the levels of vitamin B_{12} in the serum of pregnant women are somewhat reduced[20], although they do not approach those found in pernicious anæmia or in true vegetarians. The results here (Table 1) indicate not only that there is less vitamin B_{12} in the serum in the recently captive monkeys when pregnant, but also that the reduced amounts are close to those found in monkeys captive on a vegetarian diet for longer than 2 years. In the monkeys maintained in the colony, however, the levels in the pregnant animals are, significantly, situated at the upper end of the range of values for the colony as a whole. In particular, the levels in the pregnant animals in the colony are significantly higher (Table 1) than those of the breeding females that were mated without success ($P = 0{\cdot}01$–$0{\cdot}001$). In both groups of monkeys the amount of the vitamin in the serum after the termination of pregnancy was lower even than during pregnancy (Table 1).

One possible interpretation of such results is that, although the amounts of vitamin in the serum fall progressively throughout pregnancy and into the puerperium

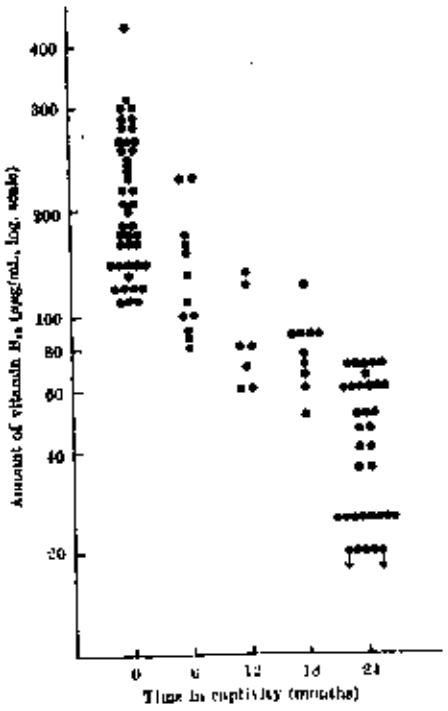

Fig. 1. Relationship between amount of vitamin B_{12} in serum and time spent in captivity

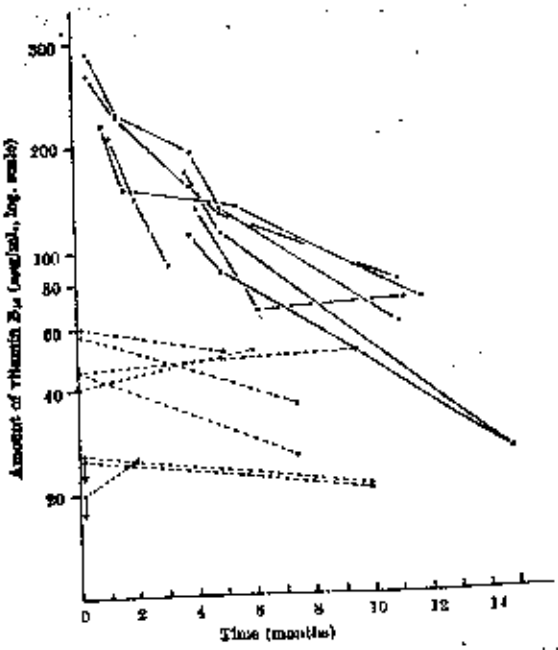

Fig. 1. Change in amount of vitamin B_{12} in serum with time spent in captivity. ——, recently captive animals; - - -, animals captive more than two years

in both groups of animals, a certain minimum level of vitamin B_{12} in the serum is necessary for conception. This minimum level appears to be such as to prevent those animals in the colony with the lowest amounts from becoming pregnant at all.

Although it can be seen that the pregnant animals in both groups have somewhat similar low levels of vitamin B_{12} in the serum, the individuals in these groups could presumably be differentiated in terms of their past, that is, pre-pregnancy, levels of the vitamin. The two groups can certainly be separated on the basis of the levels in the serum of their own progeny. Thus, in the recently captive animals the reduced levels in the serum of the mothers accompanies very high levels in the serum of the full-term fœtuses—an exaggeration of a slight trend that is known to occur in man[18] and the rat[21]. In contrast, in the animals maintained in the colony, the new-born have serum levels which are as low as those of the already deficient mothers (Table 2).

It thus appears the fœtus concentrates vitamin B_{12} at the expense of the mother and that in the animals in the colony the fœtus may be 'starved' of the vitamin. This is supported by the preliminary finding in the animals kept in the colony, that the parenteral administration of vitamin B_{12} during pregnancy has little effect on the amount in the mother but is afterwards found to have raised the level in the baby to the very high value found in the babies of recently captive animals (Table 3). Certainly babies born in the colony show marked spurts of growth after the administration of vitamin B_{12} parenterally (unpublished results).

Table 1. VARIATIONS IN VITAMIN B_{12}-LEVELS IN PREGNANCY AND THE PUERPERIUM (μμg/ml. of serum)

Status	Recently captive monkeys: No. of animals	Vitamin B_{12}-level: Mean	Vitamin B_{12}-level: Range	Monkeys in the colony: No. of animals	Vitamin B_{12}-level: Mean	Vitamin B_{12}-level: Range
Mature	15	271	110–680	19	43	< 20–70
Pregnant	18	105	50–240	8	79	60–120
Puerperal	10	62	< 20–90	12	36	20–60

Table 2. VITAMIN B_{12}-LEVELS IN MOTHERS AND INFANTS (μμg/ml. of serum)

Recently captive monkeys: No. of pairs	Mean vitamin B_{12}-level: Mothers	Mean vitamin B_{12}-level: Fœtuses	Monkeys in the colony: No. of pairs	Mean vitamin B_{12}-level: Mothers	Mean vitamin B_{12}-level: Babies
10	123	570	8	38	47

Table 3. EFFECT OF INJECTIONS OF THE VITAMIN ON THE AMOUNT IN THE SERUM (μμg/ml.)

Non-pregnant monkeys in the colony: Before injection	Non-pregnant monkeys in the colony: After injection	Pregnant monkeys in the colony: Before injection	Pregnant monkeys in the colony: After injection: Mother	Pregnant monkeys in the colony: After injection: Baby
25	310	70	80	800
25	330	25	100	640
60	680	60	130	570

These findings would suggest in addition that the serum levels of vitamin B_{12} do not necessarily provide an unequivocal index of the nutritional state of the animal. For while the pregnant monkeys in both groups have low levels of the vitamin in the serum, the recently captive group appears to be able to mobilize body stores of the vitamin for transfer to the fœtus in a way denied to the animals kept in the colony.

Normally in man very little of the vitamin B_{12} in the serum is in the 'readily available' form, whereas in many animals as much as 50 per cent of the 'total' vitamin is 'freely available' (for example, as measured by Kato[22] using *Lactobacillus leichmanni* as the bioassay organism). All the adult rhesus monkeys were comparable with man in this respect, the 'readily available' vitamin being usually less than 20 μμg/ml. of serum as estimated by our technique. In six full-term fœtuses of recently captive monkeys, however, the 'readily available' vitamin in the serum was very high (mean 334 μμg/ml.), whereas the new-born of the animals in the colony resembled the adult. Because the significance of the terms 'total' and 'readily available', as applied to the experimental animal, are not clear, it is difficult to comment further on this finding. It is nevertheless tempting to suppose that in some way the passage of large amounts of the vitamin across the maternal-fœtal barrier in the normal rhesus monkey is associated with this shift from one form of the vitamin to the other, and that in the abnormal animal this mechanism is upset.

Preliminary findings (Table 4) suggest that similar reductions in the amounts of vitamin B_{12} in the serum may also occur during captivity in some other primates. Thus in a single patas monkey, a single baboon, and a single douroucouli, all of which had been in captivity for many years on a vegetarian diet, the amounts of vitamin B_{12} in the serum were found to be 100 μμg/ml. or less. This figure, contrasting with a mean of 382 in four recently captive patas monkeys, is below the range of normal man, other omnivorous animals or recently captive rhesus monkeys, and is well within the range of the rhesus monkeys kept in the Birmingham colony. (The finding that the amount of vitamin B_{12} did not fall in talapoin monkeys during captivity may reflect the fact that these animals receive a diet which includes animal foods—mealworms and locusts—and which in this respect probably resembles their natural diet, while the finding of very high levels in captive bushbabies and pottos suggests that some additional mechanism, such as coprophagy, may occur in these prosimians.)

Table 4. VITAMIN B_{12}-LEVELS IN SOME OTHER PRIMATES (μμg/ml. of serum)

Recently captive: Species	Recently captive: Vitamin B_{12}-level	Captive for long periods: Species	Captive for long periods: Vitamin B_{12}-level
Erythrocebus patas	450	*E. patas*	100
E. patas	470	*Aotes trivirgatus*	190
E. patas	310	*Pogio cambis*	60
E. patas	320		
Cercopithecus talapoin	380	*C. talapoin*	240
C. talapoin	800	*C. talapoin*	280
C. talapoin	700	*C. talapoin*	340
C. talapoin	1,100	*C. talapoin*	570
Perodicticus potto	4,000+		
Galago crassicaudatus	4,000+		
G. crassicaudatus	4,000+		
G. crassicaudatus	2,000+		

Nevertheless, since low levels of vitamin B_{12} in rhesus monkeys appear to be associated with reduced fertility it is conceivable that the well-known difficulties of breeding many primates in captivity may stem, in part at least, from low levels of vitamin B_{12} in the serum. Certainly defects in many biologically critical reactions of vitamin B_{12} (for example, deoxyribose synthesis, or the reactivation of —SH enzymes[11]) might well be expected to affect the production of viable gametes, the nature of the uterine environment, and the differentiation and growth of the embryo and foetus.

I thank Mrs. S. M. Collyer and Miss M. Preston of the Department of Clinical Pathology, Queen Elizabeth Hospital, Birmingham, for carrying out the microbiological assays.

[1] Ross, G. I. M., *J. Clin. Path.*, 5, 259 (1952).
[2] Mollins, D. L., and Ross, G. I. M., *J. Clin. Path.*, 5, 129 (1952).
[3] Ken, S. K., *Proc. Roy. Soc.*, B, 146, 351 (1962).
[4] Okuda, K., *Nature*, 195, 293 (1962).
[5] Frank, O., Baker, H., and Sobotka, H., *Nature*, 197, 490 (1963).
[6] Smith, A. D. M., *Brit. Med. J.*, i, 1655 (1962).
[7] Emerson, G. A., *Proc. Soc. Exp. Biol. N.Y.*, 76, 302 (1949).
[8] Latner, A. L., and Raine, L., *Nature*, 180, 1107 (1957).
[9] Booth, M. A., and Spray, G. H., *Brit. J. Haematol.*, 6, 288 (1960).
[10] Krohn, P. L., Oxnard, C. E., and Chalmers, J. N. M., *Nature*, 197, 186 (1963).
[11] Jacobson, W., and Lutwak-Mann, C., *J. Endocrinol.*, 14, 19 (1956).
[12] Lophovsky, S., Barsan, H. J., Bouthillet, R., Peacharz, R., Singman, D., Dimick, M. K., and Robbins, R., *Amer. J. Physiol.*, 165, 79 (1951).
[13] Frederick, G. L., *Vitamin B_{12} and Intrinsic Factor*, 580 (Ferdinand Enke Verlag, Stuttgart, 1962).
[14] Watson, A. A., *Lancet*, ii, 644 (1962).
[15] Busch, E., *Anim. Breed. Abstr.*, 25, 44 (1957).
[16] Adams, J. F., *Scot. Med. J.*, 3, 21 (1958).
[17] Sharp, A. A., and Witts, L. J., *Lancet*, ii, 779 (1962).
[18] Smith, A. D. M., *Lancet*, ii, 934 (1962).
[19] Shinton, N. K., *Clin. Sci.*, 18, 389 (1959).
[20] Young, J. E., Barrows, Jun., C., Okuda, K., and Chow, B. F., *Obstet. Gynaec.*, 14, 449 (1959).
[21] Chow, B. F., Barrows, L., and Ling, C. T., *Arch. Biochem.*, 34, 151 (1951).
[22] Kato, N., *Nutr. Abstr. Rev.*, 31, 99 (1961).
[23] Beck, W. S., *New England J. Med.*, 265, 708 (1962).

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SOME OBSERVATIONS ON THE METABOLISM OF VITAMIN B_{12} BY JAMAICAN CHILDREN

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The following work was undertaken as part of an investigation of the nutritional status of children in Jamaica, B.W.I. A paper by Mackay, Patrick and Stafford describing this investigation is in preparation. A dietary survey had indicated that these children had a low intake of animal protein and it was thought possible that they might be deficient in vitamin B_{12}. The following experiments were carried out to investigate this possibility.

The experiments included (1) the determination of the plasma levels of vitamin B_{12} in children who had received supplements of the vitamin by mouth and in unsupplemented children; (2) an attempt to "saturate" two children by repeated intravenous administrations of vitamin B_{12}; (3) determination of the plasma levels of vitamin B_{12} and the percentage of excretion of the vitamin by children to whom a test dose of the vitamin had been administered.

The children were almost all of mixed African descent and ranged in age from 8 to 15 years. Equal numbers of boys and girls were used whenever possible.

METHODS

Crystalline vitamin B_{12} (Merck) was used as a standard in [illegible] assays and this material was used for the intravenous in[illegible]ons of the children. The dietary supplementation was [illegible] out by administering to each child daily, except

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Saturday and Sunday, a troche containing 100 μg of vitamin B_{12} to be taken by mouth. Assays for the vitamin B_{12} content of urine and plasma were carried out by the use of *Lactobacillus leichmanii* 4797 according to the method of Skeggs et al. ('50). A total of at least 10 test tubes containing graded amounts of the solution to be assayed were used in each determination. Urine samples were diluted with water to an appropriate degree and then used directly in the assay. Eight experiments on the determination by this method of B_{12} added to normal urine gave recoveries of 94 to 104%. The amounts of B_{12} added were from 0.01 to 0.10 μg per 100 ml of urine. Two methods were used for the determination of vitamin B_{12} in blood. The first method was used only to follow the changes in the concentration of the vitamin in the plasma after a test dose had been administered intravenously. The method consisted simply of withdrawing 0.2 ml of blood from the finger tip by means of a blood pipette, delivering it into 2.0 ml of 0.9% saline, centrifuging, removing the supernatant, washing the cells with one more 2.0 ml portion of saline and making the extracts to 10.0 ml. The plasma solution was then used directly in the assay as described by Skeggs et al. ('50). The procedure included autoclaving the plasma in the medium, a procedure which, according to Scheid and Schweigert ('51), liberates most of the vitamin B_{12} bound to protein. Six experiments on the determination by this method of B_{12} added to plasma gave recoveries of 94 to 107%. The amounts of B_{12} added were from 0.1 to 0.8 μg per 100 ml of plasma. This method was not of sufficient sensitivity to measure accurately the B_{12} content of normal sera and for this purpose the method of Rosenthal and Sarett ('52) was employed. This procedure involves removal of the plasma protein after bound vitamin B_{12} is liberated by boiling in an acetic acid buffer. A few determinations of the vitamin B_{12} activity of whole blood or red blood cells were carried out and these were done simply by adding an appropriate amount of water (usually 100 volumes) and using the hemolysate directly in the assay procedure.

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RESULTS AND DISCUSSION

Thirty-eight children from the same village were divided into two groups, comparable with respect to age, sex and economic condition. Each child of the first group received dietary vitamin B_{12} supplementation over a period of 9 months. The average total consumption of B_{12} per child was 13.9 mg. The second group of children received placebos for the same length of time. Determinations of the vitamin B_{12} concentration of the serum of each child were carried out during the last month of the supplementations. The average concentration of vitamin B_{12} in the serum of the unsupplemented children was 0.021 μg ± 0.010 μg per 100 ml, with a range of 0.008 to 0.043 μg per 100 ml. The corresponding figures for the supplemented group were 0.033 ± 0.020 μg per 100 ml with a range of 0.007 to 0.90 μg per 100 ml. Thus the supplemented children had significantly higher levels ($P = 0.034$) of serum B_{12}. It is interesting to note the comparatively small increase in B_{12} activity of the plasma which occurred as a result of such massive B_{12} supplementation in the diet. This is in agreement with the findings of Chow et al. ('51) and Smith ('52) that almost all of an oral dose of vitamin B_{12} administered to rats is eliminated in the feces and very little is retained. The values found in the group of unsupplemented children were very similar to those reported by Rosenthal and Sarett ('52) for healthy American adults.

An attempt was made to "saturate" two children by repeated intravenous injections of vitamin B_{12}. Each child received 1.5 μg of B_{12} per kilogram of body weight by intravenous injection on the morning of the first, 5th, 8th, 12th and 15th days of the experiment. Blood was withdrawn just *before* each injection for determination of its B_{12} content and urine was collected for two periods after the injection: zero to 5 hours and 5 to 7 hours. The average results of this experiment are given in table 1. The vitamin B_{12} activity in the whole blood of the children before the injection seemed significantly higher than that in the plasma of normal children quoted above and indeed it was found that the inclusion of protein in the as-

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say always produced some vitamin B_{12} activity. It is doubtful whether this activity represents actual vitamin B_{12} or is a non specific effect of the protein. In any case it seems clear that the resting levels of B_{12} in the blood were significantly raised by the injections but there was no corresponding increase in the excretion of B_{12} after each test dose. Only a very small percentage of the B_{12} was excreted in the urine following the 5th hour after the injection and it was found in this and other experiments that 90 to 95% of the total amount of B_{12} excreted was excreted within the first two hours after the injection. The pattern of urinary excretion of B_{12}

TABLE 1

Vitamin B_{12} activity in blood and urine of children after repeated injections of vitamin B_{12}

NO. OF INJECTIONS	B_{12} ACTIVITY IN WHOLE BLOOD	PERCENTAGE OF THE DOSE EXCRETED IN THE URINE	
	μg %	*0–5th hour*	*5th–7th hour*
0	0.041	...	...
1	0.070	38	0.05
2	0.13	43	0.12
3	0.10	41	0.10
4	0.12	38	0.15
5	0.12	33	0.15

found in these experiments seems different from that reported by Chesterman et al. ('51) by being somewhat more rapid and more complete. This is probably due to the fact that in these experiments the vitamin B_{12} was administered intravenously whereas Chesterman administered the B_{12} intramuscularly.

Preliminary experiments indicated that practically all of the vitamin B_{12} activity found in the blood after an intravenous injection of B_{12} was in the plasma. Apparently, little if any of the injected B_{12} entered the cells of the blood. Plasma levels of vitamin B_{12} and percentage excretion after a test dose of the vitamin were followed in two groups of children. Each of the first group of 5 children was judged to be failing in growth by reference to the Wetzel grid (Wetzel, '44). Each

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of the second group of children was judged of normal development by the same method. After the initial tests each child of the first group received dietary B_{12} supplementation over a period of 9 months. The average total consumption of B_{12} over this period was 14.9 mg per child. The tests were then repeated on each child of this group. The test dose was 1.5 μg of B_{12} per kilogram of body weight administered intravenously. Table 2 presents the average results obtained, together with results obtained by the same method from three normal adults.

From these data it appears that:

(a) there was no significant difference in the amounts of vitamin B_{12} excreted in the urine by the three groups of children.

(b) the "resting" vitamin B_{12} activity of the plasma of the retarded children was raised by the administration of the dietary supplements of B_{12}.

(c) the test dose of B_{12} caused consistently higher plasma B_{12} levels in the retarded children after they had received vitamin B_{12} dietary supplementation, but the *rate* of removal of B_{12} from the plasma appeared to be substantially unaltered.

(d) although the "normal" children showed higher plasma B_{12} levels after the test dose than did the "retarded" children, the differences are of doubtful significance.

(e) most of the injected B_{12} is removed from the plasma within two hours and this is in accord with the pattern of urinary excretion of B_{12}. However, a small proportion of the injected B_{12} appears to remain in the plasma for some time, there being very little decrease in B_{12} activity in plasma from the 4th to the 6th hour, although the B_{12} level at this time is higher than the level before the injection of B_{12}.

SUMMARY

1. A group of 19 Jamaican children who had received dietary vitamin B_{12} supplementation over a period of 9 months

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TABLE 2

Vitamin B_{12} levels in plasma and percentage excretion after a test dose (1.5 mg per kilogram of body weight of vitamin B_{12})

GROUP	NO. IN GROUP	PLASMA B_{12} LEVELS (μG PER 100 ML OF BLOOD)						PERCENTAGE OF DOSE EXCRETED IN URINE	
		Before test dose	Time after injection of test dose					Time after injection of test dose	
			10 min.	*1 hour*	*2 hours*	*4 hours*	*6 hours*	*0–5th hour*	*5th–7th hour*
Normal adults	3	0.02	0.38	0.19	0.14	0.11	0.09	50	0.03
Normal children	6	0.03 ± 0.01	0.40 ± 0.06	0.17 ± 0.03	0.12 ± 0.04	0.10 ± 0.03	0.09 ± 0.02	40 ± 6	0.03
Retarded children	5	0.03 ± 0.01	0.35 ± 0.07	0.14 ± 0.03	0.11 ± 0.02	0.08 ± 0.02	0.08 ± 0.01	37 ± 6	0.03
Retarded children receiving dietary supplementation of B_{12}	5	0.06 ± 0.02	0.43 ± 0.13	0.19 ± 0.03	0.17 ± 0.05	0.13 ± 0.04	0.13 ± 0.04	35 ± 6	0.03

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had significantly higher plasma B_{12} concentrations than did a control group. The control group of children had plasma B_{12} concentrations which were very similar to those reported for normal American adults by Rosenthal and Sarett ('52).

2. An attempt to saturate two children by repeated intravenous injections of vitamin B_{12} resulted in higher "resting" plasma levels of the vitamin. The percentage of the vitamin which was excreted after each test dose did not, however, rise significantly.

3. Plasma levels and urinary excretion of vitamin B_{12} after a test dose of the vitamin were compared in (a) children of retarded growth, (b) the same children after dietary B_{12} supplementation, (c) children with normal growth. No marked differences were observed between the groups in the urinary excretion of B_{12} or in the rate of removal of B_{12} from the plasma. The "resting" level of vitamin B_{12} in the plasma was, however, raised by the B_{12} supplementation.

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LITERATURE CITED

CHESTERMAN, D. C., W. F. J. CUTHBERTSON AND H. F. PEGLER 1951 Biochem. J., *48*: Proc. ii.

CHOW, B. F., C. ROSENBLUM, R. H. SILBER, D. T. WOODBURY, R. YAMAMOTO AND C. A. LANG 1951 Proc. Soc. Exp. Biol. Med. N. Y., *76*: 393.

ROSENTHAL, H. L., AND H. P. SARETT 1952 J. Biol. Chem., *199*: 433.

SCHEID, H. E., AND B. S. SCHWEIGERT 1951 Ibid., *193*: 299.

SKEGGS, H. R., H. M. NEPPLE, K. A. VALENTIK, J. W. HUFF AND L. D. WRIGHT 1950 Ibid., *184*: 211.

SMITH, L. 1952 Brit. Med. Bull., *8*: 203.

WETZEL, N. C. 1944 "Growth" in "Medical Physics." Otto Glasser, Ed., Year Book Publishers, Inc. Chicago, 535-567.

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ABSORPTION OF VITAMIN B_{12} BY THE GUINEA-PIG: THE ROLE OF THE ILEAL MITOCHONDRION

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Although the mechanism of vitamin B_{12} absorption has been intensively studied for nearly two decades, little is known about the events that take place during transport of the vitamin through the ileal mucosa. This review is concerned with recent studies on the processes which occur within the ileal enterocyte during the absorption of vitamin B_{12}.

Dietary vitamin B_{12} is released from protein complexes and bound to intrinsic factor in the upper part of the gastro-intestinal tract. The vitamin B_{12}–intrinsic factor complex (B_{12}–IF) passes to the ileum where it is attached to specific receptors on the brush border of the ileal enterocytes (Donaldson, Mackenzie and Trier, 1967). The uptake of B_{12}–IF by the ileal receptor is a rapid, energy-independent process and has a specific requirement for Ca^{++} ions and a neutral pH (Donaldson, Mackenzie and Trier, 1967; Herbert, 1968; Mackenzie and Donaldson, 1969a). In contrast, entry of vitamin B_{12} into the epithelial cell is a slow, energy-requiring process (Sullivan, Herbert and Castle, 1963). It has not been established, however, whether intrinsic factor itself gains entry to the cell or whether B_{12}–IF complex is split at the brush border membrane. In a recent review of vitamin B_{12} absorption, Mackenzie and Donaldson (1969b) suggested that B_{12}–IF was split at the brush border to give an active fragment which may be smaller than vitamin B_{12} itself. This active fragment, they suggested, enters the epithelial cell to be resynthesized to vitamin B_{12}. Wilson (1963) had previously proposed that B_{12}–IF entered the cell by pinocytosis, which would allow the powerful lysosomal proteases to cleave the complex, liberating free vitamin B_{12}. A third hypothesis has recently been proposed by Gräsbeck (1967). He suggested that the ileal receptor represents an incomplete carrier for vitamin B_{12}. Intrinsic factor or a fragment thereof 'completes' the carrier, in a functional or structural sense, and thus facilitates entry of the vitamin into the cell.

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An observation, first made by Booth and co-workers in 195., and subsequently confirmed by others (Doscherholmen *et al.*, 1957; Wilson, 1963), may be relevant to this problem of B_{12}–IF cleavage. They showed that there is a time lag, approximately 3 hours in man, in the passage of the vitamin B_{12} across the enterocyte, and it has been generally assumed that B_{12}–IF is cleaved during this period of intramucosal delay (Mackenzie and Donaldson, 1969b). Studies were therefore performed to determine the subcellular localization of vitamin B_{12} in the ileal mucosa during this period of delay; particular attention was paid to ileal brush borders and lysosomes. It was first necessary, however, to determine the subcellular location of endogenous vitamin B_{12} in guinea-pig ileum.

Localization of endogenous vitamin B_{12} in ileal mucosa

Ileal mucosa from fasting male guinea-pigs was homogenized and the subcellular organelles separated by differential centrifugation. The technique of Hübscher, West and Brindley (1965) was used to prepare the principal subcellular fractions. More purified subcellular organelles were prepared according to the methods in the cited references: brush borders (Eichholz and Crane, 1965), nuclei (Widnell, Hamilton and Tata, 1967) and mitochondria (Parsons, Williams and Chance, 1966; Shakespeare, 1969). Full details, including morphological and biochemical characterization of the subcellular fractions, are published elsewhere (Hoffbrand and Peters, 1969; Marsh, Peters and Brown, 1971; Peters, 1970; Peters and Hoffbrand, 1970). Vitamin B_{12} was extracted and assayed microbiologically, using *Euglena gracilis* (Anderson, 1965).

TABLE 1

CONCENTRATION OF ENDOGENOUS VITAMIN B_{12} IN ILEAL SUBCELLULAR ORGANELLES

Fraction	Vitamin B_{12} concentration (pg/mg protein)
Ileal homogenate	2080
Crude brush border[1]	980
Purified brush borders[2]	0
Purified nuclei[3]	10
Combined mitochondria/lysomal fraction[1]	5940
Partially purified mitochondria[4]	9900
Partially purified lysosomes[4]	4900
Microsomes[1]	556
Cytosol[1]	1150

[1] Hübscher, West and Brindley (1965).
[2] Eichholz and Crane (1965).
[3] Widnell, Hamilton and Tata (1966).
[4] Shakespeare (1969).

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Table 1 shows the concentration of vitamin B_{12} in the original ileal homogenate and in the various subcellular fractions. Vitamin B_{12} was absent, or present in only trace amounts, in purified brush borders and nuclei. The small amount of vitamin B_{12} present in the crude brush border fraction prepared by the technique of Hübscher, West and Brindley (1965) could be due to mitochondrial contamination. The combined mitochondrial/lysosomal fraction contained approximately 50 per cent of the vitamin B_{12} and subfractionation showed that the highest concentration was in the partially purified mitochondria.

The mitochondria were disrupted after swelling in hypotonic phosphate buffer and the inner and outer mitochondrial membranes separated by density gradient centrifugation (Parsons, Williams and Chance, 1966). There was a sevenfold concentration of the vitamin in the inner membrane compared to the outer membrane. This observation suggests that vitamin B_{12} is within the mitochondrion rather than adsorbed on to its outer surface. The degree of concentration of vitamin B_{12} in the inner membranes, however, was less than that of the marker enzyme, cytochrome oxidase, suggesting that vitamin B_{12} is not as firmly bound as this enzyme to the mitochondrion.

Previous studies on the subcellular localization of vitamin B_{12} have also indicated that it is concentrated in mitochondria (Swendseid, Bethell and Ackerman, 1951; Newman *et al.*, 1962). Chromatographic analysis of the cobalamins extracted from guinea-pig intestinal mitochondria have shown that 5′-deoxyadenosylcobalamin (coenzyme B_{12}) is the principal vitamin B_{12} derivative; an unidentified polar cobalamin is a minor component (Hoffbrand *et al.*, 1970; Peters *et al.*, 1971). The localization of 5′-deoxyadenosylcobalamin in this organelle is consistent with the known mitochondrial localization of methylmalonyl CoA mutase, the only mammalian enzyme known to have a specific requirement for this vitamin B_{12} analogue (Gurnani, Mistry and Johnson, 1960). A recent abstract by Newmark (1970) has suggested that vitamin B_{12} is localized in the lysosome in rat kidney. The kidney actively metabolizes propionate (Weidemann *et al.*, 1970) and is a potent source of methylmalonyl CoA mutase (Lengyel, Mazumder and Ochoa, 1960) and therefore would be expected to show at least partial localization of vitamin B_{12} in the mitochondrion. The vitamin B_{12} found in the partially purified lysosomes in the present study (Table 1) can largely be ascribed to mitochondrial contamination of the fraction, though it may also represent mitochondria which have undergone autophagy by lysosomes during the normal course of their turnover (de Duve and Wattiaux, 1966).

Distribution and subcellular localization of ^{57}Co-vitamin B_{12} during absorption

Male guinea-pigs were fed approximately 10 ng (a physiological dose) of high specific activity ^{57}Co-cyanocobalamin and were sacrificed at timed intervals during the following 24 hours. Fig. 1 shows the percentage of the

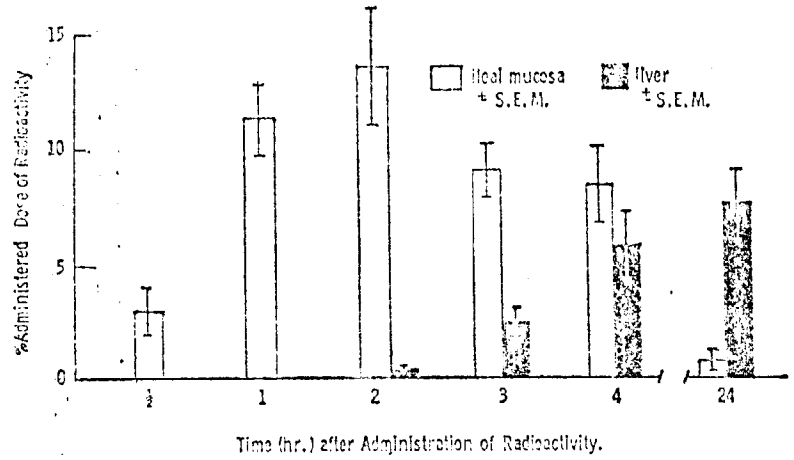

FIG. 1. The proportion of a 10-ng dose of ^{57}Co-cyanocobalamin in ileum and liver at ½, 1, 2, 3, 4 and 24 hours after administration.

administered dose of radioactivity in the ileal mucosa and in the liver at successive times after feeding the labelled vitamin. Radioactivity was present in the ileal mucosa for at least 1 hour before any of the labelled vitamin appeared in the liver confirming that there is a mucosal delay in the guinea-pig. The ileal mucosa was homogenized and subcellular fractionation was carried out. A full report of this work has been published elsewhere (Peters and Hoffbrand, 1970; Peters, Hoffbrand and Booth, 1970).

Figure 2 shows the specific activity of the subcellular fractions at successive times after feeding the labelled cyanocobalamin. The continuous heavy line represents the specific activity of the homogenate and acts as a reference. Thirty minutes after feeding cyanocobalamin, radioactivity was concentrated in the brush border fraction. This localization probably represents initial binding of the B_{12}–IF to the brush border. One and two hours after feeding vitamin B_{12} there was an approximately twentyfold increase in specific activity of the purified mitochondrial fraction. This was the only fraction which showed concentration of radioactivity during the period of intramucosal delay. In particular, there was no significant localization of vitamin B_{12} in the lysosomal subfraction at any time during the experimental procedure. These studies therefore do not support Wilson's pinocytotic hypothesis (Wilson, 1963).

Between the second and fourth hours there was a decline in the mitochondrial radioactivity coincident with the transfer of the labelled cobalamin into the animal, as shown by the hepatic radioactivity. Transfer of labelled vitamin B_{12} from the mitochondrion to the portal plasma appeared to occur via the cytoplasm of the enterocyte, since there was a significant increase in specific activity of the soluble fraction at 3 and 4 hours after feeding.

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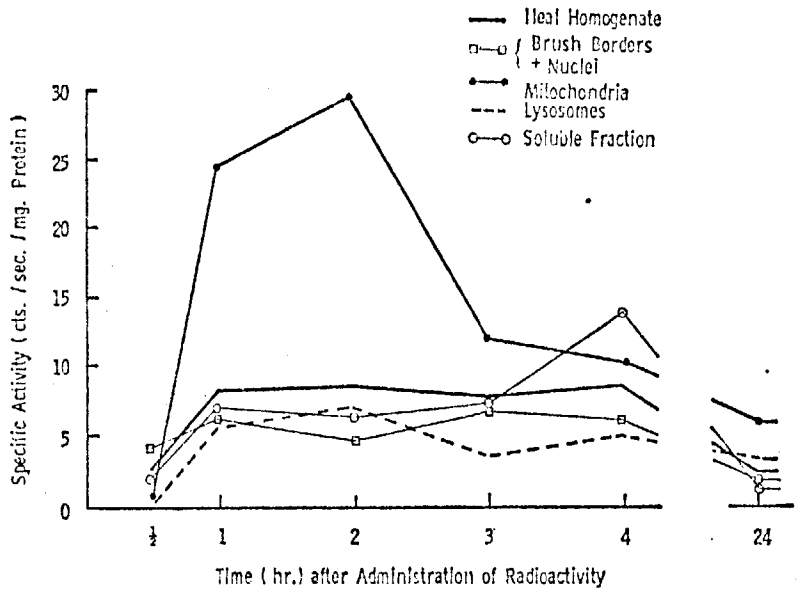

FIG. 2. The specific activity of the ileal homogenate and the subcellular fractions at ½, 1, 2, 3, 4 and 24 hours after feeding 10 ng ^{57}Co-cyanocobalamin.

Examination of subcellular distribution of radioactivity in the ileal mucosa 24 hours after administration of cyanocobalamin revealed a pattern similar to that found for endogenous vitamin B_{12}. Nearly 50 per cent of the activity was present in the combined mitochondrial/lysosomal fraction and there was a fourfold concentration of radioactivity in this fraction. The remainder of the radioactivity was almost equally divided between the brush border and soluble fractions. Although the animals were shown by total body counting and by measuring colonic radioactivity to have absorbed 25–30 per cent of the 10 ng oral dose, only about 6 per cent was found in the liver after 24 hours. On the other hand, animals killed two to four weeks after the administration of oral cyanocobalamin were found to have 80–90 per cent of the absorbed dose in the liver. Thus, the absorbed radioactivity accumulates in the liver, the main body store for vitamin B_{12}, relatively slowly. Similar results were found in the elegant study of Rosenblum and co-workers (1963) in the dog. They found slow uptake of absorbed radioactivity in the liver and although cyanocobalamin was administered, these workers showed that the major form of radioactive vitamin B_{12} in the liver was hydroxocobalamin or 5′-deoxyadenosylcobalamin.

Studies of the subcellular localization of labelled vitamin B_{12} as it is progressively stored by the liver indicate that there is increasing concentration of radioactivity in the mitochondria (Strength, Alexander and Wack, 1959; Newman *et al.*, 1962). It appears then that the mitochondrion is the principal

storage organelle for vitamin B_{12} in both hepatic and ileal cells. In [illegible]ation, the vitamin appears to enter ileal mitochondria during absorption and to reside there for the period of intra-mucosal delay. To determine whether or not the absorbed vitamin B_{12}, like endogenous vitamin B_{12}, is associated with the inner mitochondrial membrane fraction the following experiment was performed. Three guinea-pigs were fed labelled cyanocobalamin and were killed 2 hours later; the ileal mitochondria were subfractionated into inner and outer membrane fractions. Table 2 shows the specific activity of

TABLE 2

INTRA-MITOCHONDRIAL LOCALIZATION OF LABELLED VITAMIN B_{12} TWO HOURS AFTER FEEDING 10 ng ^{57}Co-CYANOCOBALAMIN

Fraction	Specific activity (cps/mg protein)
Ileal homogenate	16·6
Disrupted mitochondria	28·0
Inner mitochondrial membranes	21·4
Outer mitochondrial membranes	4·2

the labelled vitamin in the original homogenate, in disrupted mitochondria and in the membrane subfractions. Although there was some loss of radioactivity from the mitochondria during the fractionation procedure the inner membranes had a significantly higher specific activity than the outer membranes. This suggests that the labelled vitamin B_{12} enters the mitochondrial matrix during absorption rather than being merely adsorbed onto its outer surface.

Other groups have recently studied the subcellular localization of vitamin B_{12} in the ileum during absorption. Weisberg, Rhodin and Glass (1968), using electron microscope autoradiography, demonstrated localization of radioactive vitamin B_{12} to mucus in the goblet cells and to the brush border membrane early in the absorptive phase, and to the Golgi apparatus and rough endoplasmic reticulum during the later stage. However, these workers studied the absorption of rather large doses of labelled cyanocobalamin from tied ileal loops in the dog. They also claim that vitamin B_{12} absorption in the dog is not intrinsic factor mediated (Yamaguchi, Weisberg and Glass, 1969). Recent autoradiographic studies on the uptake of B_{12}–IF by guinea-pig ileal homogenates are, however, consistent with mitochondrial localization (Weisberg, 1970).

Rosenthal, Cutler and Sobieszczanska (1970) have also investigated the subcellular localization of labelled cyanocobalamin in the ileal mucosa of the guinea-pig. They concluded that the vitamin was firmly bound to particulate fractions during absorption. However, they did not isolate brush

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borders, separate mitochondria from lysosomes, or fully characterize the subcellular fractions.

The unexpected finding of mitochondrial localization of vitamin B_{12} during its absorption poses a number of questions. It certainly does not support the hypothesis that pinocytosis and lysosomes are involved in vitamin B_{12} absorption. It neither supports nor discredits the suggestion that B_{12}–IF is split at the brush border, although our results suggest that the brush border phase of vitamin B_{12} absorption is short. Possible explanations for the mitochondrial delay, some of which are under active investigation, are considered below.

Possible role of mitochondria as a transcellular 'ferry'

Mitochondria in living tissues constantly alter in shape and size partly due to swelling and contraction associated with oxidative phosphorylation (Lehninger, 1962, 1965). Mitochondria have been observed to move through mammalian cells (Tobioka and Biesele, 1956; Frédéric, 1958) and it has been suggested that vitamin B_{12} may be carried in this fashion through the enterocyte by mitochondria (Chappell, J. B., personal communication). Unless the movement of mitochondria is an ordered process, however, it is difficult to explain why there is an abrupt rise and fall in mitochondrial radioactivity. Moreover, the increase in radioactivity in the soluble fraction towards the end of the period of intramucosal delay would suggest that mitochondria discharge the labelled vitamin B_{12} into the cytoplasm rather than directly through the plasma membrane. More information is needed on mitochondrial kinetics in the enterocyte before any conclusions on this hypothesis can be reached.

Role of mitochondria in the metabolism of cyanocobalamin

Recent studies of Okuda and his colleagues have indicated that neither methylcobalamin (Okuda *et al.*, 1969a, b) nor 5′-deoxyadenosylcobalamin (Okuda, Yashima and Takamatsu, 1969) are extensively degraded during absorption although both are absorbed by an intrinsic factor-mediated process. These observations do not support the hypothesis of Mackenzie and Donaldson (1969b) that vitamin B_{12} is hydrolysed to a small 'active fragment' at the brush border.

Latner and his colleagues, however, suggested that the cyanocobalamin might be converted to 5′-deoxyadenosylcobalamin during transport through the ileal enterocyte (Latner and Hodson, 1962; Latner, Hodson and Smith, 1962). Rosenthal, Cutler and Sobieszczanska (1970) postulated that this process might occur during the binding of the vitamin B_{12} to subcellular particles. Neither group produced direct experimental data to support the theory. We have investigated the hypothesis by determining (a) whether all three vitamin

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B_{12} analogues show mucosal delay and mitochondrial localization, (b) the effect of the metabolic inhibitor, fluoroacetate, on this localization and (c) the forms of vitamin B_{12} in ileal mucosa after feeding cyanocobalamin (Hoffbrand *et al.*, 1970; Peters *et al.*, 1971; Peters, Quinlan and Hoffbrand, 1971).

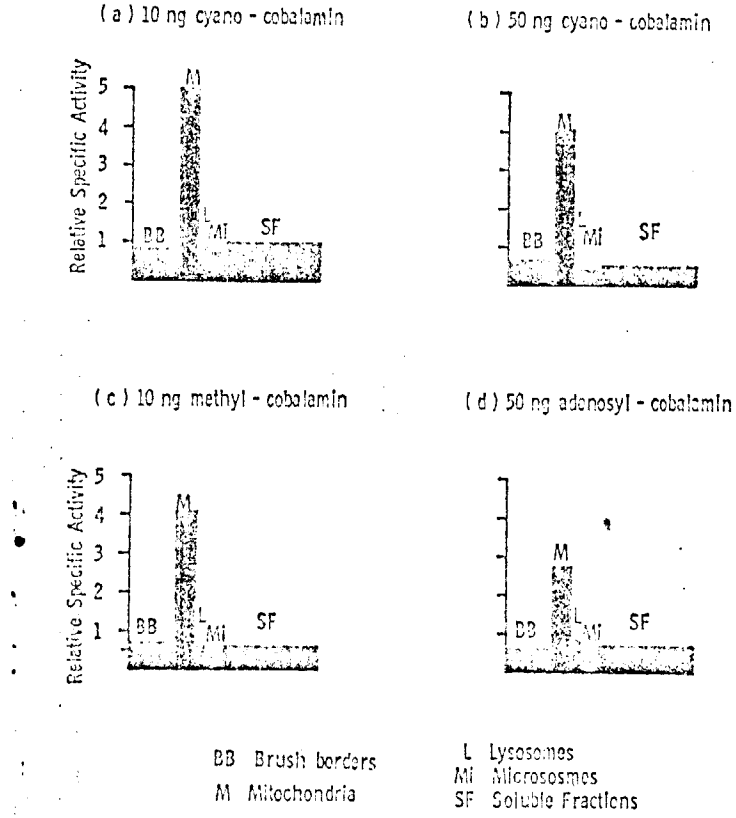

FIG. 3. The relative specific activities of the subcellular fractions of ilea mucosa 2 hours after feeding (a) 10 ng ^{57}Co-cyanocobalamin, (b) 50 ng ^{57}Co-cyanocobalamin, (c) 10 ng ^{57}Co-methylcobalamin, (d) 50 ng ^{57}Co-5′-deoxyadenosylcobalamin. The width of each column indicates the proportion of protein in that fraction.

(a) Labelled cyanocobalamin, methylcobalamin and 5′-deoxyadenosylcobalamin were fed to guinea-pigs which were killed 2 hours later. These three analogues had a similar gastro-intestinal transit time with a delay in transport across the ileal mucosa. The methyl and 5′-deoxyadenosyl analogues were, however, less well absorbed than cyanocobalamin. The ileal mucosa was subjected to subcellular fractionation. Fig. 3 shows the results of these experiments. In this figure the relative specific activity in each subcellular fraction is compared to that in the original homogenate. The width of each column is proportional to the amount of protein in that particular fraction. The subcellular distribution of the three analogues was similar; the labelled

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vitamin was concentrated only in the mitochondrial fraction. These results suggest that mitochondrial localization is a phenomenon common to all vitamin B_{12} analogues and imply that this localization is not solely concerned with conversion of cyanocobalamin to 5′-deoxyadenosylcobalamin.

(b) Latner and Hodson (1962) suggested that fluoroacetate inhibits the synthesis of 5′-deoxyadenosylcobalamin from cyanocobalamin. We found that doses of fluoroacetate similar to those used by Latner in the rat markedly inhibited the gastro-intestinal transit-time in the guinea-pig. Smaller doses, however, neither inhibited uptake nor influenced the subcellular distribution of labelled cyanocobalamin in the ileum. These observations are consistent with those of Glass and Castro-Curel (1964) who showed that fluoroacetate did not inhibit the uptake of B_{12}–IF by guinea-pig ileal homogenates.

These findings do not support the hypothesis that conversion of cyanocobalamin to 5′-deoxyadenosylcobalamin during absorption is obligatory.

(c) More direct evidence on the role of the ileum in the interconversion of different forms of vitamin B_{12} was obtained using chromatography and bioautography to separate and quantitate the different cobalamins (Linnell, Mackenzie and Matthews, 1969; Linnell *et al.*, 1969). Guinea-pigs were fed labelled cyanocobalamin and sacrificed at timed intervals. Aliquots of the ileal homogenates and mitochondria prepared from each animal were extracted, chromatographed and the amount of each individual cobalamin estimated. Figure 4 summarizes the results. There was a steep rise in the concentration of labelled cyanocobalamin in the homogenate to a peak at 1 hour followed by a decline. In contrast, the amount of labelled 5′-deoxyadenosylcobalamin in the homogenate rose throughout the experimental period. The form of these two curves suggests a precursor/product relationship with cyanocobalamin being converted to the 5′-deoxyadenosyl analogue. The finding of labelled 5′-deoxyadenosylcobalamin in the ileum 1 hour after feeding labelled cyanocobalamin indicates that the conversion occurs in the mucosa, rather than elsewhere in the body with subsequent transport of the 5′-deoxyadenosylcobalamin back to the ileum. Figure 4 also shows the amount of labelled 5′-deoxyadenosylcobalamin in ileal mitochondria. The form of this curve suggests that the conversion of cyanocobalamin to 5′-deoxyadenosylcobalamin occurs in the mitochondrion.

Several groups have studied interconversion of cyanocobalamin to 5′-deoxyadenosylcobalamin (Fenrych, Pawelkiewicz and Magas, 1962; Peterkofsky and Weissbach, 1964; Vitols, Walker and Huennekens, 1966). In particular, Gorna (1965) has demonstrated that rat intestinal mucosa can perform this interconversion *in vitro*. She demonstrated that a particulate fraction (probably mitochondria) isolated from liver is capable of carrying out the reaction.

Conversion of cyanocobalamin to 5′-deoxyadenosylcobalamin in the mucosa is by no means complete since labelled cyanocobalamin (as well as labelled 5′-deoxyadenosylcobalamin) can be detected in guinea-pig portal

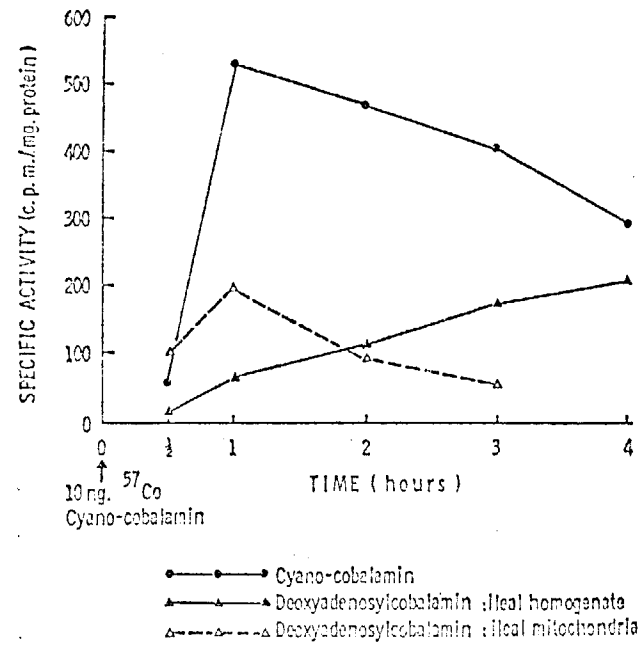

FIG. 4. The specific activity of the cyanocobalamin in ileal homogenate, and 5′-deoxyadenosylcobalamin in ileal homogenate and ileal mitochondria, at ½, 1, 2, 3 and 4 hours after feeding 10 ng ^{57}Co-cyanocobalamin.

plasma after feeding labelled cyanocobalamin (Hoffbrand *et al.*, 1970). Parallel studies have shown that significant amounts of a physiological dose of cyanocobalamin are also absorbed unchanged in man (Linnell *et al.*, 1969). Thus interconversion of vitamin B_{12} analogues appears to be an unlikely explanation for mucosal delay in the absorption of the vitamin.

Site of cleavage of B_{12}–IF complex

Although vitamin B_{12} is bound to intrinsic factor when it is attached to the brush border, it emerges into portal blood no longer bound to intrinsic factor (Cooper and White, 1968). As mentioned earlier, the site of this cleavage is unknown but in view of the prolonged association of the absorbed vitamin B_{12} with the mitochondrion it seemed possible that this organelle releases vitamin B_{12} from intrinsic factor.

Proteases, active at neutral pH, have been demonstrated within mitochondria (Alberti and Bartley, 1969; Fitzpatrick and Pennington, 1969). It has also been shown that both native and foreign proteins may accumulate in renal tubular cell mitochondria (Rouiller, 1960); the structural and func-

tional similarities of the renal tubular cell and the enterocyte are well recognized. Inulin (molecular weight 5000) is probably the largest molecule which may enter the mitochondrion *in vitro* (Werkheiser and Bartley, 1957); crucial to this discussion is the question of whether the B_{12}–IF complex can enter the enterocyte. Several workers have demonstrated the presence of intrinsic factor activity in ileal homogenates (Boass and Wilson, 1964; Ukyo and Cooper, 1965; Rothenberg, 1968), but these studies do not distinguish between adsorption on to the brush border or intracellular localization of intrinsic factor. The recent elegant studies of Hines, Rosenberg and Harris (1968) appeared to show that intrinsic factor remains on the surface of the brush border while vitamin B_{12} enters the epithelial cell. Unfortunately these workers used hog intrinsic factor and guinea-pig everted sacs and we, like other workers (cf. Glass, 1963), have had difficulty in confirming that hog intrinsic factor facilitates vitamin B_{12} absorption in the guinea-pig.

Rosenblum and Geoffroy (1969) and Yamaguchi, Rosenthal and Glass (1970) have studied the absorption of a double-labelled B_{12}–IF complex (^{57}Co-cyanocobalamin, ^{51}Cr-intrinsic factor) by guinea-pig and man respectively. Both authors showed that although significant amounts of vitamin B_{12} were absorbed, less than 1 per cent of the ^{51}Cr was absorbed. These studies are consistent with the hypothesis that intrinsic factor remains attached to the brush border membrane but they do not exclude the possibility that intrinsic factor enters the ileal enterocyte and is then shed into the intestinal lumen when that cell is sloughed off at the villus tip.

We have attempted to determine whether the B_{12}–IF enters the epithelial cell and in particular whether it is present in the mitochondrial fraction, using a double-labelled complex (^{57}Co-cyanocobalamin, ^{125}I-intrinsic factor). Human intrinsic factor—^{57}Co-cyanocobalamin was purified by a modification of the method of Mackenzie, Donaldson and Schilling (1969) and was iodinated by the method of McConahey and Dixon (1966). This double-labelled B_{12}–IF was further purified by ion-exchange chromatography and fed to guinea-pigs. The ileal mucosa was subjected to subcellular fractionation and the concentration of both isotopes determined in each subcellular fraction. Thirty minutes after feeding double-labelled B_{12}–IF, significant amounts of both isotopes were found attached to the brush border membrane. At 1 hour, although there was significant localization of radioactive vitamin B_{12} to the mitochondrion, no concentration of the intrinsic factor label was noted. These preliminary experiments suggest that the B_{12}–IF complex is split at the brush border and only the vitamin B_{12} gains entry to the enterocyte.

Vitamin B_{12} binders in the ileum

Recently-absorbed vitamin B_{12} is attached in portal plasma to a specific transport protein, transcobalamin II (Hall and Finkler, 1966). This is a β-globulin of molecular weight 38 000 (Hom, 1969) and it is possible that the

ileal mitochondrion is the site of attachment of the absorbed vitamin B_{12} to this transport protein. The site of synthesis of transcobalamin in man and the guinea-pig is unknown although in the mouse the liver is the principal source (Tan and Hansen, 1968). The chief tissue binding protein for vitamin B_{12} is transcobalamin I which has a molecular weight of 121 000 (Hom, 1969) and is thought to be responsible for the transport of the vitamin out of cells (Hall, 1969).

We have made preliminary observations on the nature of the vitamin B_{12}

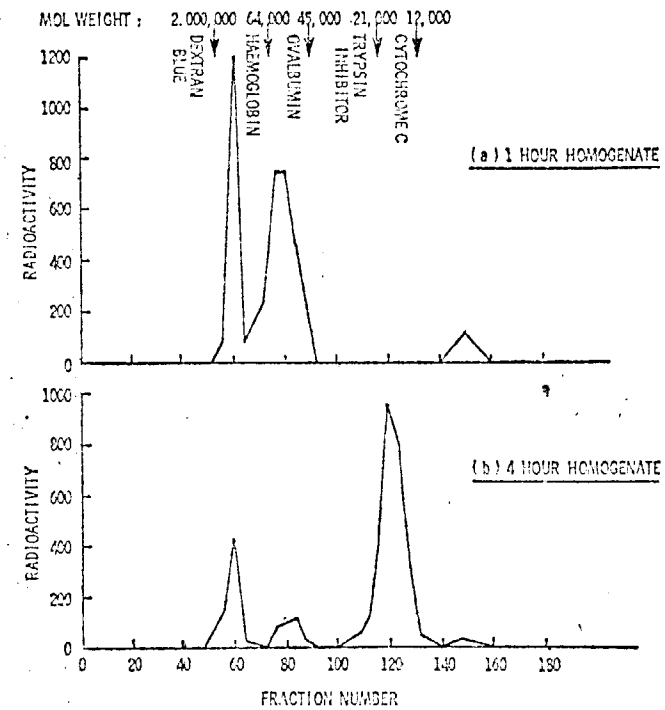

FIG. 5. Gel filtration studies (on Sephadex G-100, 100 × 2·5 cm) of supernatants prepared from ileal mucosa (a) 1 hour and (b) 4 hours, after feeding labelled cyanocobalamin.

binders in ileal mucosa at different stages during absorption of cyanocobalamin. Ileal mucosa from guinea-pigs which had been fed labelled cyanocobalamin 1 or 4 hours previously was homogenized, sonicated and centrifuged at 100 000*g* for 60 minutes. The supernatant was chromatographed on Sephadex G-100 by the method of Hom (1969). Figure 5 shows the elution pattern of radioactive B_{12} for (a) the 1-hour and (b) the 4-hour supernatants. At 1 hour there were two major labelled components, an excluded peak with a molecular weight at greater than 100 000 and a second peak with molecular weight approximately 50 000. It is noteworthy that the molecular weight of intrinsic

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factor is 50 000–60 000 (Gräsbeck, 1969). In the 4-hour supernatant the size of each of these two peaks was significantly decreased and most of the radioactivity was associated with a third peak of molecular weight 15 000–20 000. Only small amounts of free vitamin B_{12} were detected. These findings suggest that B_{12}–IF initially binds to a large molecular weight receptor on the brush border and that vitamin B_{12} subsequently becomes attached to a lower molecular weight protein within the mucosa.

There have been other preliminary reports on the nature of the ileal binding proteins during the absorption of labelled cyanocobalamin. Frentz and Miller (1966) found that absorbed labelled vitamin B_{12} was attached at 4 hours to a protein of higher molecular weight than at 1 hour. Rosenthal, Cutler and Sobieszczanska (1970) found that vitamin B_{12} could be more easily dialysed from subcellular fractions prepared 5 hours after feeding labelled cyanocobalamin compared with 1½ hour fractions. Okuda and Fujii (1970) have recently reported gel filtration data very similar to those found in the present study. They noted three separate peaks which were tentatively identified as (1) B_{12}–IF-receptor complex, (2) B_{12}–IF and (3) a B_{12}-tissue binder. Homogenates prepared 5 hours after feeding the labelled vitamin B_{12} had large amounts of peak 3 compared with earlier homogenates. Unfortunately the molecular weight of this peak is not recorded. The nature of this third binder is not clear; in our studies it does not appear to correspond to either transcobalamin II (molecular weight 38 000) or transcobalamin I (molecular weight 121 000). Further study of the identity, molecular weight and site of synthesis of vitamin B_{12} binders in both the ileum and portal plasma is clearly necessary. It is relevant to note that a low molecular weight vitamin B_{12} binder has been demonstrated in human plasma (Gizis and Meyer, 1968) and in liver (Hedbom, 1961).

Conclusions

The mechanisms involved in the transfer of vitamin B_{12} across the enterocyte are complex and poorly understood. The mitochondria appear to play an important role in this process as well as acting as the principal site of storage of vitamin B_{12}. Vitamin B_{12}-intrinsic factor complex is apparently split at the brush border and probably only the vitamin B_{12} enters the mitochondrion. Although this organelle converts part (approximately 20 per cent) of the absorbed cyanocobalamin to 5′-deoxyadenosylcobalamin, a large proportion of a physiological dose of cyanocobalamin is absorbed unchanged. During its delay in the ileal enterocyte, where it is largely confined to the mitochondrion, the labelled vitamin appears to be transferred to a relatively low molecular weight tissue binding protein. Further studies of the structural and functional relationshps of the various tissue and plasma vitamin B_{12}-binding proteins appear to be warranted.

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REFERENCES

Alberti, K. G. M. M. and Bartley, W. (1969), *Biochem. J.*, **111**, 763.
Anderson, B. B. (1965), Ph.D. thesis, University of London.
Boass, A. and Wilson, T. H. (1964), *Amer. J. Physiol.*, 207, 27.
Booth, C. C., Chanarin, I., Anderson, B. B. and Mollin, D. L. (1957), *Brit. J. Haemat.*, 3, 253.
Cooper, B. A. and White, J. J. (1968), *Brit. J. Haemat.*, **14**, 73.
De Duve, C. and Wattiaux, R. (1966), *Ann. Rev. Physiol.*, 28, 435.
Donaldson, R. M., Mackenzie, I. L. and Trier, J. (1967), *J. Clin. Invest.*, 46, 1215.
Doscherholmen, A., Hagen, P. S., Lium, M. and Olin, L. (1957), *J. Clin. Invest.*, 36, 1551.
Eichholz, A. and Crane, R. K. (1965), *J. Cell Biol.*, 26, 687.
Fenrych, W., Pawelkiewicz, J. and Magas, S. (1962), *Bull. Acad. Polon. Sci. Ser. Sci. Biol.*, 10, 117.
Fitzpatrick, K. and Pennington, R. J. (1969), *Biochem. J.*, **111**, 29P.
Frédéric, J. (1958), *Arch. Biol. (Liège)*, 69, 167.
Frentz, G. D. and Miller, O. N. (1966), *Clin. Res.*, **14**, 432.
Gizis, E. and Meyer, L. M. (1968), *Nature*, **217**, 272.
Glass, G. B. J. (1963), *Physiol. Rev.*, 43, 529.
Glass, G. B. J. and Castro-Curell, Z. (1964), *Ann. N.Y. Acad. Sci.*, **112**, 904.
Gorna, M. (1965), *Roczn. Nauk. WSWF Poznan*, 10, 9.
Gräsbeck, R. (1967), *Scand. J. clin. Lab. Invest.*, 19, Suppl. 95, 7.
Gräsbeck, R. (1969), In: *Progress in Hematology*, New York and London, Grune and Stratton. Ed. Brown, E. B. and Moore, C. V., p. 233.
Gurnani, S., Mistry, S. P. and Johnson, B. C. (1960), *Biochim. Biophys. Acta*, 38, 187.
Hall, C. A. (1969), *Brit. J. Haemat.*, **16**, 429.
Hall, C. A. and Finkler, A. E. (1966), *Proc. Soc. Exp. Biol. Med.*, **123**, 55.
Hedbom, A. (1961), *Biochem. J.*, 79, 469.
Herbert, V. (1968), *Gastroenterology*, 54, 110.
Hines, J. D., Rosenberg, A. and Harris, J. W. (1968), *Proc. Soc. Exp. Biol. Med.*, **129**, 653.
Hoffbrand, A. V., Linnell, J. C., Matthews, D. M. and Peters, T. J. (1970), *J. Physiol.*, 208, 66P.
Hoffbrand, A. V. and Peters, T. J. (1969), *Biochim. Biophys. Acta*, **192**, 479.
Hom, B. L. (1969), *Biochim. Biophys. Acta*, **175**, 20.
Hübscher, G., West, G. R. and Brindley, O. N. (1965), *Biochem. J.*, 97, 629.
Latner, A. L. and Hodson, A. W. (1962), *Biochim. Biophys. Acta*, 56, 185.
Latner, A. L., Hodson, A. W. and Smith, P. A. (1962), *Lancet*, **2**, 230.
Lehninger, A. L. (1962), *Physiol. Rev.*, **42**, 467.
Lehninger, A. L. (1965), *The Mitochondrion: Molecular Basis of Structure and Function*, New York, Benjamin.
Lengyel, P., Mazumder, R. and Ochoa, S. (1960). *Proc. Nat. Acad. Sci.*, 46, 1312.
Linnell, J. C., Mackenzie, H. M. and Matthews, D. M. (1969), *J. Clin. Path.*, **22**, 506.

LINNELL, J. C., MACKENZIE, H. M., WILSON, J. and MATTHEWS, D. M. (1969), *J. Clin. Path.*, 22, 545.
MACKENZIE, I. L. and DONALDSON, R. M. (1969a), *J. Lab. Clin. Med.*, 74, 986.
MACKENZIE, I. L. and DONALDSON, R. M. (1969b), *Fed. Proc.*, 28, 41.
MACKENZIE, I. L., DONALDSON, R. M. and SCHILLING, R. F. (1969), *J. Clin. Invest.*, 48, 516.
MCCONAHEY, P. J. and DIXON, F. J. (1966), *Int. Arch. Allergy*, 29, 185.
MARSH, M. N., PETERS, T. J. and BROWN, A. C. (1971), *Gut*. In press.
NEWMAN, G. E., O'BRIEN, J. R. P., SPRAY, G. H. and WITTS, L. J. (1962), In: *Vitamin B_{12} and Intrinsic Factor*, Stuttgart, Enke. Ed. Heinrich H. C., p. 424.
NEWMARK, P. (1970), *Proc. XIII Intern. Congr. Haematol.*, Munich, p. 160.
OKUDA, K. and FUJII, T. (1970), *Proc. XIII Intern. Congr. Haematol.*, Munich, p. 158.
OKUDA, K., YASHIMA, K. and TAKAMATSU, M. (1969), *J. Lab. Clin. Med.*, 74, 218.
OKUDA, K. YASHIMA, K., TAKARA, I., KITASAKI, T., KURASMIGA, M. and TAKAMATSU, M. (1969a), *Vitamins (Japan)*, 40, 224.
OKUDA, K., YASHIMA, K., TAKARA, I., KITSASAKI, T., MOROKUMA KINOSHITA, A. and TAKAMATSU, M. (1969b), *Vitamins (Japan)*, 40, 232.
PARSONS, D. F., WILLIAMS, G. R. and CHANCE, B. (1966), *Ann. N.Y. Acad. Sci.*, 137, 643.
PETERKOFSKY, A. and WEISSBACH, H. (1964), *Ann. N.Y. Acad. Sci.*, 112, 622.
PETERS, T. J. (1970), *Biochem. J.*, 120, 195.
PETERS, T. J. and HOFFBRAND, A. V. (1970), *Brit. J. Haemat.*, 19, 369.
PETERS, T. J., HOFFBRAND, A. V. and BOOTH, C. C. (1970), *Proc. 7th Int. Congr. Clin. Chem.*, Geneva/Evian, 1969, Vol. 4, Basel, Karger.
PETERS, T. J., LINNELL, J. C., MATTHEWS, D. M. and HOFFBRAND, A. V. (1971), *Brit. J. Haemat.*, 20, 299.
PETERS, T. J., QUINLAN, A. and HOFFBRAND, A. V. (1971), *Brit. J. Haemat*, 20, 123.
ROSENBLUM, C. and GEOFFROY, R. F. (1969), In: *Proc. Intern. Congr. on Radio-Isot. in Pharmacol.*, London, Wiley, Ed. Waser, P. G. and Glasson, B., p. 349.
ROSENBLUM, C., REIZENSTEIN, P. G., CRONKITE, E. P. and MERIWETHER, H. T. (1963), *Proc. Soc. Exp. Biol. Med.*, 112, 262.
ROSENTHAL, H. L., CUTLER, L. and SOBIESZCZANSKA, W. (1970), *Amer. J. Physiol.*, 218, 358.
ROTHENBERG, S. P. (1968), *J. Clin. Invest.*, 47, 913.
ROUILLER, CH. (1960), *Int. Rev. Cytol.*, IX, 227.
SHAKESPEARE, P. (1969), quoted by HOFFBRAND, A. V. and PETERS, T. J. (1969), *Biochim. Biophys. Acta*, 192, 479.
STRENGTH, D. R., ALEXANDER, W. F. and WACK, J. P. (1959), *Proc. Soc. Exp. Biol. Med.*, 102, 15.
SULLIVAN, L. W., HERBERT, V. and CASTLE, W. B. (1963), *J. Clin. Invest.*, 42, 1443.
SWENSEID, M. E., BETHELL, F. H. and ACKERMAN, W. W. (1951), *J. Biol. Chem.*, 190, 791.
TAN, C. H. and HANSEN, H. T. (1968), *Proc. Soc. Exp. Biol. (N.Y.)*, 127, 740.
TOBIOKA, M. and BELSLE, J. J. (1956), *J. Biophys. Biochem. Cytol.*, Suppl. Vol. 2, 219.
UKYO, S. and COOPER, B. A. (1965), *Amer. J. Physiol.*, 208, 9.
VITOLS, E., WALKER, G. A. and HUENNEKENS, F. M. (1966), *J. Biol. Chem.*, 241, 1455.
WEIDEMANN, M. J., HEMS, R., WILLIAMS, D. L., SPRAY, G. H. and KREBS, H. A. (1970), *Biochem. J.*, 117, 177.
WEIBERG, H. (1970), *Clin. Res.*, 18, 391.
WEISBERG, H., RHODIN, J. and GLASS, G. B. J. (1968), *Lab. Invest.*, 19, 516.
WERKHEISER, W. C. and BARTLEY, W. (1957), *Biochem. J.*, 66, 79.
WIDNELL, C. C., HAMILTON, T. H. and TATA, J. R. (1967), *J. Cell. Biol.*, 32, 766.
WILSON, T. H. (1963), *Physiologist*, 6, 11.
YAMAGUCHI, N., ROSENTHAL, W. S. and GLASS, G. B. J. (1970), *Amer. J. Clin. Nutr.*, 23, 156.
YAMAGUCHI, N., WEISBERG, H. and GLASS, G. B. J. (1969), *Gastroenterology*, 56, 914.

DISCUSSION

Dr White: Have you done the control experiment of taking normal ileal homogenate, adding ^{57}Co-cyanocobalamin and seeing what happens to it?

Dr Peters: Yes. If one takes ileal mucosa from a fasted animal and adds either B_{12} or B_{12}–IF to the mucosa and then performs the fractionation procedure one gets 95 per cent of the radioactivity in the soluble fraction; uptake by the subcellular is only 1 or 2 per cent. I think the mitochondrial localization is a true *in vivo* phenomenon, not an *in vitro* artefact.

Dr White: Have you tried any experiments with mitochondria *in vitro*?

Dr Peters: If you mean incubating mitochondria with B_{12}–IF, no, we have not done this. We felt that it was first necessary to determine whether intrinsic factor entered the cell, but it would, of course, be of interest to know whether mitochondria can split the B_{12}–IF complex.

Dr Chanarin: Is your iodine-labelled IF active in the Schilling test?

Dr Peters: We have not tested it with the Schilling test in man. If, however, one prepares brush borders from ileum and jejunum of guinea-pigs and incubates them with dual-labelled B_{12}–IF one can demonstate that ileal brush borders, but not jejunal brush borders, take up the ^{125}I-labelled complex. We do not, of course, say that our intrisic factor is pure; we would estimate the purity at perhaps 20 per cent, and there are probably many other iodinated proteins present, but we think that these are digested and absorbed higher in the gut.

Dr Cooper: I think your double-labelled experiments are extremely interesting and you appear to be very close to solving the problem that many people have been arguing about for a long time, provided that you are able to demonstrate that the molecules with the iodine on them really do work: your answer to Dr Chanarin's question partially answers this. When we put an 8000*g* supernatant of human intestine homogenate, obtained 10 hours after feeding radioactive B_{12}, through Sephadex (Cooper, 1968), we did not see a fraction which would correspond to a molecular weight of 15 000 such as yours. We did, however, find two other fractions which we did not identify. I wonder if your observation is consistent; have you found similar results in other animals?

Dr Peters: We have only done this in guinea-pigs using human IF. We did expect to find a higher molecular weight binder, about 38 000 MW, corresponding to TC II, but there was a report in *Nature* of a relatively low molecular weight binder in plasma of 15 000 MW (Gizis and Meyer, 1968). Certainly I think it is well worth studying these tissue binders in greater detail.

Dr Cooper: Transcobalamin II filters through Sephadex with a slightly variable mobility, and when Hom (1969) filtered TC II through Sephadex he found its mobility to be less consistent than that of TC I. I doubt, however, that it would ever filter with an apparent molecular weight of 20 000. Does TC II run there in your column?

Dr Peters: We have not run TC II through our column.

Dr Newmark: Following on the question of how good your preparation is, how comparable are the subcellular distribution results when you give the double-labelled preparation to the results obtained when giving free B_{12} orally, which would presumably be bound to proper intrinsic factor?

Dr Peters: We have not done the full series of experiments with both free B_{12} and dual-labelled B_{12}–IF. The results using ^{57}Co–B_{12} were means of four or five animals at each time interval. We have only done the comparative experiments using dual-labelled B_{12}–IF in a small number of animals.

Dr Lindstrand: Do you think that cyanocobalamin goes unchanged through the

mucosa or is there any conversion into coenzyme within the mucosa? Can the mucosa thus supply its own requirement for coenzyme?

Dr Peters: We think there is some conversion but we do not know whereabouts in the cell it occurs. Gorna (1965) has shown that rat small intestine *in vitro* can convert cyanocobalamin to deoxyadenosylcobalamin so it seems that gut is capable of this conversion, though the activity of the enzyme in gut was less than in liver. It is also interesting that in her subcellular fractionation studies Gorna showed that a particulate fraction from liver could bring about this conversion and we would estimate that this particulate fraction corresponds to mitochondria.

Dr Lindstrand: When I mix intestinal mucosa or plasma with cyanocobalamin, straight away I get some radioactivity that will go the same distance as coenzyme on chromatography, so I am always wary of saying that there is any conversion. Have you checked on this?

Dr Peters: We have not done any experiments *in vitro* ourselves but the work Gorna did involved fairly rigorous characterization using column chromatography, not simply thin-layer chromatography.

Dr Lindstrand: It would probably be satisfactory with a column.

May I take up another question? We have done total gastrectomies in guinea-pigs and have found that the animals absorb approximately 70 per cent of a dose of B_{12} given without intrinsic factor. Do you think there could be another mechanism for B_{12} absorption not involving intrinsic factor?

Dr Peters: Have you shown in fact that these animals are not producing IF, because IF can be produced by the duodenum, and recent reports have suggested that it may be produced by the pancreas?

Dr Lindstrand: We have removed the salivary glands but we have not removed the pancreas. We are looking for pancreatic IF now, but nevertheless we are a little bothered to find B_{12} absorption without a stomach. We found this also in rabbits—they have an enormous amount of B_{12} binder in the gastric fundus, but if you remove the whole stomach the rabbit will still absorb B_{12} perfectly well.

Dr Peters: We believe that we are studying an intrinsic factor-mediated mechanism in that it shows an ileal delay in B_{12} absorption. Dr Schjönsby, from Bergen, working in Professor Booth's laboratory, showed that uptake of B_{12}–IF by isolated guinea-pig ileal brush borders was blocked with IF antibody.

Professor Herbert: That was just what I was going to suggest—you can eliminate the need to purify intrinsic factor (which would probably take you two or three years) in order to prove that the mechanism is intrinsic factor-mediated simply by feeding with antibody and demonstrating a total wipe-out of the hot iodine uptake by the ileal mucosa.

Dr Peters: Has Dr Lindstrand done this?

Dr Lindstrand: No, we have not. We have been more interested in the production of intrinsic factor and the special cell type in different species; each species seems to have its own special mechanism for B_{12} absorption. We have found the rat quite a good animal to work with because it seems to have clear-cut intrinsic factor-mediated B_{12} absorption—the intact animal shows about 70 per cent absorption while removal of the fundus reduces absorption to about 2 per cent.

Dr Peters: We would have liked to have used the rat but the subcellular fractionation technique does not work with rat mucosa so we had to use the guinea-pig.

REFERENCES

COOPER, B. A. (1968), *Amer. J. Physiol.*, **214**, 832.
GIZIS, E. and MEYER, L. M. (1968), *Nature*, **217**, 272.
GORNA, M. (1965), *Roczn. Nauk. WSWF Poznan*, **10**, 9.
HOM, B. L. (1969), *Biochim. Biophys. Acta*, **175**, 20.

1983

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Nature 217(5123), 67-68

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Vitamin B_{12} Content of Aqueous Humour

THE vitamin B_{12} content of the aqueous humour of the eye does not seem to have been estimated before. Samples of aqueous humour and blood were obtained at operation in sixteen patients having cataract extraction; the serum and aqueous concentrations of vitamin B_{12} were determined by biological assay using *Euglena gracilis*. The results are presented in Table 1. It is interesting that the average value (29·9 pg/ml.) is of the same order as that found in cerebrospinal fluid (21 pg/ml.) by Kidd *et al.*[1].

There was no significant statistical correlation between the concentrations in the blood and the aqueous ($r = 0{\cdot}097$, $P \gg 0{\cdot}10$), but it must be admitted that the method of assay is not very accurate at low concentration. It is also emphasized that all the patients had cataracts and so most were elderly. It does not seem justified to risk paracentesis and removal of aqueous humour from normal human eyes in order to obtain more "physiological" values. Samples of aqueous humour were obtained through a cannula and dry-sterilized syringe in the early stages of the operation—before it, several instillations of guttae 2 per cent homatropine and cocaine 4 per cent hydrochloric acid and often 10 per cent phenylephrine had been given, and also a retrobulbar injection of 1·5 per cent xylocaine with adrenaline 1 : 200,000. Sparine (25 mg) was given by mouth on the evening before opera- ; 1 h before the operation 50 mg of pethidine with g of phenergan were injected intramuscularly. Great was taken to reduce contamination of the cannula with blood from the operative field to an absolute minimum; it was probably negligible.

It is possible that abnormally low values of vitamin B_{12} in the aqueous humour could be found in patients suffering from "tobacco amblyopia", in view of the low concentrations of serum vitamin B_{12} found in this disease[2] and the improvement following treatment with vitamin B_{12} (refs. 2 and 3). It is unlikely that there will be an opportunity to obtain samples of aqueous humour from patients with that disease, but the data available in normal patients were further analysed according to the smoking habits of the subjects. The mean aqueous concentrations of vitamin B_{12} were 20·2 pg/ml. for the smokers and 34·3 pg/ml. for the non-smokers ($t = 1{\cdot}12$, $P > 0{\cdot}10$). The difference is not regarded as being more than suggestive, but it will be seen that the five smokers showed aqueous concentrations of B_{12} of less than 30 pg/ml., whereas eight of the eleven non-smokers had concentrations greater than 25 pg/ml. If confirmed, this observation may be relevant in the elucidation of the pathogenesis of tobacco amblyopia.

Table 1. AQUEOUS B_{12} DETERMINED BY *Euglena* ASSAY

Number	Age	Sex	Smoking habits	Serum B_{12} (pg/ml.)	Aq. hum. B_{12} (pg/ml.)
1	87	F	—	420	58
2	67	F	—	224	32
3	58	M	40/day	434	29
4	63	M	—	269	49
5	75	F	—	339	42
6	73	F	—	194	35
7	87	F	—	313	12
8	52	F	10/day	93	14
9	75	F	—	123	60
10	45	M	Heavy pipe	218	15
11	74	F	—	135	29
12	53	F	10/day	161	28
13	87	M	Heavy pipe	128	15
14	87	M	—	252	14
15	74	F	—	454	18
16	72	F	—	564	28

Ranges, 12–60 pg/ml. Crude mean, 29·9 pg/ml.

The underlying reason for this investigation, apart from intrinsic interest, was the suggestion (mentioned by Phillips and McKenzie[4]) that the mechanism of tobacco amblyopia, or any toxic amblyopia, is a toxic effect on the retina of, say, cyanide or other toxin, which enters the eye with the aqueous humour and affects the retina, especially the macular area, from its vitreous surface. There is evidence for a flow of fluid from vitreous to retinal blood vessels and through retina to choroid[5]. This might explain why the retina is so selectively affected by certain toxins; the blood-aqueous barrier is not so high or so selective as the blood-retina barrier. Similar considerations might apply to the blood–cerebrospinal fluid barrier and blood–brain barrier. The response of the levels of aqueous B_{12} to injected hydroxocobalamin and cyanocobalamin is being studied, together with thiocyanate levels.

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[1] Kidd, H. M., Gould, C. E. G., and Thomas, J. W., *Canad. Med. Assoc. J.*, **88**, 876 (1963).

[2] Heaton, J. M., McCormick, A. J. A., and Freeman, A. G., *Lancet*, ii, 286 (1958).

[3] Chisholm, I. A., Bronte-Stewart, J., and Foulds, W. S., *Lancet*, ii, 450 (1967).

[4] Phillips, C. I., and McKenzie, A. D., *Brain* (in the press).

[5] da Cunha-Vaz, J. G., and Maurice, D. M., *J. Physiol.*, **191**, 467 (1967).

1984

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Ann. rheum. Dis. (1970), **29**, 533

Absorption of vitamin B_{12} in rheumatoid arthritis

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Since auto-immunity may possibly be a common denominator in pernicious anaemia, thyroid dysfunction, and the rheumatic diseases, the suggested increased incidence of pernicious anaemia in rheumatoid arthritis (Partridge and Duthie, 1963) is of considerable interest. Their figure of 1·4 per cent., which was five times higher than in their patients with other musculoskeletal disorders, was based on a retrospective study of the case notes of some 8,000 patients. However, the diagnosis of pernicious anaemia was often presumptive, as many patients had not had a marrow examination or had not been shown to be achlorhydric or to have a malabsorption of vitamin B_{12}. In those cases in which vitamin B_{12} malabsorption had been demonstrated, the effect of intrinsic factor had not been assessed. Further retrospective studies of the incidence of pernicious anaemia in rheumatoid arthritis are likely to produce similar incomplete data. It is widely accepted, however, that the incidence of latent pernicious anaemia is considerably higher than that of the overt disease (Witts, 1960), and it would therefore be expected that a population with a high incidence of pernicious anaemia would also show a high incidence of latent malabsorption of vitamin B_{12}. Couchman, Bieder, Wigley, and Glenday (1968) reported malabsorption of vitamin B_{12} in fifteen out of 52 cases of rheumatoid arthritis, but based their assessment largely on the result of a single Schilling test without further and confirmatory investigations. In the study reported here, vitamin B_{12} absorption was measured in 100 patients with rheumatoid arthritis with a full investigation of all those who had an abnormal Schilling test result, to confirm and to define their malabsorption of the vitamin.

Patients and methods

100 unselected but consecutive patients with definite or classical rheumatoid arthritis were studied, of whom 66 were women. All had been admitted to the Oxford Regional Rheumatic Diseases Research Centre, Stoke Mandeville Hospital, for medical or surgical reasons. No patient with a history of gastrointestinal disorder or operation was included, an exception being made in six patients with symptoms of peptic ulceration. Only one patient gave a family history of pernicious anaemia. Sjögren's syndrome, sometimes associated with poor gastric secretion (Buchanan, Cox, Harden, Glen, Anderson, and Gray, 1966), was found in another patient, but her symptoms were mild.

86 per cent. of the patients were over the age of 40 years. Since the incidence of any complication increases with the duration of the primary disease, it should be noted that in 77 per cent. the duration of the rheumatoid arthritis was 5 years or more and in 53 per cent. 10 years or more (Table I).

Table I *Age and duration of disease*

Age (yrs)	*No. of cases*	*Duration of disease (yrs)*	*No. of cases*
Under 30	3	Less than 5	23
31 to 40	11	5 to 9	24
41 to 50	25	10 to 14	25
51 to 60	23	15 to 19	13
61 to 70	28	20 to 29	11
Over 70	10	30 and over	4

The absorption of ^{57}Co-labelled vitamin B_{12} was measured by the urinary excretion method (Schilling, 1953), normal renal function being established by a creatinine clearance and blood urea estimations. In any patient with an abnormal Schilling test, the test was repeated in case the abnormality arose from incomplete urine collection. Where the results were again abnormal, the hepatic uptake of ^{57}Co-B_{12} (Glass, Boyd, Gellin, and Stephanson, 1954) was measured using a shielded but uncollimated Burndept scintillation counter. Approximate quantitation of the uptake was measured by counting the administered material immediately after ingestion with the counter over the epigastrium. The effect of intrinsic factor of vitamin B_{12} absorption was also assessed by the Schilling method.

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Results

Of the 100 patients investigated, 92 showed completely normal absorption of vitamin B_{12} with an excretion of 12 per cent. or more of the dose in a 24-hour urine collection.

Of the remaining eight, five had repeatedly abnormal Schilling tests of between 4 and 8 per cent., but in each case hepatic uptake was normal, ranging from 42 to 62 per cent. of the dose (Table II).

Table II *Patients with repeatedly abnormal Schilling tests not suffering from pernicious anaemia*

Patient no.	*Schilling (per cent.)*			*Hepatic uptake of $^{57}CoB_{12}$*	*Histamine test meal*
	Part I		*Part II**		
1	6	4	—	42	Normal
2	7	8	4	44	Normal
3	7	7	7	48	Normal
4	4	4	9	62	Normal
5	7	8	—	42	Normal

*plus intrinsic factor

The normal hepatic uptake in these five patients was in contrast to their low urinary excretion of the labelled vitamin B_{12} but this did not appear to be due to any obvious defect in the renal function (Table III).

Table III *Renal function in patients with repeatedly abnormal Schilling tests and normal hepatic uptake of $^{57}CoB_{12}$*

Patient no.	*Blood urea*	*Creatinine clearance*
1	29	108
2	22	99
3	26	116
4	30	101
5	41	90

The remaining three patients were all females (Table IV) with histamine-fast achlorhydria. Low Schilling tests, well within the pernicious anaemia range, were confirmed in two of the three patients by a low hepatic uptake; one patient died of pneumonia before this test could be performed.

(1) In the first patient a diagnosis of overt pernicious anaemia was based on megaloblastic marrow with a low serum vitamin B_{12}, a low Schilling test which improved with intrinsic factor, and the haemoglobin level responding to vitamin B_{12}. Both gastric parietal cell and intrinsic factor antibodies were present.

(2) The second patient had a low Schilling test, confirmed by a low hepatic uptake. The Schilling test returned to normal with intrinsic factor. There were no antibodies to intrinsic factor, although gastric parietal cell antibodies were demonstrable. Since the serum vitamin B_{12} was normal, this would appear to be a latent case of pernicious anaemia.

(3) The third patient had a low Schilling test on two occasions, confirmed by a low hepatic uptake. Serum vitamin B_{12} levels were between 120 and 130 pg./ml. The response to intrinsic factor was poor, however, suggesting the possibility of a malabsorption syndrome. Gastric biopsy showed mild atrophic gastritis, and d-xylose and faecal fat tests were normal, as were barium meal and follow-through *x* rays. On balance, this would appear to be another case of latent pernicious anaemia.

Discussion

The results of this study show that, of the 100 patients investigated, three showed malabsorption of vitamin B_{12} with results that lay well within the range expected in pernicious anaemia. In the remaining 97 patients, vitamin B_{12} absorption was entirely normal, with no patients showing results in the intermediate range—seen in patients with malabsorption syndrome or in those who had had partial gastrectomies or had a strong family history of pernicious anaemia. There was thus no suggestion of a sizeable group of patients in whom the vitamin

Table IV *Clinical particulars of three patients with a possible diagnosis of overt or latent pernicious anaemia*

Patient no.	*1*	*2*	*3*
Age (yrs)	59	74	57
Hb (g. per cent.)	9·1	12·4	10·9
Schilling (per cent.)	2	2	2; 2
Schilling + intrinsic factor (per cent.)	7	16	4
Hepatic uptake $^{57}CoB_{12}$		18	2
Histamine test meal	Achlorhydria	Achlorhydria	Achlorhydria
Serum vitamin B_{12} (pg./ml.)	28	265	120
Sternal marrow	Megaloblastic		
Barium test meal	Normal	Normal	Normal
Hb response vitamin to B_{12} (g. per cent.)	9 to 13	None	None
Parietal cell antibody	+	+	—
Intrinsic factor antibody	++	—	—

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B_{12} absorption had become subnormal though not yet within the pernicious anaemia range. This is in contrast to the expected high incidence of individuals with some degree of malabsorption of vitamin B_{12} which might be expected in a population with a high incidence of overt pernicious anaemia.

Of the three patients with confirmed malabsorption of vitamin B_{12}, one was an overt case of pernicious anaemia. The reported incidence of pernicious anaemia in south-east England is 0·85 per 1,000 (Scott, 1960). However, most patients with the disease are over 40 years of age and many authors report a predominance of women patients, so that the incidence in a predominantly female population, the majority of whom were over 40 years of age, could be expected to be several times higher than this. Mosbech (1952) reported that the incidence of this disease in Denmark in persons over age 40 varied between 1·7 per 1,000 (males: 50–59 years) and 4·4 per 1,000 (females: 70–79 years). Our finding of only a single case in 100 patients with rheumatoid arthritis does not therefore lend significant support to the view that pernicious anaemia is more common in patients with this disease. Our observations are also supported by those of Carter, Ardeman, Winocour, Perry, and Chanarin (1968), who found only one patient with pernicious anaemia among 92 rheumatoid arthritics screened by serum vitamin B_{12} measurements and tests for intrinsic factor and parietal cell antibody. They also reported that the incidence of gastric parietal cell antibody in their patients did not differ significantly from that expected in patients of similar age and sex.

In considering the incidence of latent pernicious anaemia in this series (those patients with definite malabsorption of vitamin B_{12} associated with histamine-fast achlorhydria but without overt megaloblastic change), comparison of our figures with other series presents some difficulties. McIntyre, Hahn, Conley, and Glass (1959) found only one malabsorber in 97 normal subjects, but only twenty of these were over 40 years of age. Witts (1960), basing his estimates on reported percentages of normal persons who have achlorhydria and the percentage of achlorhydrics showing malabsorption of vitamin B_{12}, has suggested that about 5 per cent. of the population over the age of 40 years might be expected to show some degree of malabsorption of the vitamin. The percentage in a largely female population might well be higher still, and, on this basis, the present study certainly does not suggest any increase in latent malabsorption of vitamin B_{12} in rheumatoid arthritis.

Finally, it may be considered doubtful whether the overall incidence of megaloblastic anaemia of any type is increased in rheumatoid arthritis. In the series of Partridge and Duthie (1963), the incidence of megaloblastic anaemia was 13·8 per 1,000, and it seems likely that their patients included a considerable number suffering from megaloblastic anaemia due to folic acid deficiency, a condition which is not uncommon in patients with rheumatoid arthritis (Gough, McCarthy, Read, Mollin, and Waters, (1964). Scott (1960) reported an incidence of pernicious anaemia in the Edinburgh region of two per 1,000, and Davis and Brown (1953) reported that in Glasgow the disease was three times as common among their female patients as in males. The expected incidence of pernicious anaemia alone in an elderly, predominantly female, Scottish population might thus be almost the same as the overall incidence of megaloblastic anaemia due to both vitamin B_{12} and folic acid deficiency as reported by Partridge and Duthie in their rheumatoid arthritis patients.

Summary

Of 100 patients with rheumatoid arthritis, impaired absorption of vitamin B_{12} was found in only three cases. A previous report has suggested that the incidence of pernicious anaemia in rheumatoid arthritis is five times higher than in patients with other musculoskeletal disorders. The results of the present study do not support this suggestion.

We wish to express our thanks to Dr. Ralph Wright, Nuffield Department of Clinical Medicine, University of Oxford, for the antibody studies and to Sister Olive M. Davies, whose supervision of accurate 24-hour urine collections made the investigation possible.

References

Buchanan, W. W., Cox, A. G., Harden, R. McG., Glen, A. I. M., Anderson, J. R., and Gray, K. G. (1966) *Gut*, 7, 351 (Gastric studies in Sjögren's syndrome).

Carter, M. E., Ardeman, S., Winocour, V., Perry, J., and Chanarin, I. (1968) *Ann. rheum. Dis.*, 27, 454 (Rheumatoid arthritis and pernicious anaemia).

Couchman, K. G., Bieder, L., Wigley, R. D., and Glenday, A. G. (1968) *N.Z. med. J.*, 68, 153 (Vitamin B_{12} absorption and gastric antibodies in rheumatoid arthritis).

Davis, L. J., and Brown, A. (1953) 'The Megaloblastic Anaemias', Blackwell, Oxford.

Glass, G. B. J., Boyd, L. J., Gellin, G. A., and Stephanson, L. (1954) *Arch. Biochem. Biophys.*, 51, 251 (Uptake of radioactive vitamin B_{12} by the liver in humans: test for measurement of intestinal absorption of vitamin B_{12} and intrinsic factor activity).

Gough, K. R., McCarthy, C., Read, A. E., Mollin, D. L., and Waters, A. H. (1964) *Brit. med. J.*, 1, 212 (Folic-acid deficiency in rheumatoid arthritis).

McIntyre, P. A., Hahn, R., Conley, C. L., and Glass, B. (1959) *Bull. Johns Hopk. Hosp.*, 104, 309 (Genetic factors in predisposition to pernicious anaemia).

Mosbech, J. (1952) *Acta med. scand.*, 141, 433 (Incidence of pernicious anaemia).

Partridge, R. E. H., and Duthie, J. J. R. (1963) *Brit. med. J.*, 1, 69 (Incidence of macrocytic anaemia in rheumatoid arthritis).

Schilling, R. F. (1953) *J. Lab. clin. med.*, 42, 860 (Intrinsic factor studies. II. The effect of gastric juice on the urinary excretion of radioactivity after the oral administration of radioactive vitamin B_{12}).

Scott, E. (1960) *J. Coll. gen. Pract.*, 3, 80 (Prevalence of pernicious anaemia in Great Britain).

Witts, L. J. (1960) *Acta haemat. (Basel)*, 24, 1 (The development of pernicious anaemia).

1987

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Intracellular Distribution of Radioactive Vitamin B_{12} in Rat Liver*

(Received for publication, March 31, 1971)

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SUMMARY

When radioactive cyanocobalamin (CN-[^{57}Co]B_{12}) was administered to rats by intracardiac injection, 98% of the CN-[^{57}Co]B_{12} bound to transcobalamin II immediately upon injection and was transported to the tissues in this form. The radioactive compounds extracted from liver 20 min and 72 hours after the administration of CN-[^{57}Co]B_{12} were identified either as CN-B_{12} or as a derivative of CN-B_{12}. Five minutes after injection of CN-[^{57}Co]B_{12}, 46% of the radioactivity of the liver sedimented with the microsomal fraction (P). The radioactivity in this P was associated primarily with vesicles containing 5′-nucleotidase (marker enzyme for plasma membranes) rather than glucose 6-phosphatase (marker enzyme for endoplasmic reticulum). The amount of radioactivity in P then decreased progressively to 9% of the total liver radioactivity by 72 hours, whereas the amount of radioactivity in the heavy mitochondrial and soluble fractions of the liver increased to 33% and 32%, respectively, at 72 hours. Highly purified lysosomal fractions contained little radioactivity 5 min and 72 hours after the injection of CN-[^{57}Co]B_{12}; however, at intermediate times these lysosomal fractions contained significant amounts of radioactivity (15% of the total liver radioactivity at 30 min postinjection). Approximately 80% of the radioactivity in these lysosomal fractions was present as CN-[^{57}Co]B_{12}. Mitochrondial fractions containing approximately 10% of the 5′-nucleotidase of the liver were fractionated on sucrose gradients, and the preliminary data suggest that a portion of this 5′-nucleotidase may be associated with lysosomes.

In mammals, vitamin B_{12} is transported in serum bound to specific proteins, certain of which have been shown to enhance the uptake of the vitamin by peripheral tissues (2, 3). Human serum contains two vitamin B_{12}-binding proteins, transcobalamin I and II (4). Transcobalamin I has a molecular weight of approximately 120,000 (5), the electrophoretic mobility of an α-globulin (6, 7), and binds most of the endogenous B_{12}[1] of serum (4, 8). The other binder, TC-II, has a molecular weight of approximately 35,000 (5, 9), the electrophoretic mobility of a β-globulin (10), and binds most of the B_{12} added to serum *in vitro* (11). When a physiological dose of B_{12} is given either orally or parenterally, the vitamin first appears in the serum bound to TC-II (4) which has been shown to facilitate the uptake of B_{12} by HeLa cells, human amnion cells, and human kidney cells (12). A recent study by Finkler, Green, and Hall (13) demonstrated that antibodies against TC-II will inhibit the uptake of B_{12} by HeLa cells in the presence of human serum. The proteins from rodent serum which bind B_{12} have not been as extensively studied; however, when B_{12} is added to mouse serum *in vitro*, it binds exclusively to a protein with a molecular weight and an electrophoretic mobility similar to those values for TC-II (14). Mouse serum and ascites fluid have also been shown to enhance the uptake of B_{12} by Ehrlich ascites carcinoma cells and Sarcoma 180 ascites cells (15-17). Rat serum contains a B_{12}-binding protein with a molecular weight of 35,000 and it has been demonstrated that, following the administration of an oral dose of B_{12} to rats, the vitamin first appears in the serum bound to this protein (18). By the use of inhibitors of protein synthesis, Tan and Hansen (19) demonstrated that mouse TC-II disappeared from serum along with B_{12} following the injection of the vitamin. Tan (14) suggested two possible explanations for this observation: (*a*) the TC-II-B_{12} complex binds to the cell membrane and then is destroyed upon releasing the B_{12} into the cell, or (*b*) the intact TC-II-B_{12} complex is absorbed into the cell where the TC-II is destroyed, perhaps by the lysosomes.

The intracellular distribution of B_{12} in mammalian tissues has been studied by a number of investigators. Some investigators have used microbiological techniques to measure endogenous B_{12} levels in subcellular fractions of livers from rats and mice (20-22), while other investigators have injected radioactive B_{12} into rats and determined the intracellular distribution of the radioactive vitamin in liver and kidney (21-24). The data

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[1] The abbreviations used are: B_{12}, vitamin B_{12}; TC-I and TC-II, transcobalamin I and II; CN-B_{12}, cyanocobalamin; HO-B_{12}, hydroxycobalamin; Ad-B_{12}, 5′-deoxyadenosylcobamide coenzyme; CH_3-B_{12}, methylcobalamin; E, cytoplasmic extract (postnuclear supernatant); N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, supernatant fraction.

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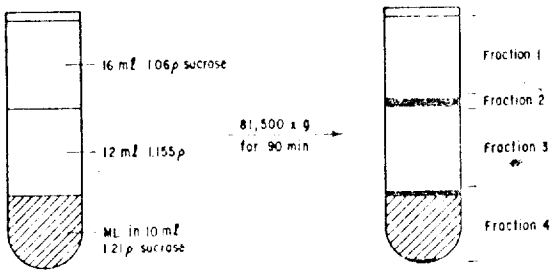

FIG. 1. Collection of fractions separated on discontinuous sucrose gradients. After centrifugation, 1.30 density sucrose was introduced into the bottom of the tube and the fractions were collected from the top of the tube. Highly purified lysosomes collect in Fraction 2 at the interface between 1.06 and 1.155 density sucrose.

from these investigations suggested that after absorption, B_{12} first concentrated in the soluble and nuclear fractions of the liver cell and then moved into the mitochondrial fraction at later times. Only a small fraction of the vitamin was found in the microsomal fraction. In contrast to these data, Tantengco *et al.* (25) reported that the microsomal and soluble fractions contained large amounts of radioactivity 15 min after the injection of CN-[^{60}Co]B_{12}. Two and 6 days after the injection of CN-[^{60}Co]B_{12}, the radioactivity had shifted from the microsomal fraction into the mitochondrial and nuclear fractions while decreasing only slightly in the soluble fraction. These previous experiments were performed using fractionation techniques which have been improved recently; furthermore, neither marker enzyme nor morphological studies were performed to determine the purity of the subcellular fractions. Methods are now available which make it possible to prepare highly purified lysosomes and mitochondria (26, 27), to further subfractionate the microsomes (28), and to isolate plasma membrane fragments (29, 30). This paper describes experiments using these improved fractionation techniques and marker enzyme studies in which the changes in the intracellular distribution of radioactive B_{12} in the livers of rats were followed at times ranging from 5 min to 72 hours after an intravenous injection of physiological doses of CN-[^{57}Co]B_{12}.

EXPERIMENTAL PROCEDURE

Animals—Female Charles River rats, weighing 200 to 250 g, were used in all experiments. All rats received Purina rat chow and water *ad libitum* until they were fasted overnight before being killed at approximately 9:00 a.m. Triton WR-1339 (Ruger Chemical Company, Irvington-on-Hudson, New York) was injected intraperitoneally at a dosage of 85 mg/100 g body wt, 3½ days before the animals were killed. Approximately 0.5 μCi of CN-[^{57}Co]B_{12} (2 to 4 ng of cyanocobalamin) in 0.9% NaCl solution was given in all experiments by intracardial injection.

Cell Fractionation—The animals were killed by decapitation. Their livers were quickly removed and weighed in beakers containing ice-cold 0.25 M sucrose. The livers were minced finely and then homogenized in 0.25 M sucrose using a smooth-walled glass homogenizer fitted with a Teflon pestle (A. H. Thomas Company, Philadelphia) driven at about 1,000 rpm by a power drill. The homogenates were fractionated into an N, an M, an L, a P, and an S by differential centrifugation according to de Duve *et al.* (31). The nuclear pellets were collected in a model PR-2 International refrigerated centrifuge using head No. 268. All other centrifugation procedures were done in either a model L3-50 or a L2-65B Spinco preparative ultracentrifuge. Highly purified lysosomes were prepared by isopycnic centrifugation from the livers of rats that had received Triton WR-1339 by the method of Trouet (26) as described by Leighton *et al.* (27). The livers were homogenized in 0.25 M sucrose and a cytoplasmic extract (postnuclear supernatant) was prepared (31). A combined ML was sedimented by subjecting the cytoplasmic extract to an integrated force of either 250,000 *g* min[2] in the No. 40 rotor or 350,000 *g* min in the No. 50.1 rotor. The ML pellet was washed once by resuspension in 0.25 M sucrose followed by resedimentation under the same conditions. The ML pellet was gently homogenized in 1.21 density sucrose in a Dounce homogenizer (Kontes Glass Company, Vineland, New Jersey) and 10 ml of the suspension were then layered in the bottom of a centrifuge tube for the SW 27 rotor. Layers of 1.15 density sucrose (12 ml) and 1.06 density sucrose (16 ml) were carefully pipetted on top and then the tube was centrifuged at 25,000 rpm for 90 min. Following centrifugation, four fractions (Fig. 1) were removed from the tube through a collecting cone fitted on top of the tube (27). The fractions were collected in graduated cylinders and their volumes recorded. Plasma membrane fractions, relatively free of other cellular constituents, were prepared from microsomal pellets by the method of Touster *et al.* (30). Briefly, the microsomal pellet from 5 g of liver was suspended by vigorous homogenization in 10 ml of 1.28 density sucrose and layered in the bottom of an SW 27 centrifuge tube. Sixteen milliliters of 1.15 density sucrose and 12 ml of 1.06 density sucrose were layered on top and then the tube was centrifuged for 16 hours at 25,000 rpm. Sucrose solutions used for the preparation of plasma membrane fragments contained 0.005 M Tris and were adjusted to a pH of 8.0 with HCl. All tissue fractionation procedures were done in the cold.

Enzyme Assays—Glucose 6-phosphatase was assayed according to de Duve *et al.* (31), and acid phosphatase was measured with β-glycerophosphate as substrate (32). Glucose 6-phosphate is hydrolyzed by both the specific glucose 6-phosphatase and the nonspecific acid phosphatase under the assay conditions; however, all glucose 6-phosphatase levels reported in this paper are corrected levels from which the contribution of acid phosphatase has been eliminated by the method of de Duve *et al.* (33). 5′-Nucleotidase was determined according to the procedure of Michell and Hawthorne (34) with AMP as substrate in the presence of 10 mM tartrate, which inhibits lysosomal acid phosphatase (35) as well as the lysosomal acid nucleotidase described by Arsenis and Touster (36). Cytochrome oxidase was measured at room temperature by the method of Cooperstein and Lazarow (37). Inorganic phosphate was determined by the method of Fiske and SubbaRow (38), and protein by the method of Lowry *et al.* (39) with bovine serum albumin as standard. All optical density measurements were made using the Gilford model 2400 spectrophotometer.

Measurement of Radioactivity—Radioactivity was measured in an Auto-Gamma spectrometer (model No. 5313, Packard Instrument Company) equipped with a sodium iodide crystal (3 × 3 inch). All samples from the fractionation experiments

[2] Centrifugation conditions are expressed in an integrated form so that forces developed during acceleration and deceleration are included. See Leighton *et al.* (27) for details of methods used to calculate *g* min.

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were counted for a sufficient period to give a standard error of less than 2%.

Paper Chromatography—All chromatography was done in the descending direction at room temperature in the dark using either Whatman No. 3MM chromatography paper or Amberlite cation exchange paper, grade WA-2 (Reeve Angel, Clifton, New Jersey). Methanol-2-butanol-0.1 M acetate buffer, pH 5.5 (1:4:12, v/v), was used as developing solvent for the ion exchange paper and water-saturated 2-butanol for the No. 3MM paper. Following chromatography of radioactive samples, the chromatograms were cut into strips (0.5 × 2 cm) which were then transferred to counting vials and counted for 10 min.

Extraction of [^{57}Co]-Vitamin B_{12} from Liver—Livers from three fasted rats that had received CN-[^{57}Co]B_{12} were homogenized at room-temperature in 3 volumes of absolute ethanol for 4 min at full speed in a Sorvall Omni-Mixer. Additional ethanol and H_2O were added to make a 1:10 (w/v) homogenate which was 80% with respect to ethanol. The homogenate was heated with stirring at 75-80° for 1 hour, filtered, and the residue re-extracted with 100 ml of 80% ethanol (v/v) for 1 hour at 75-80°. After filtering, the alcoholic extracts were combined and extracted twice with equal volumes of *n*-hexane. The ethanol was removed in a flash evaporator and insoluble material was then separated from the aqueous phase by centrifugation at 65,000 rpm for 90 min. The supernatant was decanted, the pellet was resuspended in water, and the suspension was then recentrifuged. The supernatants were combined and applied to a column (1.7 × 15 cm) of Dowex 1-X8 (hydroxyl form) which had been equilibrated with H_2O. The radioactivity was then eluted with H_2O. The radioactive effluent was extracted twice with an equal volume of diethyl ether and lyophilized. The dry residue was then dissolved in the smallest possible volume of H_2O for paper chromatography. All extraction procedures were done in a dark room.

Chemicals—Dipotassium glucose 6-phosphate, cytochrome *c* (type VI), disodium β-glycerophosphate (grade I), adenosine 5′-monophosphate (type II), Triton X-100, cyanocobalamin, and bovine serum albumin were purchased from Sigma Chemical Company. The 5′-deoxyadenosyl coenzyme B_{12} was obtained from E. R. Squibb and Sons and methylcobalamin was synthesized from CN-B_{12} by the method of Smith *et al.* (40) as outlined by Weissbach *et al.* (41). All other chemicals were reagent grade. Radioactive vitamin B_{12} (CN-[^{57}Co]B_{12}; 71 to 173 mCi per mg) of high specific activity was purchased from Philips-Duphar, Petten, Holland. The B_{12} content of the CN-[^{57}Co]B_{12} preparation was determined microbiologically using *Lactobacillus leichmannii* (42) with CN-B_{12} as standard. When the CN-[^{57}Co]B_{12} was mixed with carrier CN-B_{12} (0.1%) and chromatographed on Whatman No. 3MM paper, 95% of the radioactivity had the same R_F value as the CN-B_{12}; however, when no carrier was added, approximately 15% of the radioactivity did not chromatograph as CN-B_{12}. The significance of this minor radioactive component is not known; however, its presence under these chromatographic conditions did not interfere with the interpretation of the data (see "Results").

RESULTS

Radioactivity of Liver following Injection of [^{57}Co]Cyanocobalamin—CN-[^{57}Co]B_{12} (2 to 4 ng) was administered to rats weighing 200 g, the serum of which contained sufficient TC-II to bind approximately 4 ng of B_{12} per ml of serum (18). To verify the assumption that all of the radioactive vitamin would become bound to TC-II immediately upon injection, 4 ng of CN-[^{57}Co]B_{12} were injected intracardially and then blood withdrawn within 15 sec. The serum was separated and chromatographed on Sephadex G-100. Of the total radioactivity, 98% chromatographed with a protein having a molecular weight of approximately 35,000 (TC-II). The radioactive vitamin was removed from the circulation rapidly since the blood contained only 8% of the injected radioactivity 5 min after the injection of CN-[^{57}Co]B_{12} and less than 2% by 1 hour. The liver contained 10% of the injected radioactivity 5 min after the injection of CN-[^{57}Co]B_{12} and there was no significant change in the amount of radioactivity in the liver over the 72-hour period; therefore, the livers contained between 0.2 and 0.4 ng of the radioactive vitamin.

Chromatography of Radioactive Material Extracted from Liver—Radioactive compounds were extracted from the livers of rats at 5 min and 72 hours after the injection of CN-[^{57}Co]B_{12} (see "Materials and Methods") and prepared for paper chromatography. The extraction procedure was quite satisfactory since 93% of the radioactivity in the original homogenate was recovered in the final aqueous extract. Fig. 2 illustrates the results obtained when these extracts of liver were chromatographed on paper. Approximately 70% of the radioactivity in an extract prepared 20 min after the injection of CN-[^{57}Co]B_{12} chromatographed with either CN-B_{12} or HO-B_{12} on Whatman No. 3MM paper. A radioactive component which did not chromatograph with any of the standards accounted for about 16% of the radioactivity in the liver extract. This compound has not been identified; however, it did chromatograph in the same position as the minor radioactive component in the injected CN-[^{57}Co]B_{12} when the latter was chromatographed in the absence of carrier CN-B_{12}. Carrier CN-B_{12} was never added to the liver extracts before chromatography. The results on ion exchange paper were similar except that both the HO-B_{12} and the unidentified component remained at the origin. In all cases a small amount of radioactivity was spread diffusely along the entire chromatogram. By 72 hours, 55% of the radioactivity in the extract chromatographed with Ad-B_{12} and another 35% with HO-B_{12}, CH_3-B_{12}, and CN-B_{12}. Very little radioactive CN-B_{12} was present in the 72-hour extract, and the unidentified radioactive component in the 20-min extract was missing from the 72-hour extract. These chromatograms demonstrated that most of the radioactivity in liver following the injection of CN-[^{57}Co]B_{12} was present either as CN-B_{12}, HO-B_{12}, or Ad-B_{12}. Furthermore, the percentage of the unidentified radioactive component in the 20-min liver extract was the same as the percentage of that component in the injected CN-[^{57}Co]B_{12}, indicating that this material and CN-[^{57}Co]B_{12} were taken up by the liver at the same rate. This might be expected, since 98% of the radioactivity in the injected CN-[^{57}Co]B_{12} bound to rat TC-II *in vivo* and *in vitro*. These data plus data to be presented concerning the identity of the radioactive compounds in lysosomal fractions (see below) justify the use of ^{57}Co as a tracer for CN-B_{12} and its biologically important derivatives in this study.

Distribution of Radioactivity and Marker Enzymes in Subcellular Fractions—In all fractionation experiments, each of the subcellular fractions was assayed for cytochrome oxidase (marker enzyme for mitochondria), glucose 6-phosphatase (marker enzyme for microsomes), acid phosphatase (marker enzyme for lysosomes), and protein. The intracellular distribution of

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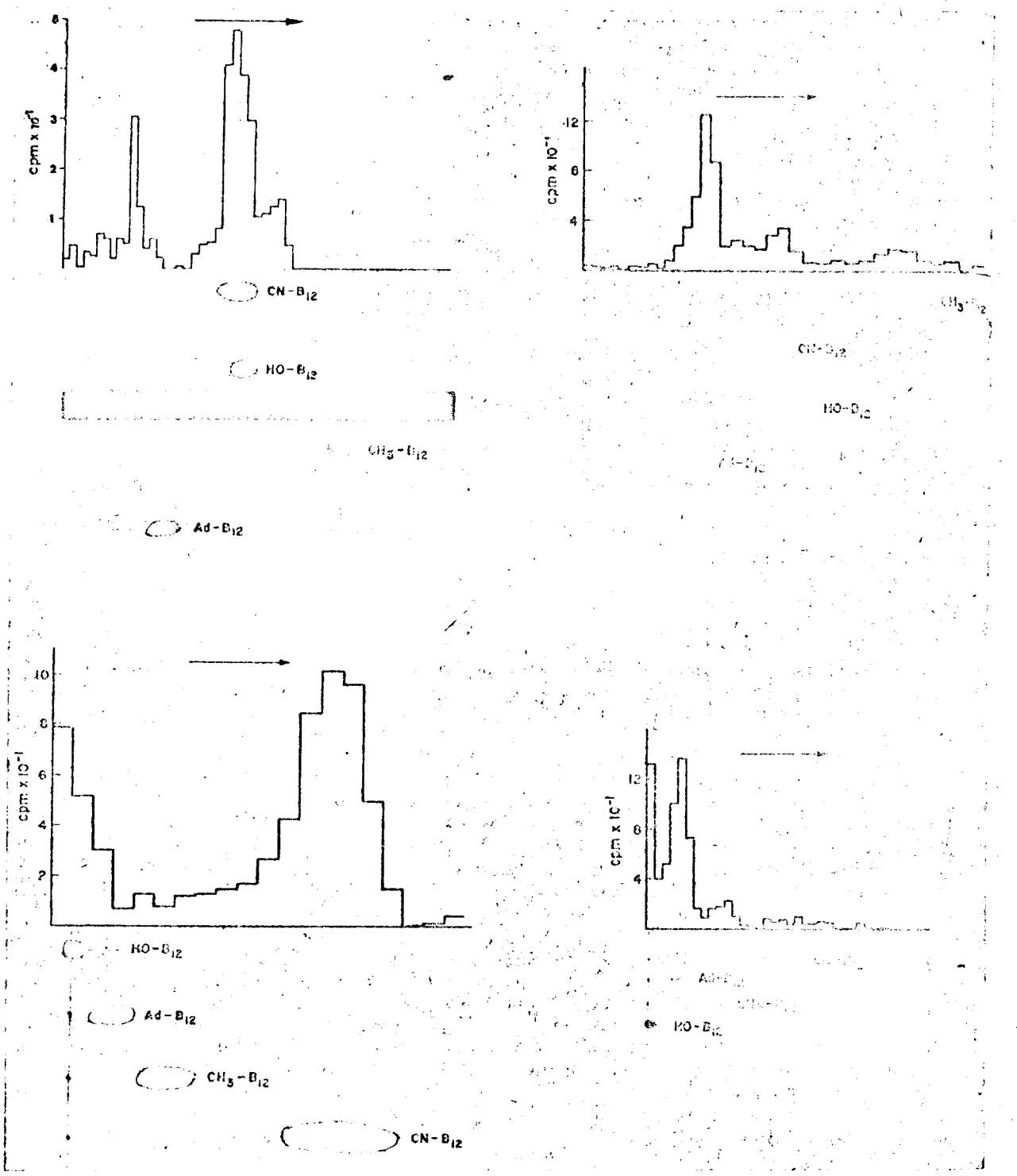

FIG. 2. Chromatography on paper of radioactive products extracted from rat liver. The radioactive sample was streaked across paper and spots of standards then applied. *Arrows* indicate direction of solvent flow. *Upper left*, extract was prepared from the livers of rats killed 20 min after receiving CN-[^{57}Co]B_{12} and was chromatographed on Whatman No. 3MM paper; *lower left*, same as upper left except that extract was chromatographed on Amberlite cation exchange paper; *upper right*, extract was prepared from the livers of rats killed 72 hours after receiving CN-[^{57}Co]B_{12} and chromatographed on Whatman No. 3MM paper; *lower right*, same as upper right except that extract was chromatographed on cation exchange paper.

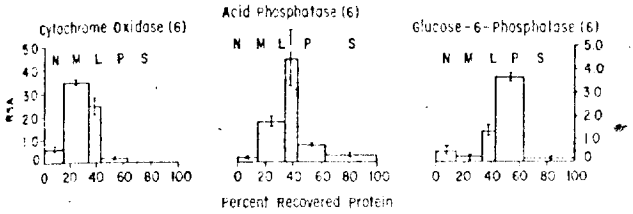

FIG. 3. Distribution profiles of enzymes. The mean relative specific activities (*RSA*) (ratio of percentage of enzyme in fraction to percentage of protein in fraction) ± standard deviations for six experiments are plotted on the *ordinate* and the mean percentage of total protein in each fraction on the *abscissa*. Recovered protein equals the sum of protein values for N, M, L, P, and S. The recovery of protein and of enzymes in these experiments ranged from 94% to 98%, where recovery is defined as the amount of protein or enzyme in N + M + L + P + S divided by the amount of protein or enzyme in E + N.

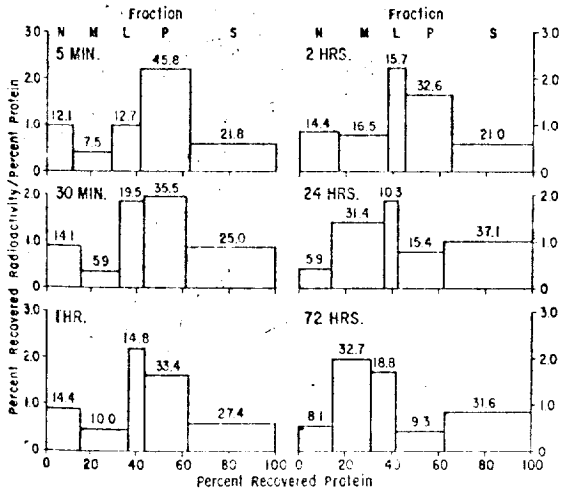

FIG. 4. Distribution profiles of radioactivity in the subcellular fractions of the liver at various times after the intravascular injection of CN-[^{57}Co]B_{12}. Data plotted as in Fig. 3. The *numbers above each block* indicate the percentage of total liver radioactivity in that fraction at the time indicated.

CN-[^{57}Co]B_{12} was studied at six different times after the injection of the radioactive vitamin and a summary of the data concerning enzyme distributions for these experiments is shown in Fig. 3. The enzymatic composition of the subcellular fractions was similar in all experiments; therefore, the amount of radioactivity in the fractions at various times after injection of CN-[^{57}Co]B_{12} can be meaningfully compared. The data concerning the distribution of radioactivity in the various subcellular fractions are shown in Fig. 4. Five min after the injection of CN-[^{57}Co]B_{12}, 46% of the radioactivity of the liver had accumulated in P which had a relative specific activity (ratio of percentage of radioactivity to percentage of protein) of 2.3. The radioactivity in P decreased with time until at 72 hours this fraction contained only 9% of the radioactivity of the liver. The radioactivity of M changed in the opposite direction increasing from 8% at 5 min to 33% at 72 hours. This is in agreement with previous studies which showed that the mitochondria of liver accumulated radioactivity following the administration of radioactive B_{12} (21, 22). The radioactivity in the supernatant fraction was relatively constant for the first 2 hours and then increased by 50% over the next 70 hours. There was little change in the amount of radioactivity in N during the time interval investigated. The percentage of total liver radioactivity in L fluctuated between 10.3 and 19.5 during the entire 72-hour period; however, the relative specific activity of the fraction increased from a low of 1.0 at 5 min to a maximum of 2.2 at 2 hours. By 24 and 72 hours the relative specific activity had decreased to 1.87 and 1.72, respectively. A part of these differences in relative specific activity may result from the slightly different amounts of protein in L pellets isolated at the various times (Fig. 4). Those fractions with a higher protein content were also higher in cytochrome oxidase activity indicating an increased number of mitochondria in these fractions. This increased number of mitochondria may contribute to the lower relative specific activity of L at 5 and 30 min when the mitochondria contained little radioactivity and probably explains the 18.8% of total liver radioactivity in L at 72 hours when the mitochondria are highly labeled. The amount of protein in L does not explain all the changes in relative specific activity since changes in protein and relative specific activity were not parallel at all times. These experiments suggested that some particles found in L (perhaps lysosomes)

TABLE I

Intracellular distribution of enzymes in livers of rats pretreated with Triton WR-1339

All values represent means ± standard deviations. Recovery = (N + ML + PS)/(E + N). See "Materials and Methods" for description of fractions and for details of fractionation procedure.

Enzyme	No. of experiments	E + N	N + ML + PS	N	ML	PS	Recovery
		units[a]	%	%	%	%	%
Protein	12	224 ± 17	100	13.9 ± 1.3	27.5 ± 1.7	58.6 ± 1.9	93.4 ± 2.5
Cytochrome oxidase	12	19.6 ± 2.9	100	7.8 ± 2.2	89.8 ± 2.6	2.4 ± 1.7	80.3 ± 8.9
Acid phosphatase (total activity)	12	7.4 ± 1.1	100	2.5 ± 0.9	47.5 ± 7.5	50.1 ± 7.8	95.4 ± 5.3
Glucose 6-phosphatase	12	17.2 ± 3.6	100	5.3 ± 2.9	10.4 ± 2.7	84.3 ± 4.5	95.6 ± 7.2
5′-Nucleotidase	3	8.3 ± 0.7	100	17.9 ± 1.0	7.8 ± 1.0	74.3 ± 2.6	91.8 ± 3.2

[a] Units are expressed in units per g of liver for enzymes and in milligrams per g of liver for protein. See de Duve *et al.* (31) for definition of units of all enzymes except 5′-nucleotidase. One unit of 5′-nucleotidase is defined as the amount of enzyme which will hydrolyze 1 μmole of substrate per min.

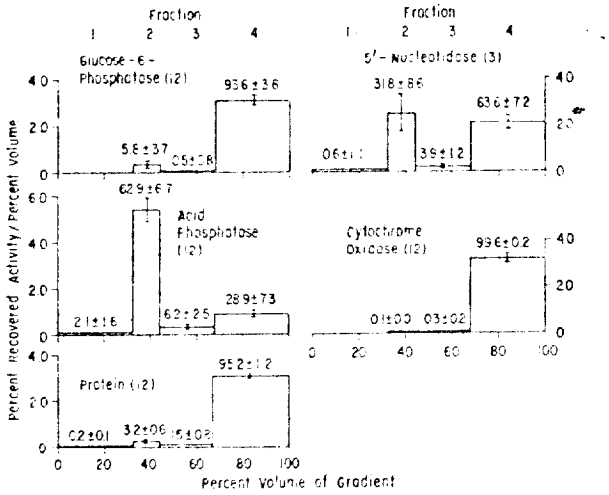

FIG. 5. Distribution profiles of enzymes and protein in gradient fractions. The *numbers above each block* indicate the means ± standard deviations for the percentage of total gradient activity in each fraction while the *values in parentheses* designate the number of experiments. The means ± standard deviations for the percentage of gradient activity in each fraction divided by the percentage of the gradient volume occupied by that fraction are plotted on the *ordinate* against mean percentage volume on the *abscissa*.

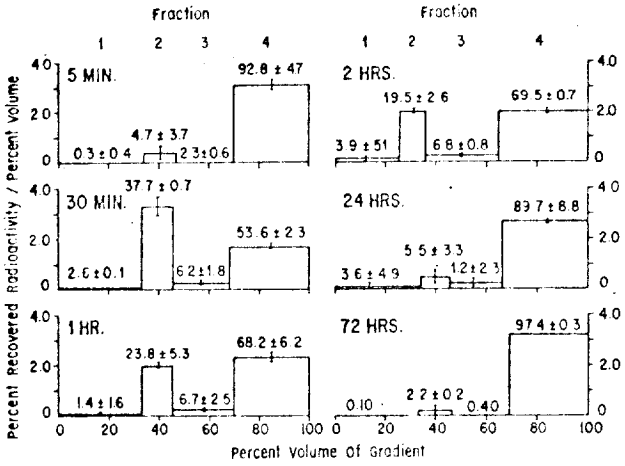

FIG. 6. Distribution profiles of radioactivity in the gradient fractions at various times after the injection of CN-[^{57}Co]B_{12}. Two experiments were done at each time and the data are plotted as in Fig. 5. *Values above each block* indicate the percentage of gradient radioactivity in that fraction at the time indicated. The MLs used for the gradients contained 4.9% of total liver radioactivity at 5 min, 17.2% at 30 min, 20.4% at 1 hour, 25.4% at 2 hours, 39.3% at 24 hours, and 57.2% at 72 hours.

may be involved in the intracellular metabolism of B_{12}; however, no conclusions were possible until highly purified lysosomal fractions were studied.

Purification of Lysosomes from Livers of Rats Receiving Triton WR-1339 and CN-[^{57}Co]Cobalamin—ML pellets were prepared from the livers of rats that had received both Triton WR-1339 and CN-[^{57}Co]B_{12}. Pretreatment of animals with Triton WR-1339 causes a selective decrease in the equilibrium density of liver lysosomes in sucrose gradients (26). This change in equilibrium density permits the separation of lysosomes from other subcellular particles found in ML by flotation through a discontinuous sucrose gradient. Fractionation experiments were done in duplicate at six times after the injection of CN-[^{57}Co]B_{12} and the data concerning enzyme studies on the initial fractions are given in Table I. The ML contained 47% of the liver acid phosphatase which was purified 1.7-fold over the homogenate. Approximately 90% of the glucose 6 phosphatase and 5′ nucleotidase of the liver was excluded from this fraction. The enzyme 5′-nucleotidase was used as a marker for plasma membrane fragments (43, 29). Fig. 5 shows the distribution of the marker enzymes in the four fractions separated from ML by density gradient centrifugation. Fraction 2, the lysosomal fraction, contained 30% of the liver acid phosphatase which was purified 34-fold over the original homogenate. Fraction 2 was virtually free of cytochrome oxidase (mitochondria), and although catalase (marker enzyme for peroxisomes) was not measured, it has been previously reported that lysosomal fractions prepared by this method are free of peroxisomes (27). Fraction 2 did contain 0.5% of the glucose 6-phosphatase and 2.5% of the 5′-nucleotidase of the liver. On the basis of this glucose 6-phosphatase activity, 10 to 20% of the protein in Fraction 2 was of microsomal origin. The source of 5′-nucleotidase in Fraction 2 is less clear since it could be due either to the contamination of the fraction with plasma membrane fragments or with intracellular vesicles derived from the plasma membrane which might also contain 5′-nucleotidase, *e.g.* pinocytotic vesicles. Some nucleotidase activity endogenous to lysosomes might also be responsible.

Fig. 6 shows the distribution of radioactivity in the gradient fractions. At 5 min, 93% of the radioactivity in the gradient was found in Fraction 4; however, this had decreased to 54% by 30 min when Fraction 2 contained 38% of the radioactivity. The amount of radioactivity in Fraction 2 then decreased with time until at 72 hours only 2% of the radioactivity of the gradient remained in Fraction 2, while the amount in Fraction 4 had again increased to a maximum of 97%. The data at 5 min and 72 hours are consistent with the results shown in Fig. 4 since the radioactivity was concentrated in P at 5 min and in M at 72 hours. Both glucose 6-phosphatase (microsomes) and cytochrome oxidase (mitochondria) are found mainly in Fraction 4, which should then contain most of the radioactivity of the gradient at these times. A comparison of the amounts of radioactivity and of glucose 6-phosphatase in Fractions 2 and 4 at 30 min, 1 hour, and 2 hours reveals that it is not possible to attribute all of the radioactivity in Fraction 2 at these times to the presence of microsomal contamination (6% of gradient glucose 6-phosphatase was found in Fraction 2 and 94% in Fraction 4) unless the fraction contained a special type of vesicle rich in glucose 6-phosphatase with sedimentation properties different from the sedimentation properties of the vesicles formed by the fragmentation of the major portion of the endoplasmic reticulum. No evidence for such a particle is now available. On the basis of the data presented, it is possible that the radioactivity in Fraction 2 may be associated with vesicles which contain 5′-nucleotidase and which are distinct from lysosomes since 32% of the 5′-nucleotidase in the gradient was found in this fraction. Of particular interest in this regard are plasma membrane fragments which might contain CN-[^{57}Co]B_{12} that had attached to the membrane during the absorptive process. Since vitamin B_{12} is rapidly absorbed by the liver, it seems likely that the

radioactivity in Fraction 2 would be highest at 5 min if the CN-[^{57}Co]B_{12} was associated with such membranes of plasma membrane origin.

These gradients were repeated using ML fractions prepared from the livers of rats that had not been pretreated with Triton WR-1339. The results are summarized in Table II. In these experiments, more than 95% of the acid phosphatase and 5′-nucleotidase of the gradient was found in Fraction 4 and less than 2% in Fraction 2. The simultaneous shift of acid phosphatase and 5′-nucleotidase from Fraction 4 into Fraction 2 following pretreatment with Triton WR-1339 indicates either that the two enzymatic activities are associated with the same particles (probably lysosomes) or that they are associated with two different particles whose densities are similarly influenced by preliminary treatment with Triton WR-1339. Wattiaux, Wiba, and Baudhuin (41) have presented data demonstrating that Triton WR-1339 first appears in the liver in pinocytotic vesicles which later fuse with pre-existing lysosomes. Pinocytotic vesicles filled with Triton WR-1339 might exhibit the sedimentation properties shown by 5′-nucleotidase in these experiments and might account not only for the 5′-nucleotidase in Fraction 2 but also for the radioactivity in that fraction. This does not seem likely since the animals used in this study were pretreated with Triton WR-1339 3½ days before the fractionations were done, and Wattiaux *et al.* (44) showed that the microsomal fraction of liver (differential centrifugation concentrates pinocytotic vesicles in this fraction) contained very little ^{131}I-labeled Triton WR-1339 4 days after intravenous administration of the labeled material. The behavior of radioactivity in these gradients was similar to that of acid phosphatase and 5′-nucleotidase; however, the percentage of gradient radioactivity in Fraction 2 at 30 min (10.6%) and 1 hour (9.1%) was greater than this percentage for the two enzymes. Nevertheless, the administration of Triton WR-1339 did cause a substantial shift of radioactivity from Fraction 4 into the lysosomal fraction (Fraction 2) at both 30 min (27.1% shift) and 1 hour (14.7% shift) (Fig. 6). On the basis of the enzyme distributions shown in Table II, it appears that some of the residual radioactivity in Fraction 2 was associated with a particle or material devoid of the enzymes studied or in which the enzymes had been inactivated. The possibility also exists that it was associated with a special particle containing only a small fraction of one of the enzymes and having unique sedimentation properties. The data available are not sufficient to identify the source of this residual radioactivity in Fraction 2.

Fractionation of Subcellular Particles on Continuous Sucrose Gradients. A modified MLP was separated from a homogenate of livers from rats that had not been injected with Triton WR-1339 but that had received CN-[^{57}Co]B_{12} 5 min before killing. The MLP pellet sedimented between 2×10^4 and 50×10^4 g-min in the 50.1 Spinco rotor and contained more microsomal material (52% of the glucose 6-phosphatase and 36% of the 5′-nucleotidase of the liver), less mitochondria (27% of the cytochrome oxidase), and about the same number of lysosomes (56% of the acid phosphatase) as a standard ML. The MLP pellet from 8.3 g of liver was suspended in 5 ml of 0.25 M sucrose and layered on top of a continuous sucrose gradient extending from a density of 1.15 to 1.25 in a SW 27 centrifuge tube. The tube was centrifuged for 3 hours at 25,000 rpm, then fractions of approximately 1.5 ml were collected into tared tubes, weighed, and their densities measured using a Bausch and Lomb refractometer. Exact volumes for the fractions were calculated from these data. An L prepared from the livers of rats 1 hour after the injection of CN-[^{57}Co]B_{12} was analyzed by the same method except that the density limits on the gradient were 1.15 to 1.27. The results of these experiments are shown in Fig. 7. A fraction rich in microsomal material was used at 5 min since earlier experiments had shown that radioactivity was concentrated in the microsomal fraction at this time. An L which contained very little microsomal material (5% of the 5′-nucleotidase of liver) and a limited number of mitochondria was used at 1 hour to facilitate the study of lysosomal radioactivity. At 5 min (Fig. 7*A*) the distribution of radioactivity was nearly identical with that of 5′-nucleotidase, demonstrating that the radioactivity was associated either with fragments of the plasma membrane or with vesicles derived from the plasma membrane. Analysis of the 1-hour gradient using L (Fig. 7*B*) revealed that slightly more than 50% of the radioactivity was present in fractions with densities greater than 1.19. Since lysosomes and peroxisomes are the only subcellular particles of liver that equilibrate at such high densities in sucrose gradients (27), and since peroxisomes have been eliminated as a site of CN-[^{57}Co]B_{12} accumulation, these data provided further evidence for the lysosomal localization of significant amounts of radioactivity at intermediate times after the injection of CN-[^{57}Co]B_{12}. The distribution of 5′-nucleotidase in the 1-hour gradient was different than that

TABLE II

Fractionation of combined heavy plus light mitochondrial fractions from livers of rats not pretreated with Triton WR-1339 on a discontinuous sucrose gradient

The discontinuous sucrose gradient used was the same as the gradient used for the fractionation of MLs from the livers of rats pretreated with Triton WR-1339 (Figs. 5 and 6). Fractionation experiments were done at three different times after the injection of CN-[^{57}Co]B_{12} and the fractions were collected as illustrated in Fig. 1. A summary of the data concerning the distribution of the marker enzymes for the three experiments is shown in the upper portion of the table and the data concerning the distribution of the radioactivity at the various times after the injection of CN-[^{57}Co]B_{12} in the lower portion of the table.

Enzyme[a]	Fraction 1	Fraction 2	Fraction 3	Fraction 4
	%	%	%	%
Cytochrome oxidase	0	0.1 ± 0.0	0.1 ± 0.1	99.8 ± 0.2
Acid phosphatase	0.1 ± 0.0	1.5 ± 0.5	0.8 ± 0.2	97.6 ± 0.6
Glucose 6 phosphatase	0.1 ± 0.1	0.7 ± 0.6	0.8 ± 0.4	98.5 ± 1.1
5′-Nucleotidase	0.7 ± 0.9	1.2 ± 0.6	2.1 ± 0.3	95.9 ± 1.7
Protein	0.1 ± 0.0	0.5 ± 0.1	0.8 ± 0.2	98.6 ± 0.4
Radioactivity[b]				
30 min post CN-[^{57}Co]B_{12}	0	10.6	2.1	87.3
1 hour post CN-[^{57}Co]B_{12}	0.6	9.1	3.0	87.3
72 hours post CN-[^{57}CO]B_{12}	0	0.5	0.1	99.4

[a] All values are reported as percentage of gradient activity in each fraction ± standard deviation.

[b] All values are reported as percentage of gradient radioactivity in each fraction at the time indicated.

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in the 5-min gradient, and, in fact, resembled the distribution of radioactivity and acid phosphatase. This most likely reflects the fact that microsomal 5′-nucleotidase, *i.e.* 5′-nucleotidase associated with plasma membrane fragments, predominated in the 5-min gradients, whereas most of these microsomal elements had been excluded from the L used for the 1-hour gradient. That fraction of the 5′-nucleotidase in the L which equilibrated around a density of 1.21 was probably associated with lysosomes since the 5′-nucleotidase which sediments in the microsomal fraction has been shown to have a median equilibrium density of 1.14 in sucrose gradients by Thines-Sempoux *et al.* (45). These authors demonstrated that fractions having a density greater than 1.19 contained virtually no plasma membrane fragments; therefore, neither the 5′-nucleotidase nor the radioactivity which peaked around a density of 1.21 when L was fractionated on the continuous gradient can be associated with plasma membrane fragments. Although tartrate was included in the assay mixture for 5′-nucleotidase to inhibit acid phosphatase (35) and lysosomal acid nucleotidase (36), it is possible that either another lysosomal phosphatase or a residual activity of one of these enzymes was responsible for the hydrolysis of AMP by these fractions containing a high concentration of lysosomes. Another possible explanation is that membranes of secondary lysosomes may contain the same 5′-nucleotidase found in plasma membranes. The peak of radioactivity at a density of 1.13 can be attributed to the presence of a second peak of lysosomes (acid phosphatase) and plasma membrane fragments (5′-nucleotidase) in this region of the gradient.

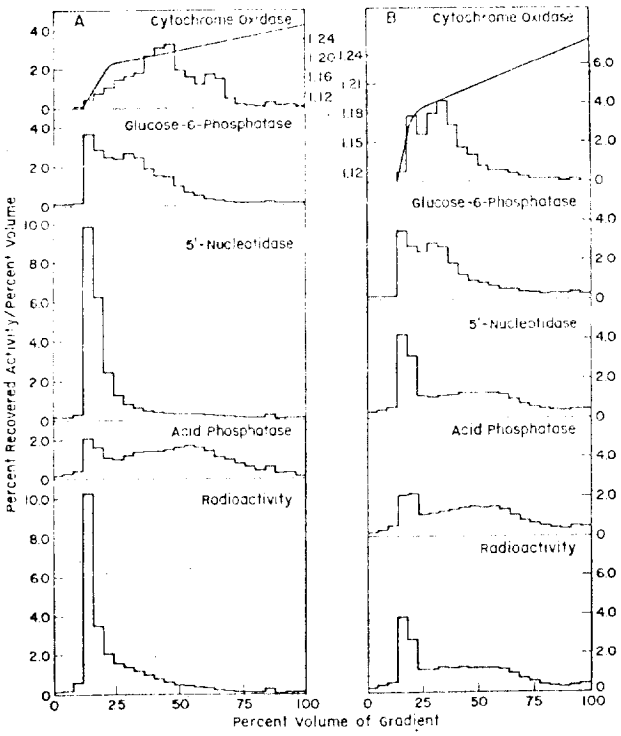

FIG. 7. Separation of subcellular particles from rat liver by isopycnic centrifugation on continuous sucrose gradients. See Fig. 5 for method used to plot data and the text for details of experimental procedures. Densities of the fractions are indicated on the *upper graphs*.

Paper Chromatography of Lysosomal Extracts—Fifteen rats that had been pretreated with Triton WR-1339 were killed 2 hours after receiving CN-[^{57}Co]B_{12}. Their livers were removed and homogenized in 0.25 M sucrose. The homogenates were combined and a ML pellet separated from the combined homogenates by differential centrifugation. The lysosomes were further purified by flotation through a discontinuous sucrose gradient. Radioactivity was extracted from the lysosomal fraction and prepared for paper chromatography as previously described (see "Material and Methods"). Fig. 8 shows the results obtained when these extracts were chromatographed on Whatman No. 3MM and ion exchange paper. On cellulose paper, 86% of the radioactivity cochromatographed with authentic CN-B_{12}. The small shoulder of radioactivity on the leading edge of the peak indicates the presence of HO-B_{12}, which has a slightly higher R_f value than CN-B_{12} in this solvent system. As in the 20 min

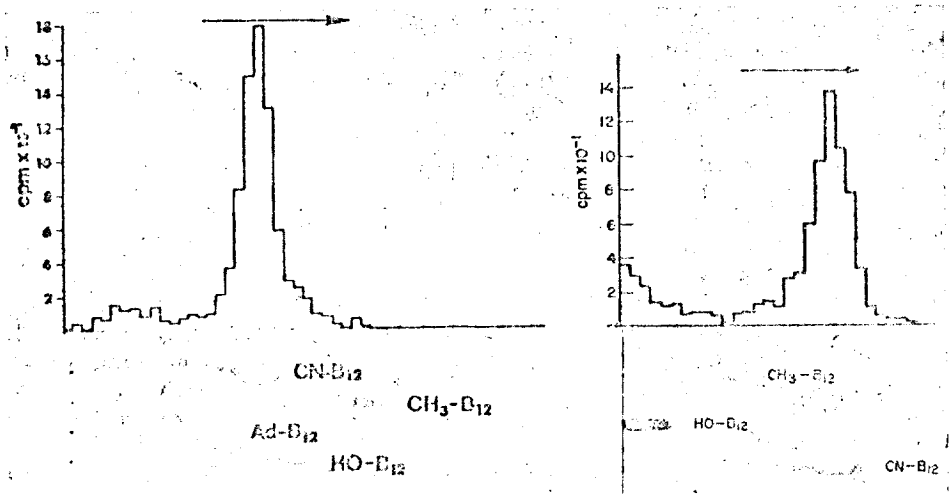

FIG. 8. Chromatography on paper of radioactive products extracted from a lysosomal fraction. *Arrows* indicate direction of solvent flow. *Left*, extract was prepared from a lysosomal fraction separated from the livers of rats that had received CN-[^{57}Co]B_{12} 2 hours previously. The fraction was then chromatographed on Whatman No. 3MM paper; *right*, same as left except that extract was chromatographed on cation exchange paper.

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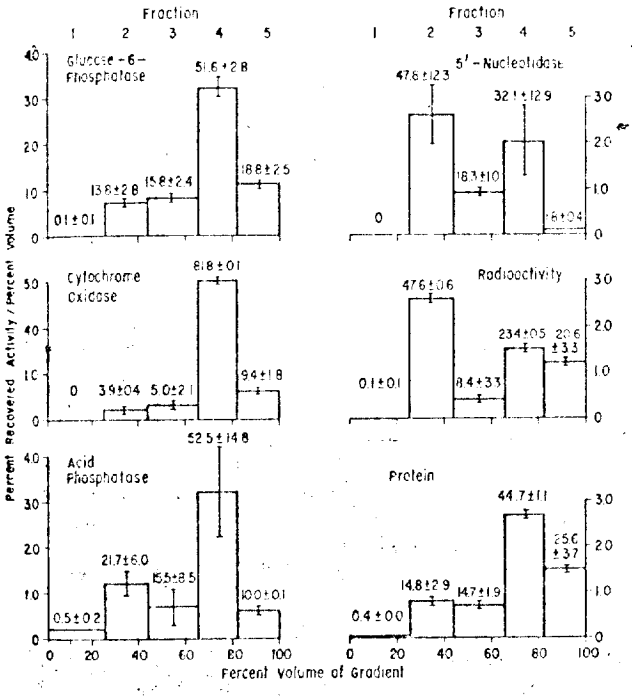

FIG. 9. Subfractionation of microsomal pellet by centrifugation through a discontinuous sucrose gradient. Data are presented as in Fig. 5. The microsomal pellet was suspended in 1.28 density sucrose and layered in the bottom of the tube. Layers of 1.15 density and 1.03 density sucrose were carefully pipetted on top of the microsomal suspension. Fraction 5 was collected from the bottom of the tube, Fraction 4 from the interface between 1.28 and 1.15 density sucrose, Fraction 3 from the 1.15 density region, Fraction 2 from the interface between 1.15 and 1.03 density sucrose, and Fraction 1 from the top of the tube. Two experiments were done and means ± standard deviations are reported. The P contained 78% of the total liver glucose 6-phosphatase, 67% of the 5'-nucleotidase, 16% of the acid phosphatase, 5% of the cytochrome oxidase, 14% of the protein, and 43% of the radioactivity.

liver extracts (Fig. 2), approximately 10% of the radioactivity had a R_F value lower than that of Ad-B_{12}. On ion exchange paper, 74% of the radioactivity cochromatographed with CN-B_{12} and another 13% remained near the origin with HO-B_{12}. These data demonstrated that 75 to 85% of the radioactivity in this purified lysosomal fraction was associated with CN-B_{12} and a smaller amount (approximately 10%) with HO-B_{12}. This eliminated the possibility that the lysosomes simply might be sequestering breakdown products of the CN-[^{57}Co]B_{12}.

Subfractionation of Microsomal Pellet—A fraction enriched in 5'-nucleotidase was separated by density gradient centrifugation from a P which had been isolated from the livers of rats that were killed 5 min after receiving CN-[^{57}Co]B_{12}. Fig. 9 shows the results of this experiment. Fraction 2 contained 48% of the gradient 5'-nucleotidase which represented 32% of the 5'-nucleotidase of the liver. The 5'-nucleotidase in Fraction 2 was purified 15-fold over the homogenate; however, there is no doubt that the plasma membrane fragments in the preparation were contaminated with other subcellular components since the fraction contained significant amounts of glucose 6-phosphatase and acid phosphatase. Fraction 2 also contained 48% of the gradient radioactivity. A comparison of the distribution of the radioactivity with that of the enzymes demonstrates that a large part of the radioactivity in the microsomal fraction must be associated with plasma membrane fragments or vesicles containing 5'-nucleotidase, although a part of the radioactivity may be present in other particles. If the major portion of the radioactivity were associated with either fragments of the endoplasmic reticulum (glucose 6-phosphatase), the lysosomes (acid phosphatase), or the mitochondria (cytochrome oxidase), Fraction 4 would contain 2 to 5 times more radioactivity than Fraction 2. The P was layered in the bottom of the tube; therefore, the radioactivity in Fraction 5 probably represents radioactivity released in a soluble form during centrifugation.

DISCUSSION

In these studies 2 to 4 ng of CN-[^{57}Co]B_{12}, which is approximately 2% of the daily requirement for B_{12} of adult rats (46), was administered intravascularly. The liver contained 10% of the injected radioactivity at 5 min postinjection and retained this amount of radioactivity for the next 72 hours. The kidneys actually concentrated the radioactivity more efficiently than the liver and, in fact, contained 45% of the injected radioactivity 4 days postinjection[3]; however, the liver was chosen for these studies because the techniques of subcellular fractionation are most refined for this tissue. The radioactive compounds extracted from livers 20 min and 72 hours after the injection of CN-[^{57}Co]B_{12} were identified as either CN-B_{12}, HO-B_{12}, or Ad-B_{12}. In agreement with previous reports (47, 48), the liver was capable of converting CN-B_{12} to Ad-B_{12}.

Five minutes after the injection of CN-[^{57}Co]B_{12}, the blood contained 8% of the injected radioactivity whereas the microsomal fraction from liver contained 4.6% of the injected radioactivity. Clearly the large amount of radioactivity associated with the microsomal fraction at 5 min cannot be explained on the basis of adsorption to the microsomes of radioactivity from the blood contaminating the liver during homogenization, since the radioactivity associated with the microsomal fraction was equivalent to the amount of radioactivity found in ½ of the total blood volume. The same type of reasoning also eliminates adsorption of radioactivity from the blood as a possible source of a significant amount of radioactivity in the lysosomal fractions; furthermore, when serum saturated with CN-[^{57}Co]B_{12} was added to a liver homogenate which was then fractionated into its subcellular components by differential centrifugation, L contained only 4% of the radioactivity.[3] Neither can adsorption of radioactivity from the soluble fraction of the liver onto the lysosomes be responsible for the results, since the radioactivity associated with the lysosomal fraction was highest at 30 min postinjection, at a time when the amount of radioactivity in the soluble fraction had not reached its maximum, and was much lower at 24 hours, when the soluble fraction contained its maximal amount of radioactivity. At early times after the injection of CN-[^{57}Co]B_{12}, a portion of the radioactivity in the soluble fraction of the liver is doubtlessly due to contamination by blood; however, at later times when the blood is virtually free of radioactivity, this factor is of little importance.

Half of the radioactivity in the liver at 5 min after the injection of CN-[^{57}Co]B_{12} sedimented in the microsomal fraction. When this microsomal fraction was subfractionated on a discontinuous sucrose gradient (Fig. 9), the distribution of the newly absorbed vitamin was similar to that of 5'-nucleotidase and distinct from

[3] Q. A. Pletsch and J. W. Coffey, unpublished data.

that of glucose 6-phosphatase, demonstrating that the vitamin was associated with fragments of the plasma membrane rather than with elements of the endoplasmic reticulum. This finding was confirmed by isopycnic centrifugation on a continuous sucrose gradient of a subcellular fraction rich in microsomal material (Fig. 7A). The amount of radioactivity in lysosomal fractions isolated from the livers of rats pretreated with Triton WR-1339 was quite small at 5 min postinjection, peaked at 30 min, and then decreased to a low level by 72 hours (Fig. 6). Based on the recovery of acid phosphatase in the lysosomal fraction (Table I and Fig. 5) and assuming that radioactivity is homogeneously distributed in all acid phosphatase containing particles, it is possible to calculate that 0.8% of the total liver radioactivity was associated with the lysosomal fraction at 5 min, 15% at 30 min, and 3% at 72 hours. These values have been corrected for the amount of radioactivity found in Fraction 2 (lysosomal fraction) of the gradient when the ML subfractionated was isolated from the livers of rats not pretreated with Triton WR-1339 (Table II). The origin of the radioactivity in this fraction is unknown since the fraction was practically devoid of all enzymes studied. Of some relevance may be the finding of House and Weidemann (19) that three plasma membrane fractions of different densities can be isolated from rat liver. 5′-Nucleotidase was not homogeneously distributed in these fractions and, in fact, one of the fractions contained very little of the enzyme. Therefore, Fraction 2 isolated on a discontinuous gradient from the livers of rats not pretreated with Triton WR-1339 may well contain membrane fragments devoid of the enzymes measured.

The lysosomal fractions prepared by flotation through a discontinuous sucrose gradient were free of mitochondrial and peroxisomal contamination, but they were contaminated with elements of the endoplasmic reticulum and with fragments of plasma membranes. Analysis of the data concerning the distribution of radioactivity and glucose 6-phosphatase in these gradients revealed that vesicles of endoplasmic reticulum origin could not be a source of any significant amount of radioactivity in the lysosomal fractions (see "Results"); however, on the basis of the data obtained from these discontinuous gradients, plasma membrane fragments could not be eliminated as a major source of radioactivity in these lysosomal fractions. This question was resolved by fractionation of a Triton-free L on a continuous sucrose gradient (Fig. 7*B*). Plasma membrane fractions isolated from rat liver by various methods have been reported to have densities ranging from 1.14 to 1.18 in sucrose gradients (29, 30, 50). Values higher than 1.18 have not been found; therefore, the radioactivity (50% of the gradient radioactivity) equilibrating at densities higher than 1.18 in the continuous sucrose gradient cannot be associated with plasma membrane fragments. The same can be said for the 5′-nucleotidase activity of these fractions. These data leave no doubt concerning the lysosomal localization in rat liver of large amounts of radioactivity at intermediate times after an injection of CN-[^{57}Co]B_{12}.

The data presented in Fig. 6 show that CN-[^{57}Co]B_{12} must have only a transient stay in the lysosomes. From the data it is not possible to determine whether all or a part of the newly absorbed B_{12} passes through the lysosomes before accumulating in either the mitochondrial or the soluble fraction, or both. Another possibility is that the radioactive B_{12} found in the lysosomes at early times may not be the same radioactive B_{12} found in the mitochondrial and soluble fractions at later times. Since 8% of the injected radioactivity was excreted in the bile within 48 hours,[4] CN-[^{57}Co]B_{12} entrapped in lysosomes may be destined for excretion. While the function of lysosomes in vitamin B_{12} metabolism is not known, the precursor-product relationship between microsomal and lysosomal radioactivity and between lysosomal and mitochondrial-soluble radioactivity suggests that lysosomes may play some role in the intracellular transport of B_{12}.

The route by which CN-[^{57}Co]B_{12} reached the lysosomes is uncertain. Data from this laboratory demonstrated that the radioactivity in a microsomal fraction isolated 5 min after injection and 70% of the radioactivity in a lysosomal fraction isolated 30 min after the administration of CN-[^{57}Co]B_{12} was associated with a protein having the same molecular weight and chromatographic properties on carboxymethylcellulose as TC-II. Since at 5 min post-injection half of the liver radioactivity was found in the microsomal fraction associated with 5′-nucleotidase, one may speculate that the vitamin was taken into the cell by pinocytosis. Pinocytotic vesicles are known to be concentrated in the microsomal fraction by differential centrifugation (44) and 5′-nucleotidase has been shown to be associated with the membrane of pinocytotic vesicles in blood capillaries (51). By analogy to the fate of other materials taken into the cell by pinocytosis (52-54), the radioactivity would be transferred into lysosomes by fusion of the pinocytotic vesicles with pre-existing lysosomes. The observations that TC-II is required for absorption of B_{12} by peripheral tissues (2) and that TC-II disappears from the circulation along with B_{12} during the absorptive process (19) would be explained if the TC-II-B_{12} complex entered the cells by pinocytosis. Presumably the pinocytotic vesicles would fuse with lysosomes to form secondary lysosomes in which TC-II would be destroyed by lysosomal proteases. The biochemical evidence presented in this paper strongly suggests that such a mechanism may play a role in the absorption of B_{12} by the liver; however, further biochemical or morphological studies using autoradiographic techniques are required to verify the existence of this pathway. Such morphological studies would also demonstrate whether CN-[^{57}Co]B_{12} is associated with lysosomes of both Kupffer cells and hepatocytes. Freeman, Gordon, and Humphrey (55) have shown that native albumin is removed from the circulation by hepatocytes rather than Kupffer cells while Benacerraf (56) has shown that denatured plasma proteins are absorbed by Kupffer cells. More recently Gregoriadis *et al.* (57) studied the fate of injected desialylated ceruloplasmin, presumably a native protein, and found that it was removed from the blood by hepatocytes exclusively. The desialylated ceruloplasmin was then degraded within the lysosomes of the hepatocytes. Since B_{12} is taken into the liver attached to an undenatured plasma protein, TC-II, it is likely that hepatocytes would remove the TC-II-B_{12} complex from the circulation more readily than the Kupffer cells.

The liver accumulated only 10% of the injected radioactive B_{12}; therefore, the importance of the mechanisms used by the liver for the uptake of B_{12} in the over-all economy of B_{12} utilization by the rat is unclear. In this regard, a recent report by Newmark, Newman, and O'Brien (58) showing that kidney lysosomes contain radioactive B_{12} at times ranging from 6 to 29 days after the administration of the radioactive vitamin is of

[4] Q. A. Pletsch and J. W. Coffey, manuscript in preparation.

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importance. In these studies, a fraction rich in lysosomes contained approximately 25% of the total kidney radioactivity over the entire time interval. Their data differ from the data obtained in this study on rat liver, since by 72 hours postinjection the lysosomal fraction of liver contained only 3% of the total liver radioactivity. No data were collected at times later than 72 hours. These time differences may be responsible for the results obtained; furthermore, Newmark *et al.* administered the vitamin intramuscularly rather than intravascularly. At time intervals longer than 6 days after the administration of radioactive B_{12}, the kidney may be presented with a constant amount of radioactive B_{12}, and this may explain the unchanging levels of radioactive B_{12} in the lysosomal fraction. Two recent reports (59, 60) indicate that lysosomes are not involved in the ileal absorption of B_{12} in the guinea pig; however, in view of the results with kidney and liver, studies designed to assess the functional significances, if any, of lysosomal B_{12} in a number of tissues are needed.

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REFERENCES

1. Pletsch, Q. A., and Coffey, J. W., *Fed. Proc.*, **28**, 628 (1969).
2. Grasbeck, R., *Progr. Hematol.*, **6**, 233 (1969).
3. Simons, K., *Progr. Gastroenterol.*, **1**, 195 (1968).
4. Hall, C. A., and Finkler, A. E., *J. Lab. Clin. Med.*, **65**, 459 (1965).
5. Hom, B. L., and Olesen, H. A., *Scand. J. Clin. Lab. Invest.*, **19**, 269 (1967).
6. Miller, A., and Sullivan, J. F., *J. Clin. Invest.*, **38**, 2135 (1959).
7. Pitney, W. R., Beard, M. F., and Van Loon, E. J., *J. Biol. Chem.*, **207**, 143 (1954).
8. Mendelsohn, R. S., Watkin, D. M., Horbett, A. P., and Fahey, J. L., *Blood*, **13**, 740 (1956).
9. Tan, C. H., Miller, O. N., and Hansen, H. J., *Fed. Proc.*, **26**, 695 (1967).
10. Hom, B. L., Olesen, H. A., and Lous, P., *J. Lab. Clin. Med.*, **68**, 958 (1966).
11. Hall, C. A., and Finkler, A. E., *Blood*, **27**, 611 (1966).
12. Finkler, A. E., and Hall, C. A., *Arch. Biochem. Biophys.*, **120**, 79 (1967).
13. Finkler, A. E., Green, P. D., and Hall, C. A., *Biochim. Biophys. Acta*, **200**, 151 (1970).
14. Tan, C. H., Ph.D. thesis, Tulane University, 1967.
15. Copper, B. A., and Paranchych, W., *Nature*, **191**, 393 (1961).
16. Paranchych, W., and Cooper, B. A., *Biochim. Biophys. Acta*, **60**, 393 (1962).
17. Coffey, J. W., Hansen, H. J., and Miller, O. N., *Arch. Biochem. Biophys.*, **110**, 117 (1965).
18. Fleintz, G. D., Ph.D. thesis, Tulane University, 1968.
19. Tan, C. H., and Hansen, H. J., *Proc. Soc. Exp. Biol. Med.*, **127**, 740 (1968).
20. Sandsteid, M. E., Bethell, F. H., and Ackermann, W. W., *J. Biol. Chem.*, **190**, 791 (1951).
21. Schmilewitz, M., Stein, Y., Stein, O., and Grossowicz, L., *Haematologica*, **2**, (1959).
22. Newman, G. E., O'Brien, J. R. P., Spray, G. H., and Witts, L. J., in H. C. Hernrich (Editor), *Vitamin B_{12} and Intrinsic Factor, Second European Symposium*. Ferdinand Enke, Stuttgart, 1962, p. 424.
23. Strength, D. R., Alexander, W. F., and Wack, J. P., *Proc. Soc. Exp. Biol. Med.*, **102**, 15 (1959).
24. Barrows, C. H., Jr., and Chow, B. F., *Proc. Soc. Exp. Biol. Med.*, **95**, 517 (1957).
25. Tantengco, V. D., Okudo, K., Gabriel, A. R., and Chow, B. F., *J. Philippine Med. Ass.*, **39**, 146 (1963).
26. Trouet, A., *Arch. Intern. Physiol. Biochim.*, **72**, 698 (1964).
27. Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S., and de Duve, C., *J. Cell Biol.*, **37**, 482 (1968).
28. Dallner, G., and Ernster, L., *J. Histochem. Cytochem.*, **16**, 611 (1968).
29. Emmelot, P., Bos, C. J., Benedetti, E. L., and Rümke, P. H., *Biochim. Biophys. Acta*, **90**, 126 (1964).
30. Touster, O., Aronson, N. N., Dulaney, J. T., and Hendrickson, H., *J. Cell Biol.*, **47**, 604 (1970).
31. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F., *Biochem. J.*, **60**, 604 (1955).
32. Gianetto, R., and de Duve, C., *Biochem. J.*, **59**, 433 (1955).
33. de Duve, C., Berthet, J., Hers, H. G., and Dupret, L., *Bull. Soc. Chim. Biol.*, **31**, 1242 (1949).
34. Michell, R. H., and Hawthorne, J. N., *Biochem. Biophys. Res. Commun.*, **21**, 333 (1965).
35. Sloat, B. F., and Allen, J. M., *Ann. N. Y. Acad. Sci.*, **166**, 574 (1969).
36. Arsenis, C., and Touster, O., *J. Biol. Chem.*, **243**, 5702 (1968).
37. Cooperstein, S. J., and Lazarow, A., *J. Biol. Chem.*, **189**, 665 (1951).
38. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
39. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951).
40. Smith, E. L., Mervyn, L., Johnson, A. W., and Shaw, N., *Nature*, **194**, 1175 (1962).
41. Weissbach, H., Peterkofsky, A., Redfield, B. G., and Dickerman, H., *J. Biol. Chem.*, **238**, 3318 (1963).
42. Skegos, H. R., in P. Gyorgy and W. N. Pearson (Editors), *The vitamins, Vol. VII*, Academic Press, New York, 1967 p. 277.
43. Goldfischer, S., Essner, E., and Novikoff, A. B., *J. Histochem. Cytochem.*, **12**, 72 (1964).
44. Wattiaux, R., Wibo, M., and Baudhuin, P., *Ciba Found. Symp. on Lysosomes*, 176 (1963).
45. Thines-Sempoux, D., Amar-Costesec, A., Beaufay, H., and Berthet, J., *J. Cell Biol.*, **43**, 189 (1969).
46. Grasbeck, R., Ignatius, R., Jarnefelt, J., Linden, H., and Mali, A., *Clin. Chim. Acta*, **6**, 56 (1961).
47. Pawelkiwicz, J., Gorna, M., Fenrych, W., and Magas, S., *Ann. N. Y. Acad. Sci.*, **112**, 641 (1964).
48. Yagiri, Y., *J. Vitaminol.*, **13**, 228 (1967).
49. House, P. D. R., and Weidemann, M. J., *Biochem. Biophys. Res. Commun.*, **41**, 541 (1970).
50. Weaver, R. A., and Boyle, W., *Biochem. Biophys. Acta*, **173**, 377 (1969).
51. Marchesi, V. T., Phil, D., and Barrnett, R. J., *J. Cell Biol.*, **17**, 547 (1963).
52. Straus, W., *J. Cell Biol.*, **20**, 497 (1964).
53. Jacques, P., *Arch. Intern. Physiol. Biochim.*, **68**, 683 (1960).
54. Mego, J. L., Bertini, F., and McQueen, J. D., *J. Cell Biol.*, **32**, 699 (1967).
55. Freeman, T., Gordon, A. H., and Humphrey, J. H., *Brit. J. Exp. Pathol.*, **39**, 459 (1958).
56. Benacerraf, B., *Publ. Am. Inst. Biol. Sci.*, **4**, 205 (1958).
57. Gregoriadis, G., Morell, A. G., Sternlieb, I., and Scheinberg, I. H., *J. Biol. Chem.*, **245**, 5833 (1970).
58. Nilsmark, P., Newman, G. E., and O'Brien, J. R. P. *Arch. Biochem. Biophys.*, **141**, 121 (1970).
59. Peters, T. J., and Hoffbrand, A. V., *Brit. J. Haematol.*, **19**, 369 (1970).
60. Peters, T. J., Quinlan, A., and Hoffbrand, A. V., *Clin. Sci.*, **37**, 568 (1969).

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Properties of the Proteins That Bind Vitamin B_{12} in Subcellular Fractions of Rat Liver[1]

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The proteins in the subcellular fractions of rat liver that bind radioactive vitamin B_{12} (B_{12}) after an intravascular injection of [^{57}Co]-cyanocobalamin ([^{57}Co]CN-B_{12}) have been characterized by chromatography on columns of Sephadex G-100 and carboxymethyl cellulose (CM-cellulose). Each fraction was studied at that time after the injection of [^{57}Co]CN-B_{12} when it contained the maximum radioactivity (*J. Biol. Chem.* 246, 4619, 1971). The radioactive B_{12} in microsomal and plasma membrane fractions isolated 5 min after the injection of [^{57}Co]CN-B_{12} was associated with a protein which cochromatographed with rat transcobalamin-II (TC-II) on columns of Sephadex G-100 and CM-cellulose. Seventy percent of the radioactivity in a highly purified lysosomal fraction isolated 30 min after the injection of [^{57}Co]CN-B_{12} was bound to this same protein. The remainder of the radioactivity in the lysosomal fraction chromatographed as free B_{12}. After incubation of the lysosomal fraction *in vitro* for 2 hr at 37°C in buffered isotonic sucrose (pH = 5.5), 70% of the radioactivity in the fraction was present as free B_{12}. Ninety percent of the radioactivity in a soluble fraction and 40% of the radioactivity in a mitochondrial fraction isolated 24 and 72 hr, respectively, after the injection of [^{57}Co]CN-B_{12} was associated with proteins having M_r greater than 100,000. The data suggest that a portion of the [^{57}Co]CN-B_{12} was taken into the cells of the liver as the intact [^{57}Co]CN-B_{12}–TC-II complex which was then further metabolized.

The extracellular fluids of mammals contain a number of proteins which specifically bind B_{12}[3]: among them are intrinsic factor (2), TC-II (3), and the "R" proteins which include TC-I (2). Transcobalamin II functions as a transport protein for B_{12} in serum (4) and has been shown to promote the uptake of B_{12} by peripheral tissues (5, 6). Transcobalamin I binds most of the endogenous B_{12} of serum (3, 7).

Few attempts have been made to characterize the intracellular proteins which bind B_{12} in rat liver. Sahli (8) prepared an ML from the livers of rats that had been fed a diet containing [^{60}Co]CN-B_{12} and studied the chromatographic properties of the B_{12}-binding proteins in the fraction. When an aqueous extract of the mitochondrial pellet was passed through a Sephadex G-100 column, 40% of the radioactivity in the column effluent was bound to a protein with a M_r of approximately 110,000 while the remaining 60% behaved as free B_{12}. Tan (9) used similar techniques to study the B_{12}-

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[3] The abbreviations used are: B_{12}, vitamin B_{12}; TC-I and TC-II, transcobalamin I and II; CN-B_{12}, cyanocobalamin; N, nuclear fraction; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, supernatant fraction; CM-cellulose, carboxymethyl cellulose.

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binding proteins in an S from rat liver and found that approximately 90% of the radioactivity in the S was bound to a protein with a M_r of 90,000–100,000. Ten percent of the radioactivity was present as free B_{12} and a small amount was associated with a protein similar in size to TC-II.

Previous experiments (10) demonstrated that, after the intravenous injection of a physiologic dose of [^{57}Co]CN-B_{12} into rats, the radioactive vitamin bound immediately to TC-II and was transported to the tissues in this form. The liver absorbed 10% of the administered [^{57}Co]CN-B_{12} under these conditions. Approximately 50% of the radioactivity in the liver 5 min after the injection of [^{57}Co]CN-B_{12} sedimented with the P. Subfractionation of this microsomal pellet on continuous and discontinuous sucrose gradients demonstrated that most of the radioactivity in the microsomal pellet was associated with fragments of the plasma membrane rather than with elements of the endoplasmic reticulum. By 72 hr 32% and 33% of the radioactivity of the liver was associated with the S and M, respectively. At intermediate times after the injection of [^{57}Co]CN-B_{12} (30 min to 2 hr), highly purified lysosomes contained approximately 15% of the liver radioactivity. The experiments described in this paper were designed to study the properties of the B_{12}-binding proteins in the subcellular fractions of rat liver at various times after the administration of [^{57}Co]CN-B_{12}.

MATERIALS AND METHODS

Animals. Female Charles River rats weighing 200–250 g were used for all experiments. All rats received Purina rat chow and water *ad libitum* until they were fasted overnight before being killed by decapitation. Those animals receiving [^{57}Co]CN-B_{12} 24 and 72 hr before being killed were allowed free access to food until 16 hr before the time of sacrifice. The animals used for the preparation of highly purified lysosomes and mitochondria were injected intraperitoneally with Triton WR-1339 at a dosage of 85 mg/100 g body weight 3½ days before the animals were killed.

Approximately 0.5 μCi of [^{57}Co]CN-B_{12} (2–4 ng of cyanocobalamin) in 0.9% NaCl was administered in all experiments by intracardial injection.

Chemicals. Triton X-100 and cyanocobalamin were purchased from Sigma Chemical Company, St. Louis, Mo and Triton WR-1339 from Ruger Chemical Company, Irvington-on-Hudson, NY. All other chemicals were of reagent grade. The [^{57}Co]CN-B_{12} (71–173 μCi/μg) was purchased from Philips-Duphar, Petten, Holland and the [^{57}Co]CN-B_{12} (0.67 μCi/μg) from E. R. Squibb and Sons, New York, NY.

Cell fractionation. Livers from fasted rats were homogenized in ice-cold 0.25 M sucrose using a smooth-walled glass homogenizer fitted with a Teflon pestle driven at about 1000 rpm by a power drill. The homogenates were separated by differential centrifugation according to the procedure of de Duve *et al.* (11) into N, M, L, P, and S fractions. Lysosomes (purified 34-fold over the homogenate on the basis of acid phosphatase content) and partially purified mitochondria were prepared from ML fractions from the livers of rats pretreated with Triton WR-1339 by the method of Trouet (12) as described by Leighton *et al.* (13). Plasma membrane fractions were obtained from microsomal pellets by the method of Touster *et al.* (14). The purity of these fractions as assessed by marker enzymes was discussed in detail previously (10). The fractions were solubilized for chromatography in buffers containing 1% (w/v) Triton X-100 by stirring at 4°C until the solutions became clear. The lysosomal fractions dissolved quickly; however, mitochondrial and plasma membrane fractions had to be stirred for 12 hr.

Column chromatography. Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ) was swelled in water for 3 days and then packed into a 2.5 × 90-cm column. The column was equilibrated with buffer (0.05 M phosphate buffer, pH 7.4, containing 1% Triton X-100 and 0.154 M NaCl) by passing the buffer through the column in an upward direction at a flow rate of 24 ml/hr using a peristaltic pump (Buchler Instruments, Fort Lee, NJ). Samples were dissolved in 4 ml of the same buffer, pumped into the bottom of the column, and then eluted in 4-ml fractions by upward flow. The CM-cellulose (Mann Research Laboratories, New York, NY) was washed successively with 0.5 N HCl, water, 1 N NaOH, water, and finally buffer (0.05 M phosphate buffer, pH 6.0, containing 1% Triton X-100 and 0.15 M NaCl) and then packed into a 2.5 × 35 cm column. Buffer was pumped upward at a flow rate of 24 ml/hr and 4-ml fractions collected. Samples (4–5 ml) were dissolved in the equilibrating buffer, dialyzed against the same buffer for 24 hr at 4°C, and then pumped into the bottom of the column. The column was washed with approximately 200 ml of equilibrating buffer and then the radioactivity bound to the column eluted using a continuous ionic strength gradient produced by introducing 0.05 M phosphate buffer,

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pH 6.0, containing 1% Triton X-100 and 2.0 M NaCl to a reservoir containing 150 ml of the equilibrating buffer. All chromatography was done at 4°C. The protein content of the fractions was measured by a modification of the method of Lowry *et al.* (15) as described by Miller (16). The Triton X-100 in the fractions produced a precipitate when the reagents for the protein determination were added. This precipitate was removed by centrifugation before the optical density was measured using a Gilford Model 2400 spectrophotometer. Radioactivity was measured in a Packard Model 3003 Auto-Gamma spectrometer equipped with three pulse-height analysis channels. When the samples contained both ^{57}Co and ^{60}Co, the contribution of the ^{60}Co to the counts in the ^{57}Co window was eliminated by applying the appropriate correction factor (12%).

RESULTS

The protein binders of B_{12} in the various subcellular fractions of liver were characterized by chromatography on columns of Sephadex G-100 and CM-cellulose. Each fraction was isolated and then prepared for chromatography at that time after the injection of [^{57}Co]CN-B_{12} when the fraction contained its maximal amount of radioactivity (10). The times chosen were 5 min, 30 min, 24 hr, and 72 hr postinjection of [^{57}Co]-CN-B_{12} for the plasma membrane fraction, lysosomal fraction, soluble fraction, and mitochondrial fraction, respectively. The solubilized subcellular fractions labeled *in vivo* with [^{57}Co]CN-B_{12} were mixed with either mouse or rat serum to which [^{60}Co]-CN-B_{12} had been added *in vitro* and the mixture then applied to the appropriate column. An excess of nonradioactive B_{12} was also added before the samples were mixed to avoid possible exchange reactions involving [^{57}Co]CN-B_{12} and [^{60}Co]CN-B_{12}. When a small amount of [^{60}Co]CN-B_{12} is added to either mouse or rat serum *in vitro*, it binds to

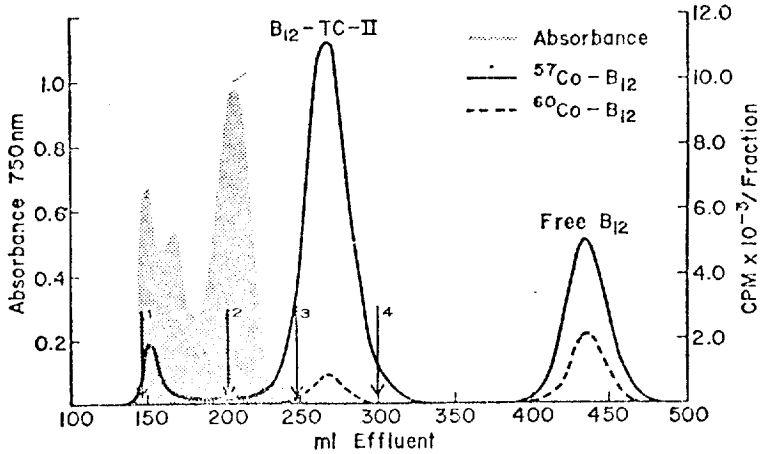

FIG. 1. Cochromatography of rat [^{57}Co]CN-B_{12}-TC-II and mouse [^{60}Co]CN-B_{12}-TC-II on a Sephadex G-100 column. One tenth milliliter of [^{57}Co]CN-B_{12} (1.0 μCi/ml) was mixed with 1.0 ml of rat serum, and then 0.1 ml of nonradioactive CN-B_{12} (10 μg/ml) added. The solution was then transferred to a tube containing 1.0 ml of mouse serum and 0.1 ml of [^{60}Co]CN-B_{12} (0.1 μCi/ml) and the final volume adjusted to 4.0 ml by addition of buffer. The 4.0-ml sample was then applied to the Sephadex G-100 column. See Materials and Methods for details of chromatographic procedures. Both the mouse and rat B_{12}-TC-II complex eluted from the column after albumin between 225 and 300 ml of effluent while free B_{12} eluted between 400 and 475 ml of effluent. Mouse serum contains 10-20 times more TC-II than does rat serum; however, the amount of radioactivity associated with the rat TC-II labeled with [^{57}Co]CN-B_{12} was large relative to the amount of radioactivity associated with the mouse TC-II labeled with [^{60}Co]CN-B_{12} because the specific radioactivity of the [^{57}Co]CN-B_{12} was 100 times higher than the specific radioactivity of the [^{60}Co]CN-B_{12}. The protein pattern is shown by the shaded area. Arrow 1 indicates the elution peak for porcine thyroglobulin; arrow 2 the elution peak for bovine serum albumin; arrow 3 the elution peak for ovalbumin; arrow 4 the elution peak for chymotrypsinogen A.

the TC-II in the serum to form a [^{60}Co]CN-B_{12}–TC-II complex which can then serve as an internal marker for TC-II in the chromatographic experiments. Mouse serum rather than rat serum was used as an internal marker in some instances for Sephadex G-100 chromatography because mouse serum contains 10–20 times more TC-II than does rat serum. Since serum from individual animals whose levels of TC-II may vary several fold was added to the solubilized subcellular fractions as an internal marker, the size of the [^{60}Co]CN-B_{12} TC-II peaks in the figures varies considerably. This has no influence on the results since the chromatographic properties of TC-II from all animals of the same species were identical. The protein profiles shown in the figures reflect the chromatographic properties of the serum proteins added *in vitro* as well as those of the protein endogenous to the subcellular fractions. A mixture of proteins containing porcine thyroglobulin (M_r 680,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 45,000), chymotrypsinogen A (M_r 25,000), and cyanocobalamin (M_r 1355) was passed through the Sephadex G-100 column in order to standardize the system. The elution volumes of these proteins are shown in Fig. 1. Figure 1 also shows that mouse TC-II labeled with [^{60}Co]CN-B_{12} cochromatographs with rat TC-II labeled with [^{57}Co]CN-B_{12} on Sephadex G-100 and justifies the use of mouse TC-II as a molecular weight marker for rat TC-II. Samples applied to CM-cellulose columns were always cochromatographed with rat serum to which [^{60}Co]CN-B_{12} had been added. Figure 2 illustrates the behavior of the rat [^{57}Co]-CN-B_{12}–TC-II complex on CM-cellulose.

Chromatography of a plasma membrane fraction. A purified plasma membrane preparation, i.e., a fraction rich in 5′-nucleotidase, was separated by density-gradient centrifugation from a microsomal pellet isolated from the livers of eight rats that were killed 5 min postinjection of [^{57}Co]-CN-B_{12}. After solubilization in 1% Triton X-100 and centrifugation at 40,000 rpm for 30 min in a Spinco No. 40 rotor, an aliquot of the supernatant fluid which contained 98% of the radioactivity originally associated with the purified plasma membranes was applied to the Sephadex G-100 column. Thirty percent of the radioactivity in this supernatant fraction was lost upon dialysis in preparation for chromatography on CM-cellulose. Figures 3 and 4 show the results obtained when these solubilized plasma membranes were chromatographed on columns of Sephadex G-100 and CM-cellulose. Ninety-eight percent of the radioactivity in the plasma membrane preparation was associated with a protein which cochromatographed on both columns with the [^{60}Co]-CN-B_{12}–TC-II complex added as an internal

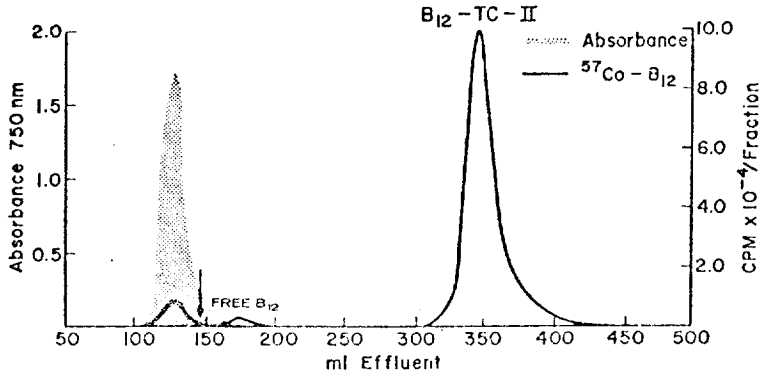

FIG. 2. Chromatography of rat [^{57}Co]CN-B_{12}–TC-II on a CM-cellulose column. One half milliliter of [^{57}Co]CN-B_{12} was added to 2 ml of rat serum which was then dialyzed against the equilibrating buffer. See Materials and Methods for experimental details. The arrow indicates the position at which the ionic strength gradient was started. The B_{12}–TC-II complex eluted between 310 and 400 ml of effluent while the small amount of free B_{12} remaining after dialysis eluted between 160 and 190 ml.

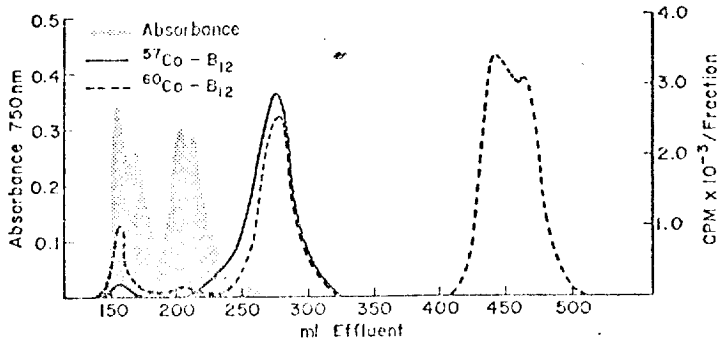

FIG. 3. Chromatography of a solubilized plasma membrane preparation on a Sephadex G-100 column. A plasma membrane fraction isolated from 6 g of liver was solubilized in 3.0 ml of buffer containing 1% Triton X-100 as described in the text and then added to a mixture containing 0.7 ml of mouse serum, 0.2 ml of [^{60}Co]CN-B_{12}, and 0.1 ml of nonradioactive CN-B_{12}. See Fig. 1 for the positions at which the B_{12}–TC-II complex and free B_{12} elute from the column. The large peak of free [^{60}Co]CN-B_{12} (400 to 500 ml of effluent) was present because an excess of [^{60}Co]CN-B_{12} was added to the mouse serum.

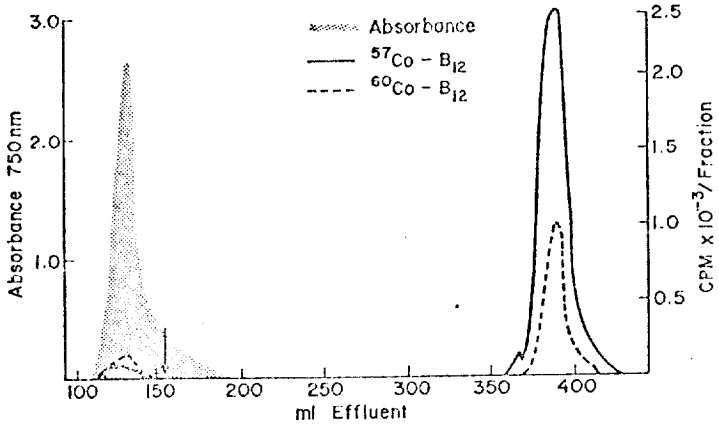

FIG. 4. Chromatography of a solubilized plasma membrane preparation on a CM-cellulose column. A plasma membrane fraction isolated from approximately 4 g of liver was dissolved in 3.0 ml of the equilibrating buffer and dialyzed as described in the text. The dialyzed preparation was added to a mixture which had been dialyzed separately containing 2.0 ml of rat serum and 0.1 ml of [^{60}Co]CN-B_{12}. One-tenth milliliter of nonradioactive CN-B_{12} was added and then a 4.0-ml aliquot applied to the column. The arrow marks the start of the ionic strength gradient. See Fig. 2 for the positions at which the B_{12}–TC-II complex and free B_{12} elute from the column.

marker. No free [^{57}Co]CN-B_{12} was present in the effluent from either column. The leading edge of the [^{57}Co]CN-B_{12}–TC-II peak in Fig. 3 is asymmetric suggesting the presence of a small amount of a [^{57}Co]CN-B_{12}–protein complex with a slightly higher molecular weight than the [^{57}Co]CN-B_{12}–TC-II complex. This may indicate some degree of interaction between the [^{57}Co]-CN-B_{12}–TC-II complex and other relatively low molecular weight molecules in the mixture; however, no attempts have been made to characterize this higher molecular weight material or to relate it to the small peak on the leading edge of the [^{57}Co]CN-B_{12}–TC-II peak from the CM-cellulose column (Fig. 4). When a crude microsomal fraction was used for these chromatographic experiments rather than a purified plasma membrane fraction, the results obtained were identical

to those illustrated in Figs. 3 and 4. It is important to establish that the [^{57}Co]CN-B_{12} TC-II complex identified chromatographically in the homogenates of rat liver was present *in vivo* and not produced artifactually during homogenization. The following experiment was done to study this possibility. Rats were injected intravenously with 0.5 μCi of [^{57}Co]CN-B_{12} and 5 min later their livers removed, and homogenized in ice-cold 0.25 M sucrose containing a large excess of nonradioactive CN-B_{12} (10 μg/ml) to saturate all B_{12}-binding sites in the homogenate. The homogenate was made 1% with respect to Triton X-100, stirred for 8 hr in the cold, and then centrifuged at high speed to remove insoluble material. When an aliquot of this extract which contained the greater part of the radioactivity of the homogenate was passed through a Sephadex G-100 column, the radioactivity eluted in association with a protein that had the molecular weight of TC-II. No free radioactive B_{12} was detected in the extract; therefore, it is unlikely that the livers of animals sacrificed 5 min after an injection of [^{57}Co]CN-B_{12} contain any free [^{57}-Co]CN-B_{12} which might interact with the TC-II in the blood contaminating the liver during homogenization. Furthermore, when the homogenate containing excess nonradioactive CN-B_{12} was fractionated into its subcellular components by differential centrifugation, the subcellular distribution of radioactive B_{12} was the same as that obtained using homogenates prepared in sucrose free of nonradioactive CN-B_{12} (10). These results indicate that homogenizational artifacts have little, if any, effect on the results reported in this paper.

Chromatography of lysosomal fractions. A fraction containing highly purified lysosomes was prepared from the livers of 19 rats that had been pretreated with Triton WR-1339. The rats had also received an injection of [^{57}Co]CN-B_{12} 30 min before sacrifice. For this large-scale preparation of lysosomes, some yield was sacrificed for the sake of convenience and purity. The nuclear pellet was washed only once, and all the fluffy layer which sediments on top of the ML pellet was removed to eliminate as much microsomal contamination as possible. The final lysosomal fraction isolated by isopycnic centrifugation on a sucrose gradient was diluted with an equal volume of 0.25 M sucrose and the intact lysosomes pelleted by centrifugation for 350,000 g min in the No. 50.1 Spinco rotor. The pellet was suspended in 9.0 ml of 0.25 M sucrose containing 0.05 M acetate buffer (pH 5.5) and divided into three aliquots. Two of the aliquots were solubilized immediately with 1% Triton X-100 for chromatography on columns of Sephadex G-100 and CM-cellulose. Ninety-two percent of the radioactivity in the original lysosomal preparation remained in the supernatant fraction after high-speed centrifugation of

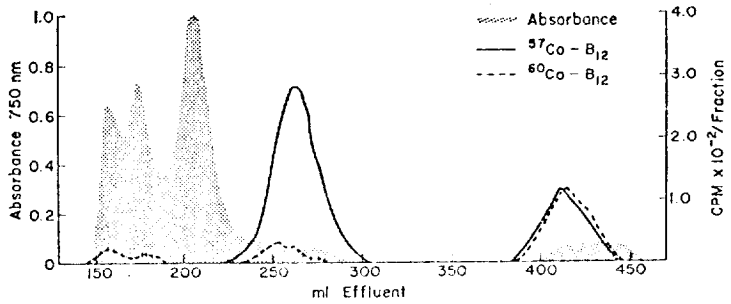

FIG. 5. Chromatography of a solubilized lysosomal fraction on a Sephadex G-100 column. A lysosomal fraction isolated from approximately 15 g of liver was solubilized in 4.0 ml of buffer containing 1% Triton X-100 and then added to 1 ml of a mixture containing 0.8 ml of rat serum and sufficient [^{60}Co]CN-B_{12} to saturate the TC-II in the serum. One-tenth milliliter of nonradioactive CN-B_{12} was added and then a 4.0-ml aliquot applied to the column. See text and legend to Fig. 1 for details.

the Triton-treated lysosomal fractions. The supernatant fluid from the first aliquot was applied to a Sephadex G-100 column without further pretreatment while that from the second aliquot was dialyzed against buffer in preparation for chromatography on CM-cellulose. Thirty-two percent of the radioactivity was lost during dialysis. Seventy-one percent of the radioactivity in the solubilized lysosomal preparation cochromatographed with the rat [^{60}Co]CN-B_{12}–TC-II complex on Sephadex G-100 while 27% eluted as free B_{12} (Fig. 5) Three peaks appeared in the effluent from the CM-cellulose column (Fig. 6). The second peak of radioactivity (12% of the radioactivity) behaved as free B_{12} while the third peak of radioactivity (55% of the radioactivity) cochromatographed with the [^{60}Co]CN-B_{12}–TC-II complex. As determined by the zirconyl phosphate assay of Hansen *et al.* (17), the radioactivity (33% of the radioactivity) in the first peak was also bound to protein. This material has not been characterized; however, it may represent a B_{12}–TC-II complex whose ionic properties have been altered by the action of lysosomal enzymes. If this is true, no extensive degradation of the TC-II occurred since all of the B_{12}–protein complex in the lysosomal fraction had the same molecular weight as the serum [^{60}Co]CN-B_{12}–TC-II complex (Fig. 5). The third aliquot (3.0 ml) of purified lysosomes was incubated *in vitro* for 2 hr at 37°C in 0.25 M sucrose adjusted to pH 5.5 with 0.05 acetate buffer. After 2 hr, the incubation was interrupted by addition of 1% Triton X-100 (final concn) which solubilized 80% of the radioactivity in the preparation. The extract was then chromatographed on Sephadex G-100 and the results are shown in Fig. 7. After incubation of the lysosomal fraction *in vitro*, 71% of of the radioactivity in the extract chromatographed as free B_{12} while 27% cochromatographed with the [^{60}Co]CN-B_{12}–TC-II complex. A comparison of these results with the results shown in Fig. 5 demonstrates that the incubation *in vitro* increased the proportion of free radioactive B_{12} in the lysosomal fraction from 27 to 70% of the total radioactivity while decreasing the amount of protein-bound B_{12} from 71 to 27%. These results indicate either that the protein(s) to which B_{12} was bound in the lysosomal fraction was degraded by lysosomal enzymes with the subsequent release of free B_{12} or that the binder(s) was denatured by the incubation conditions. The [^{57}Co]CN-B_{12}–TC-II complex of serum was stable when incubated at a pH of 5.5 for 2 hr (unpublished data).

Chromatography of the soluble fraction. A supernatant fraction was prepared from the livers of rats killed 24 hr after receiving 0.5 μCi of [^{57}Co]CN-B_{12} and an aliquot chromatographed on a Sephadex G-100 column

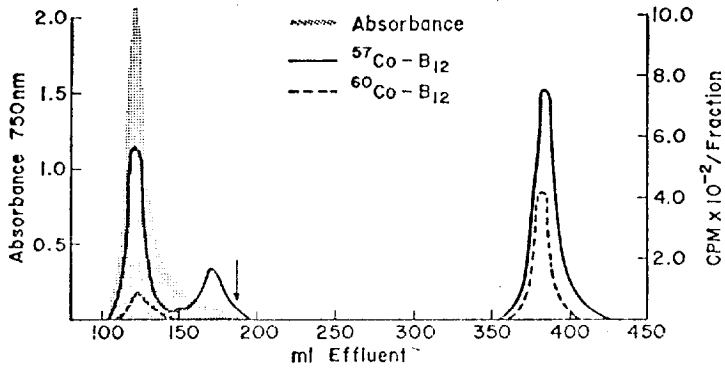

FIG. 6. Chromatography of a solubilized lysosomal fraction on a CM-cellulose column. A lysosomal fraction isolated from 30 g of liver was solubilized in 3.0 ml of the equilibrating buffer and dialyzed as described in the text. The dialyzed preparation was added to a mixture which had been dialyzed separately containing 2.0 ml of rat serum and 0.1 ml of [^{60}Co]-CN-B_{12}. After the addition of 0.1 ml of nonradioactive CN-B_{12}, a 4.0-ml aliquot was applied to the column. See text and legend to Fig. 2 for details.

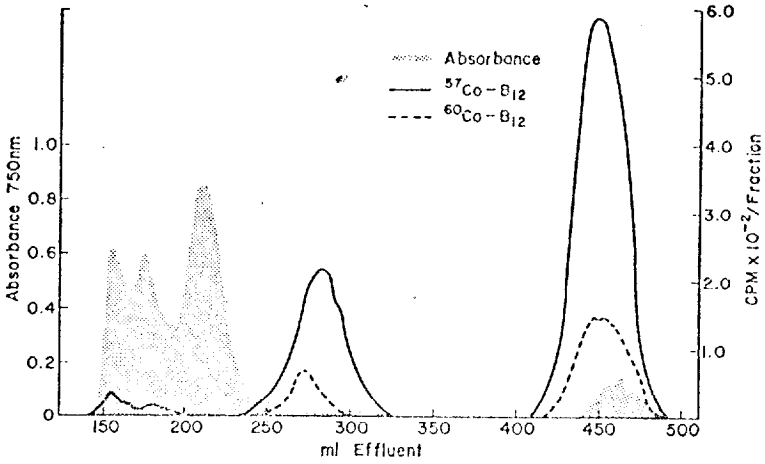

FIG. 7. Chromatography of a solubilized lysosomal fraction on a Sephadex G-100 column after incubation of the fraction *in vitro*. See text for incubation conditions. After solubilization with 1% Triton X-100, a 3.0-ml aliquot of the incubation mixture (this aliquot contained the lysosomes isolated from 22.5 g of liver) was added to a solution containing 2.0 ml of rat serum, 0.1 ml of [^{60}Co]CN-B_{12}, and 0.1 ml of nonradioactive CN-B_{12}. A 4.0-ml aliquot of the sample was then applied to the column. See text and legend to Fig. 1 for details.

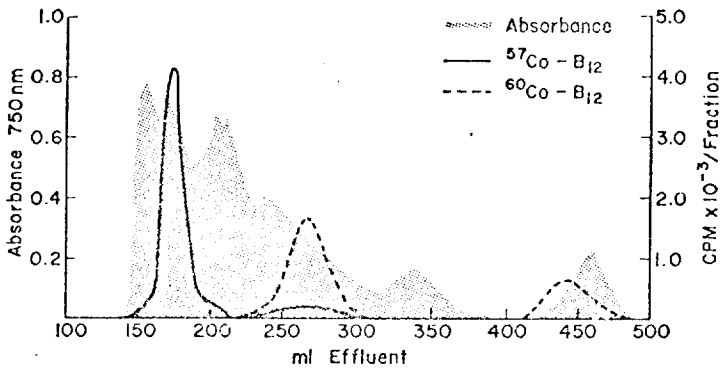

FIG. 8. Chromatography of the supernatant fraction from rat liver on a Sephadex G-100 column. A 3.0-ml aliquot of the supernatant fraction containing the soluble material from 2.7 g of liver was added to a mixture containing 0.5 ml of mouse serum, 0.3 ml of buffer, and 0.1 ml of [^{60}Co]CN-B_{12}. One-tenth milliliter of nonradioactive CN-B_{12} was added and then the 4.0-ml sample was applied to the column. See text and legend to Fig. 1 for details.

(Fig. 8). Ninety-two percent of the radioactivity eluted from the column in association with a protein having a molecular weight considerably greater than that of the B_{12}–TC-II complex. No free B_{12} was detectable in the fraction and less than 10% of the radioactivity cochromatographed with the [^{60}Co]CN-B_{12}–TC-II complex. When an aliquot of the soluble fraction was dialyzed in preparation for chromatography on CM-cellulose, only 6% of the radioactivity was lost; however, this high molecular weight B_{12}–protein complex was not retained by CM-cellulose using the chromatographic conditions employed for this study.

Chromatography of mitochondrial fraction. Seventy-two hours after an injection of [^{57}Co]CN-B_{12}, a combined ML fraction was isolated from the livers of rats pretreated with Triton WR-1339. The mitochondria were further purified by density-gradient centrifugation (13) and then solubilized by

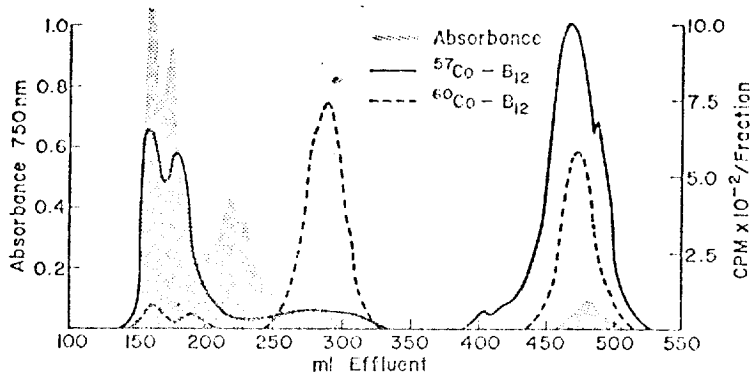

FIG. 9. Chromatography of a solubilized mitochondrial fraction on a Sephadex G-100 column. One-tenth milliliter of nonradioactive CN-B_{12} was added to a 3.0-ml aliquot of a solubilized mitochondrial fraction containing the mitochondrial material from 4.0 g of liver and then the sample added to a mixture containing 0.5 ml of mouse serum, 0.3 ml of buffer, and 0.1 ml of [^{60}Co]CN-B_{12}. The 4.0-ml sample was then applied to the column. See text and legend to Fig. 1 for details.

treatment with 1% Triton X-100. This treatment converted 87% of the radioactivity of the mitochondrial fraction into a soluble form. Sixty-two percent of the radioactivity in the mitochondrial extract chromatographed on Sephadex G-100 as free B_{12} (Fig. 9) while the remainder of the radioactivity, which was protein-bound, eluted in two peaks. One of these peaks appeared at the void volume of the column and the other eluted at the same volume as the high molecular-weight binder of the soluble fraction (Fig. 8). This mitochondrial binder is distinct from the soluble binder since dialysis of the mitochondrial extract against buffer of pH 6.0 resulted in the loss of 98% of the radioactivity. Virtually no radioactivity was lost from the soluble fraction when it was dialyzed under identical conditions. A small amount of the [^{60}Co]B_{12} eluted in association with high molecular-weight proteins which may indicate that a portion of the [^{60}Co]B_{12}–TC-II complex was binding to some of the solubilized mitochondrial proteins.

DISCUSSION

All of the B_{12} circulating in the serum of rats is bound to protein and it appears that a large proportion of the intracellular B_{12} of rat liver is also bound to protein. In this study highly purified plasma membranes and lysosomes, a mitochondrial fraction, and a supernatant fraction were prepared by differential and density-gradient centrifugation from the livers of rats that had received an intravenous injection of [^{57}Co]CN-B_{12} and then the chromatographic properties of the ^{57}Co-labeled materials in these subcellular fractions assessed. Each fraction was studied at that time after the injection of [^{57}Co]-CN-B_{12} when the fraction contained its maximal amount of radioactivity (see Results). Although rarely encountered, at least in humans, under physiological conditions (18), CN-B_{12} was used for these studies since it is the form of the vitamin most often used for therapeutic and dietary purposes. Furthermore, from a practical point of view, the availability of [^{57}Co]CN-B_{12} of high specific radioactivity permitted the use of physiological doses of the vitamin. In a recent publication, Peters *et al.* (19) reported that, after the oral administration of CN-B_{12} to guinea pigs, a portion of the CN-B_{12} is converted to 5′-deoxyadenosylcobalamin while in transit across the intestinal wall; however, the greater part of the orally administered CN-B_{12} appeared in the portal circulation unaltered.

The enzyme 5′-nucleotidase was used as a marker for plasma membrane fragments (20); however, the fraction rich in 5′-nucleotidase which was designated as the plasma membrane fraction may have contained, in addition to plasma membrane

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fragments, intracellular vesicles formed by the internalization of portions of the plasma membrane, e.g., pinocytotic vesicles. The radioactivity associated with either a microsomal fraction or a plasma membrane fraction isolated 5 min after the injection of [^{57}Co]CN-B_{12} was bound to a protein identical in size on Sephadex G-100 and in charge on CM-cellulose to rat TC-II. Approximately 70% of the radioactivity in a lysosomal fraction isolated 30 min postinjection of [^{57}Co]CN-B_{12} was associated with this same binder. The remainder of the radioactivity in the lysosomal fraction was present as free B_{12}. The lysosomes may contain enzyme(s) capable of releasing B_{12} from this protein complex since, after a 2-hr period of incubation *in vitro* in isotonic sucrose, more than 70% of the radioactivity in the lysosomal fraction was present as free B_{12} (Fig. 7). Virtually all of the radioactivity in a soluble fraction isolated 24 hr after the injection of [^{57}Co]CN-B_{12} and approximately 40% of the radioactivity in a mitochondrial fraction isolated 72 hr postinjection of [^{57}Co]-CN-B_{12} was bound to protein. However, in these fractions the radioactivity was not bound to a protein(s) with the molecular size of TC-II (M_r 35,000) but rather to proteins having molecular weights well over 100,000.

A detailed study (10) of the subcellular distribution of radioactive B_{12} at time intervals ranging from 5 min to 72 hr after the administration of [^{57}Co]CN-B_{12} yielded the following information: (1) nearly 50% of the radioactivity in the liver of a rat killed 5 min after the administration of [^{57}Co]CN-B_{12} was associated with the microsomal fraction; however, the amount of radioactivity in the microsomal fraction decreased progressively with time to a low of 9% by 72 hr; (2) the radioactivity in the microsomal pellet was associated with vesicles containing 5′-nucleotidase (marker enzyme for plasma membranes) rather than glucose-6-phosphatase (marker enzyme for endoplasmic reticulum); (3) highly purified lysosomal fractions isolated from the livers of rats killed at times ranging from 30 min to 2 hr after the injection of [^{57}Co]CN-B_{12} contained approximately 15% of the total liver radioactivity; however, at shorter (5 min) and longer (24 and 48 hr) time intervals, the lysosomal fraction contained little radioactivity; and (4) the radioactivity of the liver was localized principally in the soluble and mitochondrial fractions at 24 and 72 hr postinjection of [^{57}Co]CN-B_{12}. These data concerning the subcellular distribution of radioactive B_{12} and the data presented in this paper concerning the chromatographic properties of the B_{12}–protein complexes in the subcellular fractions are compatible with the hypothesis that a portion of the radioactive B_{12} was taken into the cells of the liver as the intact B_{12}–TC-II complex by pinocytosis. The radioactivity in the microsomal fraction would then be associated with pinocytotic vesicles containing the [^{57}Co]CN-B_{12}-TC-II complex. Pinocytotic vesicles are concentrated in the microsomal fraction by differential centrifugation (21) and the membranes of these vesicles might well contain the same 5′-nucleotidase found in plasma membranes; however, at this time few data concerning the enzymatic composition of the membranes of the pinocytotic vesicles of liver are available. 5′-Nucleotidase has been detected in the membranes of pinocytotic vesicles of blood capillaries (22) by histochemical methods; however, Eriesson (23) found that the ATPase associated with the plasma membranes of kidney cells was inactivated after the pinching-off process during the formation of endocytotic vesicles. By analogy to the fate of other proteins taken into the liver cell by pinocytosis (24–26), the vesicles containing the B_{12}–TC-II complex would fuse with preexisting lysosomes to form secondary lysosomes in which the TC-II either would be digested by the lysosomal enzymes or denatured by the acid milieu of the lysosomes with the resultant release of free B_{12}. The free B_{12} then could either diffuse or be transported into the soluble phase of the cytoplasm for further metabolism. This hypothetical scheme is also consistent with the important finding of Tan and Hansen (27) that TC-II disappears from the blood along with B_{12} after the administration of the vitamin and that the TC-II is not reutilized. Further work, perhaps morphological, is needed to verify the existence of this mechanism for the absorp-

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tion of B_{12} by the liver. While the chromatographic data presented in this paper strongly support the suggestion that the intracellular binders of B_{12} in the microsomal and lysosomal fractions of rat liver and rat TC-II are identical, more specific methods (e.g., immunologic methods) should be used to confirm these results.

The results reported in this paper are in agreement with the finding of Finkler *et al.* (28) that Sarcoma 180 cells, incubated for 2 hr *in vitro* in the presence of the B_{12}–TC-II complex, absorb the intact B_{12}–TC-II complex. This group has also demonstrated that HeLa cells in tissue culture rapidly absorb radioactive B_{12} added to the medium in the form of the B_{12}–TC-II complex and later release it into the medium as a radioactive B_{12}-protein complex having the chromatographic properties of the B_{12}–TC-I complex (29). Hom (30) injected the radioactive B_{12}–TC-II complex into humans and found that the radioactivity was absorbed rapidly by the tissue. The radioactivity reappeared in the blood at a later time bound to TC-I. The high molecular-weight binder) of B_{12} from the mitochondrial and soluble fractions of rat liver which were characterized in this study appear to have a slightly higher molecular weight than rat TC-I (M_r 120,000) (31). Further characterizations of these high molecular-weight binders are needed to determine whether they function as apoenzymes, as proteins involved in the storage of B_{12}, or perhaps as precursors of TC-I.

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We thank Dr. Hans J. Hansen for helpful discussions and suggestions during the course of this work.

REFERENCES

1. Pletsch, Q. A., and Coffey, J. W. (1971) *Fed. Proc.* **30**, 210.
2. Grasbeck, R., Simons, K., and Sinkkonen, I. (1966) *Biochim. Biophys. Acta* **127**, 47.
3. Hall, C. A., and Finkler, A. E. (1965) *J. Lab. Clin. Med.* **65**, 459.
4. Hall, C. A., and Finkler, A. E. (1966) *Proc. Soc. Exp. Biol. Med.* **123**, 55.
5. Grasbeck, R. (1969) *Progr. Hematol.* **6**, 233.
6. Simons, K. (1968) *Progr. Gastroenterol.* **1**, 195.
7. Pitney, W. R., Beard, M. F., and Van Loon, E. J. (1951) *J. Biol. Chem.* **207**, 143.
8. Sahli, S. (1968) Ph.D. Thesis, Tulane University, New Orleans, LA.
9. Tan, C. H. (1967) Ph.D. Thesis, Tulane University, New Orleans, LA.
10. Pletsch, Q. A., and Coffey, J. W. (1971) *J. Biol. Chem.* **246**, 4619.
11. deDuve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F. R. (1955) *Biochem. J.* **60**, 604.
12. Trouet, A. (1964) *Arch. Int. Physiol. Biochim.* **72**, 698.
13. Leighton, F., Poole, B., Beaufay, H., Baudhuin, P., Coffey, J. W., Fowler, S., and deDuve, C. (1968) *J. Cell Biol.* **37**, 482.
14. Touster, O., Aronson, N. N., Dulaney, J. T., and Hendrickson, H. (1970) *J. Cell Biol.* **47**, 604.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
16. Miller, G. L. (1959) *Anal. Chem.* **31**, 964.
17. Hansen, H. J., Miller, O. N., and Tan, C. H. (1966) *Amer. J. Clin. Nutr.* **19**, 10.
18. Linnell, J. C., Hoffbrand, A. V., Peters, T. J., and Matthews, D. M. (1971) *Clin. Sci.* **40**, 1.
19. Peters, T. J., Linnell, J. C., Matthews, D. M., and Hoffbrand, A. V. (1971) *Brit. J. Haematol.* **20**, 299.
20. Emmelot, P., Bos, C. J., Benedetti, E. L., and Rumke, PH. (1964) *Biochim. Biophys. Acta* **90**, 126.
21. Wattiaux, R., Wibo, M., and Baudhuin, P. (1963) *in* "Ciba Foundation Symposium on Lysosomes" (de Reuck, A. V. S., and Cameron, M. P., eds.), p. 176. Little, Brown, Boston.
22. Marchesi, V. T., Phil, D., and Barrnett, R. J. (1963) *J. Cell Biol.* **17**, 547.
23. Ericsson, J. L. E. (1969) *Exp. Cell Res.* **56**, 393.
24. Straus, W. (1964) *J. Cell Biol.* **20**, 497.
25. Jacques, P. (1960) *Arch. In. Physiol. Biochim.* **68**, 683.
26. Mego, J. L., Bertini, F., and McQueen, J. D. (1967) *J. Cell Biol.* **32**, 609.
27. Tan, C. H., and Hansen, H. J. (1968) *Proc. Soc. Exp. Biol. Med.* **127**, 740.
28. Finkler, A. E., Green, P., and Hall, C. A. (1971) *Fed. Proc.* **30**, 235.
29. Finkler, A. E., and Hall, C. A. (1967) *Arch. Biochem. Biophys.* **120**, 79.
30. Hom, B. L. (1967) *Scand. J. Haematol.* **4**, 321.
31. Stenman, V. H., Simons, K., and Grasbeck, R. (1968) *Scand. J. Clin. Lab. Invest.* **21**, 202.

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ABSORPTION, ELIMINATION AND EXCRETION OF ORALLY ADMINISTERED VITAMIN B_{12} IN NORMAL SUBJECTS AND IN PATIENTS WITH PERNICIOUS ANEMIA*

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With the Technical Assistance of
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BOSTON

A SELECTIVE defect of Vitamin B_{12} absorption is pathogenic of Addisonian pernicious anemia. The parenteral administration of crystalline vitamin B_{12} in daily doses of 1 to 5 microgm. to patients with pernicious anemia in relapse has been shown to produce a characteristic, optimal hematopoietic response.[1-5] Oral administration of crystalline vitamin B_{12} in daily doses of 5 microgm. to patients with pernicious anemia in relapse produces little or no hematopoietic response unless a source of intrinsic factor is administered either simultaneously or within six hours.[6,7] This persistent inability of patients with Addisonian pernicious anemia to absorb from the gastrointestinal tract the vitamin B_{12} in their daily food (approximately 5 to 15 microgm.) is the derangement that eventually results in a vitamin B_{12} deficiency state manifested clinically in many ways. The desirability of measuring the absorption of the dietary intake of vitamin B_{12} is evident. Efforts to do this by measurement of blood and urine with standard microbiologic-assay procedures have not succeeded because of the minute change in the amount of vitamin B_{12} in the blood or urine after ingestion of dietary quantities. It has not been possible

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to bioassay the unabsorbed portion of oral doses of vitamin B_{12} eliminated in the feces because of the presence of large amounts of material with vitamin B_{12} activity thought to be synthesized by bacteria in the lower intestine.

The biosynthesis in 1950 of cobalt^{60}-labeled vitamin B_{12} with a relatively high specific activity[8,9] has made it possible to measure extremely minute amounts of this material. In this laboratory it is possible to measure within ± 30 per cent radioactive vitamin B_{12} at a concentration of 8 micromicrogm. per milliliter. Soon after its biosynthesis, Heinle and his colleagues[10] employed radioactive vitamin B_{12} to determine the fecal elimination of a small oral dose given alone or with a source of intrinsic factor to patients with pernicious anemia. In the following year Schilling,[11] by ingeniously employing a massive parenteral "loading" dose of nonlabeled vitamin B_{12}, was able to demonstrate the absorption or relative nonabsorption of small oral doses of radioactive vitamin by measuring the urinary excretion of the labeled vitamin B_{12}. A few months later Glass et al.[12] measured the gamma-ray emission of cobalt^{60} at the body surface over the liver after ingestion of the radioactive vitamin and thus demonstrated in still another way the absorption or relative nonabsorption of vitamin B_{12}.

The experiments described below were undertaken to compare quantitatively the values for absorption of vitamin B_{12} as determined by the methods mentioned above and to evaluate these technics of measuring vitamin B_{12} absorption. Three normal persons and 3 patients with pernicious anemia in complete remission were each tested for intestinal absorption of the radioactive vitamin by measurement of fecal elimination, urinary excretion and hepatic deposition of cobalt^{60}. The diagnosis of pernicious anemia was established in the 3 patients by the demonstration of the presence of a macrocytic anemia and a megaloblastic erythroid hyperplasia of the bone marrow before therapy, as well as by histamine-fast achlorhydria. Each underwent a complete hematopoietic response after parenteral administration of vitamin B_{12}.

Methods

Fecal Elimination of Orally Administered Cobalt^{60}-Labeled Vitamin B_{12}

The same lot of radioactive vitamin was used in the preparation of all the oral test doses: dissolved crystalline cyanocobalamin cobalt^{60} with a specific activity of 0.77 microcuries per microgram. A ten-day collection of stools was obtained by having the patient defecate directly into a previously tared 4-liter screw-cap jar to which a small amount of formol had been added. All stool specimens during the ten-day period were collected in a single jar. Upon completion of the collection, an appropriate amount of distilled water was added to produce a suitable consistence for homogenization. This jar was then capped with a lid in which rotor blades were mounted and placed in an Osterizer, and the contents thoroughly homogenized. The jar and its contents were weighed. Samples approximately 25 ml. in volume were removed to suitable, previously tared vials, and then placed within a shielded Geiger-Müller counter under conditions of constant geometry. The counts per minute above background thus obtained were multiplied by the ratio of the total weight of the homogenized fecal collection to the weight of the sample counted. The total counting rate of the fecal collection was compared with the similarly determined total counting rate of the oral dose administered at the beginning of the fecal collection and expressed as the percentage

Table 1. *Absorption of Orally Administered Radioactive Vitamin B_{12}.*

Subject	Percentage in Feces	Percentage Not in Feces	Percentage in Liver	Hepatic Deposition of Ingested Radioactive Vitamin *c.p.m./microcurie*	Hepatic Deposition of Injected Radioactive Vitamin *c.p.m./microcurie*	Percentage in Urine
Normal persons:						
N.Z.	18.1	81.9		4085		10.3
A.N.	37.9	62.1		5300		7.3
F.C.	31.2	68.8	71.0	4108	5750	8.7
Patients with pernicious anemia:						
S.N.	85.3	14.7	10.4	390	3742	0.6
S.N., with added gastric juice	42.4	57.6	67.9	2541	3742	
G.A.	93.0	7.0	6.2	286	4633	0.6
G.A., with added gastric juice	47.8	52.2	58.6	2713	4633	
G.P.	95.0	5.0	8.5	334	3926	1.0
G.P., with added gastric juice	36.0	64.0	70.9	2790	3926	

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of the ingested cobalt^{60}-labeled vitamin B_{12} eliminated in the feces during the following ten-day period.

Urinary Excretion of Orally Administered Cobalt^{60}-Labeled Vitamin B_{12}

Twenty-five-milliliter aliquots of a twenty-four-hour collection of urine were counted, and the determined total urinary counting rate compared with the total counting rate of the oral dose of radioactive vitamin administered at the beginning of the urine collection. The counter described above was employed.

Hepatic Deposition of Cobalt^{60} after Oral Administration of Cobalt^{60}-Labeled Vitamin B_{12}

The crystal of a mobile scintillation counter head was placed on the skin of the subject overlying the middle of the liver in the mid-axillary line. A similar application to the lower left thigh was used to determine the background counting rate. In this manner hepatic counts per minute per microcurie of administered cobalt^{60}-labeled vitamin B_{12} were obtained before and ten days after ingestion. To obtain the hepatic counting rate resulting from "complete absorption," the procedure described above was subsequently repeated with the use of subcutaneous injection of an equal dose of radioactive vitamin. The hepatic counting rate after ingestion was compared with the hepatic counting rate after injection to determine the percentage of the orally administered radioactive vitamin that had been absorbed.

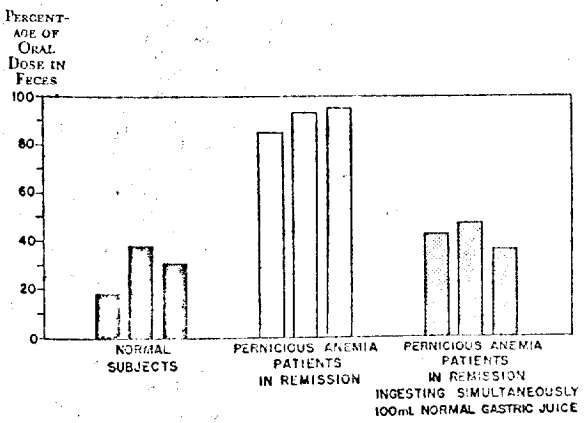

Figure 1. *Absorption of Vitamin B_{12} As Measured by Fecal Elimination of 0.5 Microgm. of Ingested Cobalt^{60}-Labeled Vitamin B_{12} in a Ten-Day Stool Collection.*

Experimental Procedures

Three patients with pernicious anemia in complete remission, and 3 normal persons were each monitored over the liver and thigh. They then received an oral dose of 0.5 microgm. of cobalt^{60}-labeled vitamin B_{12}. During the subsequent ten days stool collections were obtained in the manner stated above, and the fecal elimination of the oral dose determined. At the conclusion of this ten-day period the hepatic deposition of cobalt^{60} was ascertained. The same procedure was then repeated in the patients with pernicious anemia, 100 ml. of normal human gastric juice being administered simultaneously with the oral dose of 0.5 microgm. of radioactive vitamin. The urinary excretion was determined in the 6 subjects as follows. After a control specimen had been collected each subject was given an oral dose of 2.0 microgm. of cobalt^{60}-labeled vitamin B_{12}, and at the same time 1000 microgm. of unlabeled vitamin was injected subcutaneously. All urine passed during the next twenty-four hours was obtained from each subject, and the excretion of the orally administered vitamin determined. After the liver and thigh had been monitored, 1 normal subject and 3 patients with pernicious anemia were given a subcutaneous injection of 0.5 microgm. of cobalt^{60}-labeled vitamin B_{12}, and the hepatic deposition of the parenterally administered cobalt^{60} determined. Monitoring the injection sites before and shortly after injection, as well as ten days later, indicated that no significant activity remained at the injection site at the latter time.

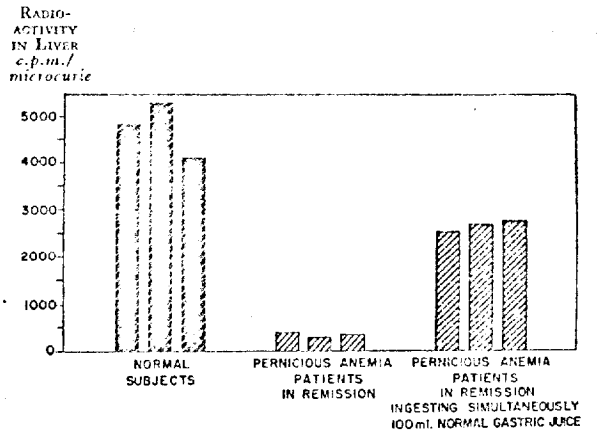

Figure 2. *Absorption of Vitamin B_{12} As Measured by Hepatic Deposition of Cobalt^{60} after Ingestion of 0.5 Microgm. of Cobalt^{60}-Labeled Vitamin B_{12}.*

Results

The percentages of oral doses of 0.5 microgm. of cobalt^{60}-labeled vitamin B_{12} eliminated in the stool during the ten-day collection period by the 3 normal subjects and the 3 patients with pernicious anemia in complete remission are shown in Table 1 and Figure 1. Whereas the former eliminated 18 to 38 per cent of the administered dose in their feces, the latter eliminated 85 to 95 per cent of the administered dose. When 100 ml. of filtered, neutralized normal gastric juice was given simultaneously with the oral dose to the patients with pernicious anemia, only 38 to 48 per cent of the administered dose was eliminated in the feces (Table 1 and Fig. 1). It is assumed that

most of the vitamin B_{12} not eliminated in the feces was absorbed. These values are shown in Table 1.

The radioactivity measured at the body surface over the liver ten days after ingestion of 0.5 microgm. is shown in Table 1 and Figure 2. These data were obtained after the same oral dose simultaneously analyzed for the percentage eliminated in the feces. Normal individuals deposited enough cobalt^{60} in the liver to produce hepatic body-surface counts above background, ranging from 4035 to 5300 counts per minute per microcurie ingested, as contrasted with patients with pernicious anemia, whose hepatic body-surface counts ranged from 286 to 390 counts per minute per microcurie. The subsequent addition of

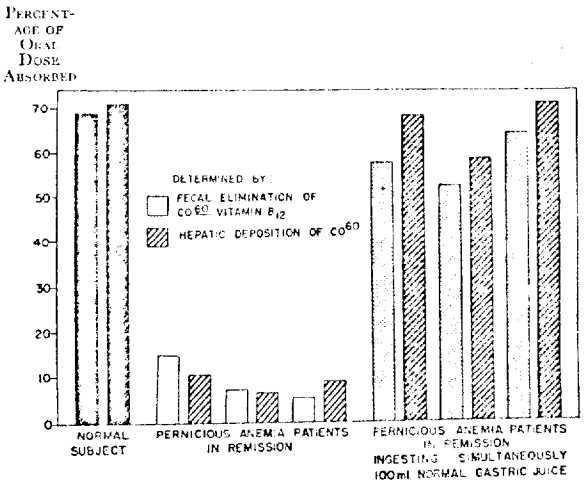

FIGURE 3. *Absorption of Vitamin B_{12} As Measured Simultaneously by Fecal Elimination of Vitamin B_{12} and Hepatic Deposition of Cobalt^{60} after Ingestion of 0.5 Microgm. of Cobalt^{60}-Labeled Vitamin B_{12}.*

100 ml. of normal gastric juice to an oral dose of 0.5 microgm. of cobalt^{60}-labeled vitamin B_{12} caused a rise of 2541 to 2790 counts per minute per microcurie. Ten days after the subcutaneous injection of 0.5 microgm. of radioactive vitamin, rises of hepatic body-surface counts varied from 3742 to 5750. No significant difference was found in hepatic deposition of cobalt^{60} between the normal persons and the patients with pernicious anemia when the vitamin was administered in this fashion. The percentage absorbed as determined by hepatic deposition is compared in Table 1 and Figure 3 with the corresponding percentage absorbed as calculated from measurements of fecal elimination. The values as determined simultaneously by the two methods are in fairly close agreement.

The twenty-four-hour urinary excretion of an oral dose of 2.0 microgm. of cobalt^{60}-labeled vitamin B_{12} administered simultaneously with a subcutaneous injection of 1000 microgm. of nonradioactive vitamin B_{12} is shown in Table 1 and Figure 4. The normal subjects excreted 7.3 to 10.3 per cent of the orally administered dose in the urine whereas the patients with pernicious anemia excreted only 0.6 to 1.0 per cent.

DISCUSSION

The small dose of 0.5 microgm. of vitamin B_{12} was selected for administration because the percentage of larger doses absorbed by normal persons decreases even when a large quantity of normal human gastric juice is ingested simultaneously. Thus, as much as 70 per cent of a 5.5-microgm. dose of orally administered cobalt^{60}-labeled vitamin B_{12} may be eliminated in the feces of a normal person.[10] At the 0.5-microgm. level the range of fecal elimination for the normal subjects (18 to 38 per cent) and the patients with pernicious anemia (85 to 95 per cent) is in good agreement with that reported in similar studies.[16] The reduction of fecal elimination of the vitamin in patients with pernicious anemia when 100 ml. of normal human gastric juice was ingested simultaneously with the cobalt^{60}-labeled vitamin B_{12} suggests that the abnormally high fecal elimination of radio-

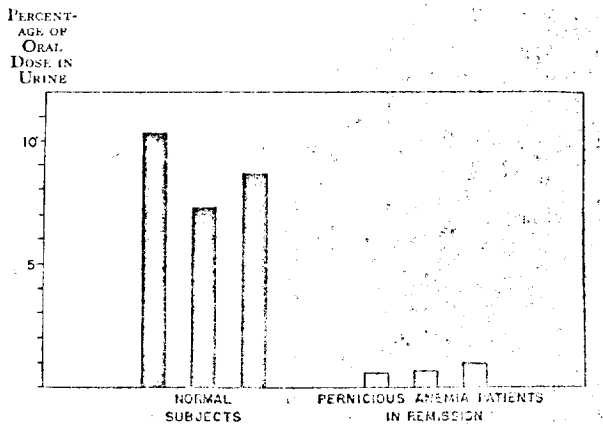

FIGURE 4. *Absorption of Vitamin B_{12} As Measured by Excretion of 0.5 Microgm. of Ingested Cobalt^{60}-Labeled Vitamin B_{12} in a Twenty-four-Hour Collection of Urine.*

active vitamin in these patients is due to a deficiency of intrinsic factor. The chief advantage of the fecal-elimination method of determining absorption is that it is a direct measurement of the amount of vitamin B_{12}, or at least of its cobalt moiety, eliminated. If the collection period is sufficiently long, the greater part of balance may be assumed to have been absorbed. The method has two minor disadvantages: ten days are required to ensure that no further radioactive vitamin will be eliminated after the collection is terminated; and the processing and counting of the stool collection takes time and is somewhat unpleasant. The major disadvantage, however, is the difficulty and uncertainty involved in obtaining a complete ten-day stool collection. To accomplish this a relatively long period of co-operation and vigilance is required from the subject during which he is greatly inconvenienced. Since the upper limit of the normal range

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(45 per cent) is not far removed from the lower limit of the pernicious anemia range (70 per cent),[10] the omission of a single critical bowel movement from the collection of a patient with pernicious anemia could easily lower the percentage of radioactive vitamin B_{12} recovered in the feces to the normal range.

The ranges of twenty-four-hour urinary excretion of orally administered vitamin B_{12} for normal persons (7.8 to 10.3 per cent) and patients with pernicious anemia (0.6 to 1.0 per cent) found in this study (Table 1 and Fig. 4) qualitatively confirm the results of Schilling[11] but are slightly at variance with his ranges for normal subjects (1.5 to 5.5 per cent) and patients with pernicious anemia (0 to 0.65 per cent). This method has the advantages of being relatively rapid and convenient in collection, processing and counting. In addition, the ranges of vitamin B_{12} excretion in the urine in normal subjects and patients with pernicious anemia are well separated from one another. The method has the disadvantages of being unsuited for measuring the percentage of the dose absorbed and of requiring a massive subcutaneous "flushing" dose of vitamin B_{12} that, by initiating maximal therapy prematurely in certain cases of pernicious anemia in relapse, may preclude other studies.

The premise that hepatic counting rates reflect the degree of absorption of cobalt^{60}-labeled vitamin B_{12} rests on the following observations of Glass[12] and our unpublished data.[13] Approximately a week after oral or parenteral administration of the radioactive vitamin to normal subjects or patients with pernicious anemia in remission the hepatic counting rate for gamma-ray emission of cobalt^{60} is maximal, and the liver is the only major site of deposition. During the subsequent thirty to one hundred days the hepatic counting rate remains above 90 per cent of the maximal rate attained at the end of the first week and at no other site is cobalt^{60} detectable. This apparently static deposition suggests that cobalt^{60} may be detached in some fashion from the vitamin B_{12} molecule and deposited in the liver while the physiologically active fraction of the vitamin B_{12} molecule travels elsewhere to perform its necessary functions. On the other hand, this may very well not be so, since normal human liver and the livers of patients with pernicious anemia who have received adequate treatment have been shown to contain sufficient amounts of potent material to keep other patients with pernicious anemia in remission for seven to thirty-six months.[14-16] Patients with pernicious anemia in relapse have had remissions persisting for two to twelve months after a single massive injection of 160 *U.S.P.* units of liver extract.[17] MacDonald, Ingelfinger and Belding[18] have noted that usually four or more years are required for pernicious anemia to develop after total gastrectomy when no vitamin B_{12} is administered parenterally. Halsted, Gasster and Drenick,[19] using the fecal-elimination method, have shown that the deficiency of intrinsic factor in patients after total gastrectomy is as complete as that existing in patients with pernicious anemia. It thus appears probable that the amount of vitamin B_{12} stored in the liver is usually more than five hundred times the daily body requirement. Under these circumstances it is understandable that, in fact, virtually all the vitamin B_{12} absorbed may be deposited in the liver and remain there for many months. The following recent observation by Pollycove, Arias and Apt[20] suggests either that cobalt^{60} is detached from an active moiety of vitamin B_{12} and deposited in the liver or that vitamin B_{12} is utilized by the liver to elaborate another substance that is utilized peripherally for erythropoiesis: after the subcutaneous injection into patients with pernicious anemia in relapse of 1 microgm. of cobalt^{60}-labeled vitamin B_{12}, there is a diagnostic reticulocyte response and a simultaneous deposition of cobalt^{60} in the liver equal to the deposition of cobalt^{60} in normal subjects or patients with pernicious anemia in remission. Since, at present, there is no direct evidence whether cobalt^{60} remains in the liver as an integral part of the vitamin B_{12} molecule, the phrase "hepatic deposition" is used here without any implication concerning the chemical status of cobalt^{60} within the liver.

The close agreement of the percentages of orally administered vitamin B_{12} absorbed as simultaneously determined by the methods of fecal elimination and hepatic deposition supports the hypothesis that the degree of hepatic deposition of cobalt^{60} corresponds to the degree of absorption of radioactive vitamin from the intestinal tract. Study of intestinal absorption of cobalt^{60}-labeled vitamin B_{12} by measurement of hepatic deposition of cobalt^{60} has the great advantage of requiring neither single nor collected specimens, and thus eliminates a most troublesome procedure and a frequent source of error. The measurements are simple, accurate and rapidly performed. This method also has the advantage of being able to quantitate the percentage of radioactive vitamin absorbed from the intestinal tract. The chief disadvantage is that a period of seven days must elapse before the measurement can be made, and a second such period is required if it is desired to determine the ratio of deposition between an oral and a parenteral dose.

All three methods of measuring absorption of vitamin B_{12} were applied to each subject to establish the degree of correlation between these methods. The data of absorption and urinary excretion on each subject are listed in Table 1 and charted in Figures 1, 2, 3 and 4. In general it is seen that intestinal absorption and urinary excretion of vitamin B_{12} are high in the normal persons and in the patients with pernicious anemia given normal human gastric juice simultaneously and low in the same patients not given the gastric juice.

These methods are especially useful in establishing

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or disproving the diagnosis of Addisonian pernicious anemia in patients with achlorhydria receiving maintenance liver or vitamin B_{12} therapy in whom the diagnosis has not been adequately established, patients with achlorhydria, whose macrocytic anemia and megaloblastic bone marrow is the result of folic acid deficiency, patients with signs and symptoms of subacute combined degeneration of the spinal cord but without significantly abnormal blood or bone-marrow findings, and the rare patient in whom, because of lack of extrinsic factor, a deficiency state identical with that of Addisonian pernicious anemia, except for the presence of intrinsic factor, develops. The findings in patients of the last two groups are presented and discussed elsewhere.[21,22]

The applicability of these methods to the assay of intrinsic-factor activity is most evident. They not only are more rapid and simple than classic assay by reticulocyte response but also possess the enormous advantage of being able to employ for intrinsic-factor assay the relatively large number of patients with pernicious anemia in remission as compared with the increasingly scarce number of untreated patients with pernicious anemia in relapse.

Summary and Conclusions

Determinations of vitamin B_{12} absorption by three methods employing radioactive (cobalt^{60}-labeled) vitamin B_{12} were obtained in normal subjects and in patients with pernicious anemia in remission. The absorption data so obtained were reasonably consistent. The ranges of vitamin B_{12} absorption in normal subjects and in patients with pernicious anemia are approximately 62 to 82 per cent and 5 to 15 per cent respectively, as determined by fecal elimination and hepatic deposition of cobalt^{60}. Urinary excretion of orally administered vitamin B_{12} was 7.3 to 10.3 per cent in normal subjects and 0.6 to 1.0 per cent in patients with pernicious anemia.

The clinical diagnostic value of these methods of measuring vitamin B_{12} absorption is indicated and discussed. These methods are especially useful in establishing or disproving the diagnosis of Addisonian pernicious anemia in the relatively large group of patients with achlorhydria who are receiving maintenance liver or vitamin B_{12} therapy but whose previous course was not adequately investigated or documented.

The advantages and disadvantages of the stool, urine and hepatic methods of measuring vitamin B_{12} absorption are discussed. The urinary-excretion method is the most rapid. The hepatic-deposition method combines quantitation of absorption with great simplicity and also with freedom from specimen collections, thus eliminating a troublesome and potentially large source of error. Measurement of the hepatic deposition of cobalt^{60} is the best method so far as simplicity, ease and accuracy are concerned. By dispensing with the necessity of resorting to clinical trial of therapeutic effects in patients with pernicious anemia in relapse, these methods should bring within comparatively close grasp the determination of the nature and exact mode of action of the intrinsic factor of Castle.

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References

1. West, R. Activity of vitamin B_{12} in Addisonian pernicious anemia. *Science* 107:398, 1948.
2. Berk, L., Denny-Brown, D., Finland, M., and Castle, W. B. Effectiveness of vitamin B_{12} in combined system disease: rapid regression of neurologic manifestations and absence of allergic reactions in patient sensitive to injectable liver extracts. *New Eng. J. Med.* 239:328-330, 1948.
3. Hall, B. E., and Campbell, D. C. Vitamin B_{12} therapy in pernicious anemia. I. Effect on hematopoietic system: preliminary report. *Proc. Staff Meet., Mayo Clin.* 23:561-570, 1948.
4. Jones, E., Darby, J., and Totter, J. R. Pernicious anemia and related anemias treated with vitamin B_{12}. *Blood* 4:627-644, 1949.
5. Mueller, J. F., Jarrold, T., Hawkins, V. R., and Viltter, R. W. Effect of vitamin B_{12} on hematologic and neurologic manifestations of pernicious anemia. *Ohio State M. J.* 46:225-229, 1950.
6. Berk, L., et al. Observations on etiologic relationship of achylia gastrica to pernicious anemia. X. Activity of vitamin B_{12} as food (extrinsic) factor. *New Eng. J. Med.* 239:911-913, 1948.
7. Hall, B. E., Morgan, E. H., and Campbell, D. C. Oral administration of vitamin B_{12} in pernicious anemia. I. Presence of intrinsic factor in Berkefeld-filtered pooled human gastric juices: preliminary report. *Proc. Staff Meet., Mayo Clin.* 24:99-107, 1949.
8. Smith, E. L., Hockenhill, D. J. D., and Quilter, A. R. J. Tracer studies with B_{12} vitamins. II. Biosynthesis of vitamin B_{12} labeled with ^{60}Co and ^{65}P. *Biochem. J.* 52:507, 1952.
9. Chaiet, L., Rosenblum, C., and Woodbury, D. T. Biosynthesis of radioactive B_{12} containing Cobalt^{60}. *Science* 111:[illegible]d, 1950.
10. Heinle, R. W., Welch, A. D., Scharf, V., McCleary, G. C., and Prasoff, W. H. Studies of excretion (and absorption) of Co^{60}-labeled vitamin B_{12} in pernicious anemia. *Tr. A. Am. Physicians* 65:214-222, 1952.
11. Schilling, R. F. Intrinsic factor studies. II. Effect of gastric juice on urinary excretion of radioactivity after oral administration of radioactive vitamin B_{12}. *J. Lab & Clin. Med.* 42:[illegible]-565, 1953.
12. Glass, G. B. J., Boyd, L. J., Gellin, G. A., and Stephanson, L. Uptake of radioactive vitamin B_{12} by liver in humans: test for measurement of intestinal absorption of vitamin B_{12} and intrinsic factor activity. *Arch. Biochem.* 51:251-257, 1954.
13. Pollycove, M., and Apt, L. Unpublished data.
14. Richter, O., Ivy, A. C., and Kim, M. S. Action of human "pernicious anemia liver extract." *Proc. Soc. Exper. Biol. & Med.* 79: 1093-1098, 1952.
15. Sebrell, W. H., and Harris, R. S. *The Vitamins.* Vol. 1. 676 pp. New York: Academic Press, 1954. P. 478.
16. Girdwood, R. H. Occurrence of growth factors for *Lactobacillus leichmannii*, *Streptococcus faecalis* and *Leuconostoc citrovorum* in tissues of pernicious anaemia patients and controls. *Biochem. J.* 52:58-63, 1952.
17. Strauss, M. B., and Dible, F. J. Duration of remission in pernicious anemia with liver therapy: efficacy of massive doses administered at one time. *J.A.M.A.* 144:1318-1320, 1950.
18. MacDonald, R. M., Jacobinger, F. J., and [illegible], H. W. Late effects of total gastrectomy in man. *New Eng. J. Med.* 237:[illegible]-[illegible], 1947.
19. Halsted, J. A., Gaston, M., and Dunlap, E. J. Absorption of radioactive vitamin B_{12} after total gastrectomy: relation to macrocytic anemia and to site of origin of Castle's intrinsic factor. *New Eng. J. Med.* 254:191-193, 1956.
20. Pollycove, M., Arias, I., and Apt, L. Unpublished data.
21. Arias, I. M., Apt, L., and Pollycove, M. [illegible] of radioactive vitamin B_{12} in normal subjects with [illegible] *Physiol.* *New Eng. J. Med.* 253:[illegible]-169, 1956.
22. Pollycove, M., Apt, L., and [illegible] due to dietary deficiency of vitamin B_{12}. *New Eng. J. Med.* 255: 164-169, 1956.

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