MONOGRAPH ON VITAMIN B_{12} #127 Vol II
Copies of articles cited in monograph summary

E32

Vitamin B12-Volume 2 #127 8/30/74

VOLUME 2

GRAS MONOGRAPH SERIES

VITAMIN B_{12}

(COPIES OF ARTICLES CITED IN MONOGRAPH SUMMARY)

prepared for

THE FOOD AND DRUG ADMINISTRATION
DEPARTMENT OF HEALTH, EDUCATION
AND WELFARE

AUGUST 30, 1974

This publication was prepared under Contract Number FDA 72-100
with the Public Health Service, Food and Drug Administration,
Department of Health, Education, and Welfare

prepared by

Tracor Jitco, Inc.

POLYCYTHEMIA FOLLOWING FOLIC ACID AND VITAMIN B_{12} TREATMENT OF ANTICONVULSANT-INDUCED MEGALOBLASTIC ANEMIA

Israel J. Med. Sci 6:49-52, 1970

J. AMIR, M.D., M. ELIAN, M.D. and A. DE VRIES, M.D., Ph.D.

Departments of Medicine D and of Neurology, Beilinson Hospital, Petah Tikva and Tel Aviv University Medical School, Israel

ABSTRACT

An epileptic patient with severe anticonvulsant-induced megaloblastic anemia developed polycythemia consequent to combined folic acid and vitamin B_{12} treatment. The polycythemia disappeared following withdrawal of the antianemic drugs. Noteworthy was the association of the megaloblastic anemia with severe leukopenia and thrombocytopenia, and low serum folic acid and iron values.

Polycythemia has been reported to develop following treatment of megaloblastic anemia; about 35 cases have been reported (1–4). All of these patients, except one, appear to have had Addisonian pernicious anemia, they were achlorhydric, responded to treatment with vitamin B_{12} or liver extract and showed no signs of other conditions associated with megaloblastic anemia. In 1963 Grønback and Larsen (5) described a patient who developed polycythemia following folic acid treatment for megaloblastic anemia induced by an anticonvulsant drug. The rarity of this condition prompted the present report of a similar case, to our knowledge the second of its kind.

CASE REPORT

S.I., a 19-year-old Ashkenazi Israel-born unmarried male was first seen in the Department of Medicine D on 28 October 1966. At the age of five he had begun to suffer from epileptic seizures of various types: general convulsions, right-sided Jacksonian fits and short-lasting psychomotor seizures. On several occasions he went into status epilepticus lasting for a whole day. In 1959 when he was twelve years old, the seizures increased in frequency, appearing daily despite the administration of anticonvulsive treatment which included hydantoin, LUMINAL® and MYSOLINE®. In 1965 hydantoin treatment was discontinued because of gingival hypertrophy. Since then he received Luminal, Mysoline and DIAMOX®. His family history was noncontributory.

A week prior to admission he had diarrhea and fever. He did not receive antibiotics, and although the diarrhea subsided in four days, his temperature remained high and he became pale and weak. On admission he was in a state of prostration and had pronounced pallor. His temperature was 39 C, his pulse 104/min, weak and regular, his blood pressure 90/70 mm Hg. The gingivae were swollen and appeared inflamed, the tongue was smooth and cherry red. Neck and chest findings were not remarkable. The abdomen was soft; liver and spleen were not palpable. Neurological examination was negative. Chest X-ray was normal. Laboratory examinations showed hemoglobin, 3.9 g/100 ml; red blood cell count (RBC),

10^6/mm^3; platelet count, 31,000/mm^3; reticulocytes, 2.3%; white blood cell count (WBC), 1,500/mm^3 with 26% polymorphonuclear granulocytes, 8% band forms, 60% lymphocytes and 6% monocytes. The peripheral blood smear showed numerous macrocytes, anisocytosis, poikilocytosis, Cabot's rings and several normoblasts. Many of the granulocytes showed multisegmented nuclei. The bone marrow showed hyperplasia of the red cell series with numerous megaloblasts, and maturation arrest of the white cell series at the myelocyte stage; a few megakaryocytes were seen with platelets around them. Bone marrow hemosiderin appeared slightly reduced. Erythrocyte sedimentation rate (ESR) was 85/105 mm (Westergren); blood urea, 35 mg/100 ml; electrolytes normal. Serum iron was 13 μg/100 ml, the unsaturated transferin was 270 μg/100 ml, serum vitamin B_{12} undetectable [microbiological assay based on the growth-promoting activity of the vitamin on a mutant strain of *Escherichia coli* (6)]; blood folic acid, 28 ng/ml [assayed with *Lactobacillus casei* ATCC No. 7469 (7), normal range 100 to 300 ng/ml]. Gastric analysis showed free acid. Schilling test was normal. The EEG was compatible with epilepsy.

Six units of blood were transfused within the first three days and treatment with parenteral vitamin B_{12} (1,000 μg/week), oral folic acid (30 mg/day) and ferrous sulfate (0.8 g/day) was instituted. The prostration disappeared following the first blood transfusion and the blood pressure rose to 120/80 mm Hg. Temperature became normal within four days and the reticulocyte count rose to 18% after 10 days of treatment (antibiotics had not been given). Additional examinations showed a normal X-ray study of the gastrointestinal tract and a fecal fat excretion of 5 g in 24 hr. After one month the patient's hemoglobin was 12.5 g/100 ml; RBC, 4.5×10^6/mm^3; WBC, 6,000/mm^3 with a normal differential count; the platelet count was 250,000/mm^3. The peripheral blood smear was normal; no normoblasts were found. The serum iron was 89 μg/100 ml. ESR was 12/24 mm (Westergren). In a repeated bone marrow biopsy, no megaloblasts were seen and maturation of the white cell series had become normal.

The patient was discharged on 30 November 1966 and remained under the observation of the Hematology Clinic. He continued to receive treatment with Luminal, Mysoline and Diamox, as well as parenteral vitamin B_{12} (200 μg/month) and oral folic acid (15 mg/day). Ambulatory examination three months later revealed a normal appearance of the tongue with adequate papillae.

One year later the patient began to complain of headache and dizziness, and pruritus after a hot bath. Examination on 10 November 1967 revealed an enlarged firm spleen, palpable 2 cm below the costal margin. The liver was not palpated. His hemoglobin was 17 to 19 g/100 ml; hematocrit, 54 to 59%; RBC, 5.5 to 6.7×10^6/mm^3; WBC, 9,500/mm^3; platelets 350,000/mm^3; ESR 1/3 mm (Westergren). The leukocyte alkaline phosphatase score was 180 (normal range 40 to 70). Blood volume examination showed a red blood cell mass of 44 ml/kg body wt (normal 28±2 ml/kg) and a plasma volume of 45 ml/kg (normal 40±2 ml/kg). An i.v. pyelogram was normal. Vitamin B_{12} and folic acid treatment was discontinued and phlebotomy performed four times, 400 ml each, during a period of eight months. The patient responded well, his headache, dizziness and pruritus disappeared. His hemoglobin level on recent examination (April 1969) was 15 g/100 ml; his red blood cell mass 31 ml/kg, his plasma volume 39 ml/kg. Routine hematologic values and peripheral blood smear were normal; the leukocyte alkaline phosphatase score was 60.

DISCUSSION

A variety of hematological complications may appear during anticonvulsive treatment: aplastic anemia (8, 9), leukopenia (10), thrombocytopenia (11), Coombs' positive hemolytic anemia (12), systemic lupus erythematosus (13). In 1954 Badenoch reported a hitherto undescribed form of megaloblastic anemia in two patients receiving an anticonvulsant drug (14). Since then some hundred cases have been described (15).

The patient described here had anticonvulsant-induced megaloblastic anemia. Consistent with this diagnosis were the gingival changes, the megaloblastic bone marrow, the macrocytosis and normoblastosis, the multisegmented nuclei of the granulocytes, the low serum vitamin B_{12} with free gastric acid and a normal Schilling test. Unusual was the association of the megaloblastic anemia with the severe leukopenia and thrombocytopenia,

and the decreased blood folic acid and serum iron values. No adequate explanation was found in the described patient for the latter two abnormal laboratory findings: patient's febrile disease associated with diarrhea prior to admission was of very short duration and, even on thorough interrogation, there was no history of prolonged inadequate food intake.

Anticonvulsant-induced megaloblastic anemia usually responds satisfactorily to folic acid treatment, even though the anemia may have been associated with a normal blood folic acid value (16). Possibly, in such cases the utilization of folic acid in the tissue is interfered with and large amounts of administered folic acid would be required to overcome the tissue deficit and to increase intestinal absorption impaired by the inhibitory action of anticonvulsant drugs on the intestinal polyglutamate depolymerizing enzyme (16).

In the patient described here, it is impossible to decide which of the two therapeutic agents, folic acid or vitamin B_{12}, has been active in restoring a normal hematological condition, although according to the literature (16) folic acid would be the most likely candidate. Hausser et al., reviewing the literature, found that folic acid never failed to correct the megaloblastic anemia when administered alone (16).

Regarding the mechanism underlying the development of the polycythemia following antianemic treatment in our patient, erythropoietin levels unfortunately were not measured. Also, in the literature on polycythemia following pernicious anemia, relevant data are lacking. Noteworthy is the disappearance of polycythemia in some of the reported cases consequent to discontinuation of the antianemic treatment (2, 5); in others, however, the polycythemia persisted. In our patient, fourteen months after discontinuing the antianemic treatment and seven months after the last phlebotomy, the polycythemia has disappeared, both hemoglobin level and red cell mass having become normal. It is too early to judge whether the polycythemia will not reappear in our patient. In this respect, it should be mentioned that, although his red blood cell mass has become normal, the splenomegaly persists. The possibility has to be considered that continued anticonvulsant therapy without associated antianemic treatment may again induce anemia. In that case the problem of finding a balanced anticonvulsant-antianemic treatment would arise.

Noteworthy is the absence of leukocytosis and thrombocytosis during the polycythemic phase. In the described cases of polycythemia following treatment of pernicious anemia, leukocytosis or thrombocytosis or both were almost constant features (2–5).

Received for publication 10 August 1969

REFERENCES

1. ROSNER, F., RUBENBERG, M. L. and ALTER, A. Pernicious anemia, polycythemia vera and acute myelogenous leukemia in same patient. *N.Y. St. J. Med.* **67**: 2119, 1967.
2. CHALMERS, J. N. and RICHARDS, U. Megaloblastic anemia alternating with polycythemia vera. *Brit. med. J.* **1**: 540, 1961.
3. KENNY, J. J. and BERMAN, H. J. Development of polycythemia vera after treatment for pernicious anemia: report of a case. *J. Amer. Geriat. Soc.* **11**: 632, 1963.
4. DOUGLAS, A. S. and RIFKIND, B. M. Megaloblastic anemia in association with polycythemia vera. *Scot. med. J.* **9**: 469, 1964.
5. GRØNBAK, P. and LARSEN, J. V. Polycythemia vera following treatment of megaloblastic anemia with folic acid. *Acta med. scand.* **174**: 781, 1963.
6. GROSSOWICZ, N., ARONOVITCH, J. and RACHMILEWITZ, M. Determination of vitamin B_{12} in human serum by a mutant strain of *Escherichia coli*. *Proc. Soc. exp. Biol. (N.Y.)* **87**: 513, 1954.
7. GROSSOWICZ, N., MANDELBAUM-SHAVIT, F., DAVIDOFF, R. and ARONOVITCH, J. Microbiologic determination of folic acid derivatives in blood. *Blood* **20**: 609, 1962.
8. ROBINS, M. M. Aplastic anemia secondary to anticonvulsants. *Amer. J. Dis. Child.* **104**: 614, 1962.
9. UNDERWOOD, L. C. Fatal bone marrow depression after treatment with acetozolamide (Diamox). *J. Amer. med. Ass.* **161**: 1477, 1956.
10. LIVINGSTONE, S. and BOKS, L. L. Use of the dione drugs in the treatment of epilepsy in children. *New Engl. J. Med.* **253**: 138, 1955.

11. REICHELDERFER, T. E., PEARSON, P. H. and LIVINGSTONE, S. Thrombocytopenic purpura occuring in association with paradione and dilantin sodium therapy. *J. Pediat.* 43: 43, 1953.
12. SNAPPER, I., MARKS, D., SCHWARTZ, L. and HOLLANDER, L. Hemolytic anemia secondary to mesantoin. *Ann. intern. Med.* 39: 619, 1953.
13. BENTON, J. W., TYNES, B., REGISTER, H. B., JR., ALFORD, C. and HOLLEY, H. C. Systemic lupus erythematosus occuring during anticonvulsive drug therapy. *J. Amer. med. Ass.* 180: 115, 1962.
14. BADENOCH, J. The use of labelled vitamin B_{12} and gastric biopsy in the investigation of anemia. *Proc. roy. Soc. Med.* 47: 426, 1954.
15. WINTROBE, M. M. "Clinical hematology," 6th edn. Philadelphia, Lea and Febiger, 1967, p. 552.
16. KIRBOE, F. and PLUM, C. M. Megaloblastic anemia developing during treatment of epilepsy. *Acta med. scand.* 179: 349, 1966.
17. HAUSSER, E., BERGOZ, R. and CROCI, D. L'anémie mégaloblastique provoquée par les médicaments antiépileptiques. *Nouv. Rev. franç. Hémat.* 4: 221, 1964.

MAIMONIDES ON THE IMMEDIATE GASTRIC RESORPTION OF ALCOHOL

היותר מפליג מכל הדברים שיזון מהר פתאום הוא היין. ועניין מהר: אחר שיקח אותו בזמן מועט; ואמרו פתאום: שיהיה אחר לקוחתו משלים הגוף מזונו כולו ולא ימשכהו מעט מעט, אבל בפעם אחת.

(פרוש הרמב"ם לפרקי היפוקרט, II, 18)

Wine above all other nourishment acts most quickly and suddenly. Quickly means a short time after intake; suddenly means the body takes nourishment completely and not gradually, but all at once.

(*Commentary to Hippocrates' Aphorisms, II, 18*)

Brit. Med. Jour. 4:167-168

Anon

TODAY'S DRUGS

With the help of expert contributors we print in this section articles on drugs in current use.

Vitamin B_{12}

Therapy with vitamin B_{12} is required in the treatment of vitamin B_{12} deficiency states and in patients who have permanently lost all ability to absorb vitamin B_{12} from the diet. On a normal mixed diet an adult may absorb up to 2 or 3 μg. of vitamin B_{12} from each meal, and if two or three adequate meals are taken each day between 4 and 8 μg. of the vitamin B_{12} can be absorbed.

Failure to absorb vitamin B_{12} may arise from *gastric causes*, such as pernicious anaemia, in which an atrophic process results in loss of adequate numbers of functioning gastric parietal cells, inadequate secretion of intrinsic factor, and hence impaired vitamin B_{12} absorption. Total gastrectomy always results in failure of absorption of vitamin B_{12}, and this is the case in some 30% of patients after partial gastrectomy, though only 5% to 6% in time get a megaloblastic anaemia due to vitamin B_{12} deficiency. Failure to absorb vitamin B_{12} may also be due to *intestinal causes*, such as disease or resection of the ileum or a disease process which results in an abnormal intestinal bacterial flora ("anatomical" abnormalities of the small gut), particularly small-intestinal diverticulosis, regional ileitis, strictures, and non-emptying loops such as after some Polya gastrectomies. The treatment in some of these cases may in the first instance be surgical, but in others treatment with vitamin B_{12} may be more important.

The diet taken by strict vegetarians or lactovegetarians often [illegible] to vitamin B_{12} deficiency, but this is a very rare cause [illegible]megaloblastic anaemia, probably because many of the prepared vegetable foods have a sufficiently high bacterial content to provide enough vitamin B_{12}.

Treatment with vitamin B_{12} should be given to patients with megaloblastic anaemia shown to be due to deficiency of the vitamin; prophylactically to patients after a total gastrectomy; and on occasion a patient may be seen—for example, after a partial gastrectomy—who has severe vitamin B_{12} malabsorption due to intrinsic factor deficiency: such a patient, too, should be started on vitamin B_{12} therapy. A further use is in neurological and ophthalmic practice, such as amblyopia and Leber's atrophy. But a lot more vitamin B_{12} is prescribed than could possibly be utilized for these legitimate reasons, because it is widely used as a "tonic." This has no scientific basis.

Aims in Vitamin B_{12} Therapy

In giving vitamin B_{12} in a deficiency state we set out to achieve:

(1) An optimal haematological and clinical response.

(2) A return of a normal blood picture and a normal clinical state.

(3) Maintenance of a normal serum level of vitamin B_{12}.

(4) A return to normal vitamin B_{12} stores, particularly in the liver.

It is clear that objectives 1 and 2 are the minimum, and indeed most schedules of therapy are also associated with the [illegible]enance of normal serum levels of vitamin B_{12}. Short of [illegible]biopsy it is difficult to determine whether repletion of [illegible] stores of vitamin B_{12} has taken place, and the extent to which modern vitamin B_{12} therapy achieves this is unknown.

Minimal Amount to Achieve a Response

The least amount of vitamin B_{12} that starts off a rise in the reticulocyte count may be about 0.1 μg. daily, but for an optimal response about 2 μg. must be supplied daily by a parenteral route. With such a dose the reticulocyte peak expected from the degree of anaemia will be reached on day 5, 6, or 7 after the start of therapy (day 0) and the red cell count (or its equivalent) will exceed 3 million/cu. mm. in the third week. As a single injection of vitamin B_{12} 80 μg. has been found to give marginally better rises in the red cell count than 40 μg., but 160 μg. produces no additional improvement.

Forms of Vitamin B_{12}

Vitamin B_{12} is available in three forms:

(1) Cyanocobalamin.

(2) Hydroxocobalamin.

(3) Combined with other substances.

The results in Table I show that hydroxocobalamin is retained in the body to a far greater extent than cyanocobalamin. Indeed, once the plasma binding capacity has been saturated cyanocobalamin is excreted by the kidney at a rate dependent only on the rate of blood flow through the glomeruli. Hydroxocobalamin binds more avidly to proteins, is cleared more slowly from the injection site, binds much more strongly to serum proteins, and greater amounts reach the liver as compared to cyanocobalamin.

TABLE I.—*Urinary Loss of Vitamin B_{12} After Parenteral Injection* (%)

Dosage μg	Cyanocobalamin	Hydroxocobalamin
100	45	8
500	70	23
1000	85	23

Preparations of cyanocobalamin linked to tannin in sesame oil/aluminium monostearate complex and as a zinc-tannate complex have been marketed. They do not offer any advantages over hydroxocobalamin. Thus of the preparations available hydroxocobalamin is easily the most satisfactory and the most economical. In some disorders, such as tobacco amblyopia and Leber's atrophy, hydroxocobalamin in large dosage may assist in transient detoxication of cyanide, though controlled evidence on these aspects is not available.

The normal body content of vitamin B_{12} is 3 mg., and the body stores can be replaced satisfactorily by four injections of 1,000 μg. hydroxocobalamin given at weekly intervals.

Maintenance should supply the amount normally lost from the body each day. This may vary from 2 to 5 μg. daily; so, assuming 4 μg. vitamin B_{12} as the daily requirement, this is met by an injection of 250 μg. of hydroxocobalamin monthly or 500 μg. every two months. Others have suggested that 4 injections of 1,000 μg. hydroxocobalamin on alternate days given once a year is adequate. All these schemes are likely to be fully adequate provided the patient does not default.

Another firm belief is that patients with vitamin B_{12} neuropathy require more vitamin B_{12} than those with only haematological manifestations. There is no good evidence upon which to base this view, and the best studies show that the amount of vitamin B_{12} that maintains a full haematological remission also results in the most rapid remission of neurological lesions.

313

Toxic Reactions

Vitamin B_{12} is remarkably free of side-effects. Whereas at least 7% of patients given liver injections became sensitive to other proteins in the extract reactions are very rare with vitamin B_{12}. Nevertheless, occasional cases of allergic sensitivity to vitamin B_{12}, anaphylactoid reactions, and a single fatality in a 48-year-old Indian has been reported.

Other Modes of Vitamin B_{12} Therapy

There are other ways of giving vitamin B_{12} to patients. Oral liver therapy is of historical interest only, but parenteral injections of liver concentrates are still used. Their action in pernicious anaemia depends only on their vitamin B_{12} content, which may be as little as 1 to 2 μg./ml. with some preparations. It is not surprising that haematological relapses have been recorded in pernicious anaemia in patients maintained on purified liver extracts.

Vitamin B_{12} may be given by mouth in very large amounts, when some will be absorbed by a mechanism, possibly passive diffusion, which is independent of the availability of intrinsic factor. Thus, if 500 to 1,000 μg. oral B_{12} is given daily an average 5 to 10 μg. will be absorbed. This method has proved a satisfactory alternative form of therapy in the rare patient sensitive to vitamin B_{12} by injection. Administration of 1,000 μg. weekly by mouth, however, fails to elevate serum vitamin B_{12} levels in pernicious anaemia.

Vitamin B_{12} may be given orally together with hog intrinsic factor, when an adequate amount will be absorbed in patients with pernicious anaemia. Unfortunately most patients treated in this way relapse because antibodies develop to hog intrinsic factor, and vitamin B_{12} so linked is no longed absorbed. This mode of therapy had a vogue, particularly in the Scandinavian countries in the 1950s, but it is not widely used today. Vitamin B_{12} inhaled as an aerosol is also readily absorbed, but this mode of administration is also not widely practised.

Response to Therapy

It is important when vitamin B_{12} is given for the first time to a patient suspected of having pernicious anaemia that the character of the response be assessed both clinically and by the changes in the blood. Absence of an optimal response suggests either that the diagnosis is wrong or that there are factors which have been missed which have interfered with the response, such as intercurrent infection of the chest or of the urinary tract, or a neoplasm of the stomach or elsewhere.

With an optimal response there is a dramatic change in the clinical state within 24 to 36 hours—perhaps most striking being the rapid return of an often voracious appetite. The serum iron level falls abruptly from elevated levels to low levels within 24 to 48 hours. A painful tongue is relieved in 48 hours, regeneration of papillae is evident in four to seven days, and it becomes normal in two to three weeks. Unexplained pyrexia may disappear in a few days. The patient goes into positive nitrogen balance. There may be some rise in blood uric acid levels.

The reversal of vitamin B_{12} neuropathy is related to the duration of the process. In all cases progress is arrested. Improvement continues for about six months and little more can be expected after the first year. The earliest evidence of response is loss of muscle tenderness and increase in strength of muscles. Symptoms of less than two to three months' duration usually disappear completely. Sphincter control is soon regained. Lost tendon reflexes invariably return.

The haematological response consists in a rise in the reticulocytes to a peak at day 5 to 7, and the approximate height of the peak is as shown in Table II.

TABLE II.—*Reticulocyte Response to Vitamin B_{12}*

Initial Red Cell Count/mm³	Expected Reticulocyte Peak %
1·0	40
1·5	30
2·0	20
2·5	15
3·0	10

More important is the response in the red cell count, which, as indicated, should exceed 3 million by the fifteenth day.

When the response has been suboptimal there is little or no clinical benefit, and the rise in reticulocyte count is late—day 8 to 12 or later—and is not as high at its peak as one expects from the degree of anaemia. On occasion such responses can even be entirely unrelated to any form of specific therapy, but may follow, for example, an oral dose of histidine given as part of the test of formiminoglutamic acid (Figlu) excretion. In some cases there may be no response whatsoever to vitamin B_{12} therapy, and in the presence of a megaloblastic process this must be regarded as good evidence that the process is due to folate deficiency.

Effects of a Biguanide (Metformin) on Vitamin B_{12} and Folic Acid Absorption and Intestinal Enzyme Activities

P. BERCHTOLD, A. DAHLQVIST,
A. GUSTAFSON & N.-G. ASP
Clinical Laboratory of Metabolic Research, First Medical Service,
Sahlgren's Hospital, University of Gothenburg, Gothenburg,
and Research Department I, E-Blocket, University Hospital,
Lund, Sweden

Berchtold, P., Dahlqvist, A., Gustafson, A. & Asp, N. G. 1971. Effects of a Biguanide (Metformin) on Vitamin B_{12} and Folic Acid Absorption and Intestinal Enzyme Activities. *Scand. J. Gastroent.* 6, 751-754.

The effects in vivo of a biguanide (Metformin) on intestinal enzyme activities and vitamin B_{12} and folic acid absorption can be summarized as follows. 1. The enzyme activities of the brush border of the mucosal cell of the upper intestine were influenced. 2. The disaccharidase activities were reduced, although not to such an extent as to be expected to impair disaccharide absorption. 3. Vitamin B_{12} absorption was significantly reduced, as measured by Schilling test. 4. Serum folic acid levels were significantly reduced, possibly due to a decreased intestinal absorption of dietary folic acid.

Key-words: Absorption; biguanide; enzyme activity; folic acid; intestine small; vitamin B_{12}

P. Berchtold, M. D., Dept. of Pharmacology, University of Southern California, Medical School, 2025 Zonal Av., Los Angeles, Ca. 900 33, USA

In previous studies disturbances in intestinal absorption during biguanide treatment have been observed. Effects have been demonstrated on the absorption of glucose (6, 12, 14), d-xylose (3, 16), amino acids (6), vitamin B_{12} (3, 18), and probably fat (3).

In order to elucidate the pathogenesis of this drug-induced malabsorption, we have studied the activity of disaccharidases and some related enzymes (hetero beta-galactosidase and acid beta-galactosidase) (1, 7) in intestinal biopsy specimens obtained before and after 2-10 weeks treatment with a biguanide (Metformin). Also, in another group of subjects, serum vitamin B_{12} and serum and whole blood folic acid levels have been studied before and after 2 months on Metformin.

MATERIALS AND METHODS

In five apparently healthy volunteers (4 males and 1 female, age range 25-52 years, mean age 39.4 years), a biopsy of the proximal jejunal mucosa was performed with a Watson capsule before and after 2-10 weeks on Metformin, 3 g daily. The biopsy specimens were analyzed for the following enzyme activities: maltase, isomaltase, sucrase, trehalase, lactase, hetero beta-galactosidase, and acid beta-galactosidase, according to methods of assay previously described (1, 7).

Vitamin B_{12} absorption was studied by the Schilling test with intrinsic factor (15), using a 24-hour specimen twice.

In another group of 6 hyperlipidemic subjects, 4 males and 2 females (age range 26-56 years, mean age 44.8 years), serum levels of vitamin B_{12} (13) and serum and whole blood levels of folic acid (10) were studied before and after 2 months on 1.5 g Metformin daily. Each subject served as his own control.

RESULTS

The results of the assay of enzyme activities in intestinal biopsy specimens and of the Schilling

Table I. Schilling test and enzyme activities in intestinal biopsy specimens before (B) and after (A) Metformin treatment

Pat.	Age (yrs)	Time of treatment (weeks)		Schilling test %	Biopsy specimen enzyme activities in units/g protein (normal values in parentheses*)						
					Maltase (266 ± 70)	Isomaltase (97 ± 30)	Sucrase (87 ± 29)	Trehalase	Lactase (44 ± 22)	Acid β galactosidase	Hetero β galactosidase
H.A.	29	10	B	16.8	353	101	84	29	34	– **	– **
			A	0.8	140	46	36	15	18	3.9	3.0
T.R.	47	2	B	15.3	355	133	99	34	39	4.2	0.1
			A	5.5	135	52	35	15	19	4.1	1.0
D.C.	25	5	B	19.0	183	81	50	24	34	4.1	2.2
			A	7.2	213	61	62	31	51	4.0	0.8
N.E.	50	2	B	17.7	201	78	49	22	34	4.0	2.0
			A	5.5	213	82	57	25	52	4.3	2.8
H.J.	52	2	B	19.2	269	84	67	35	53	5.3	4.9
			A	4.2	165	62	82	30	51	5.6	5.8
Mean			B	17.6	272	95	70	29	39	4.4***	2.3***
			A	4.6	173	61	54	23	38	4.5***	2.6***

* Mean ± SD (n = 22) ** Not analyzed. *** H.A. not included.

test, before and after Metformin treatment, are given in Table I. Among the intestinal disaccharidase activities, the mean values of maltase, isomaltase, and sucrase showed a tendency to decrease. This decrease was not of a magnitude that could be expected to impair the disaccharide absorption. The trehalase and lactase activities were not decreased. All these disaccharidases are located in the brush border of the mucosal cells. The acid beta-galactosidase, which is a lysosomal enzyme, and the hetero beta-galactosidase of the cytoplasm of the epithelial cells, were not influenced in their acivities by Metformin.

The mean value for the absorption of radioactive vitamin B_{12} in the Schilling test decreased significantly ($p<0.001$). One subject even reached a pathologically low value after 10 weeks of treatment (Table I).

Folic acid in serum decreased significantly ($p<0.01$) after two months on Metformin treatment, while serum vitamin B_{12} and folic acid levels in whole blood did not change significantly (Table II).

DISCUSSION

It appears generally accepted that the effects of Metformin include influence on the intestinal absorption. A high absorption rate for Metformin has been proven (2), and the highest concentration of biguanides has been detected in the upper portion of the intestine.

Table II. Means ± SD of serum vitamin B_{12}, serum and whole blood folic acid in 6 subjects before (B) and after (A) treatment for 2 months with 1.5 g Metformin daily (range of normal values in parentheses)

	Vitamin B_{12} serum (180–700 pg/ml)	Folic acid in whole blood (23–110 ng/ml)	Folic acid in serum (3.0–13.0 ng/ml)
B	398 ± 115	109 ± 41	6.33 ± 0.83
A	405 ± 74	70 ± 21	4.83 ± 0.57
p	n.s.	n.s.	0.01

The effects, however, of a biguanide on the intestinal wall cells have not earlier been investigated. In the present study the observed influence on the mucosal cell enzyme activities was a reduction, although a moderate one, in some of the disaccharidase activities (maltase, isomaltase and sucrase). It is of interest to note that these three activities belong to the same enzymological group and are closely located in the mucosal cell (4, 8).

The mean value of the lactase activity, known in intestinal diseases (4) to be more easily and severely affected than the other disaccharidases, was not influenced by the Metformin treatment. All these disaccharidases are located in the brush border of the mucosal cell.

Acid beta-galactosidase and hetero beta-galactosidase, located in the lysosomes and cytoplasm of the epithelial cells respectively, were not altered by Metformin treatment. Together these results suggest that the effects of Metformin are on the brush border of the epithelial cells of the upper portion of the intestine, and furthermore that the enzyme activities of the brush border are only partly involved.

The Schilling test, being a sensitive indicator of the absorption in the lower intestine, was significantly decreased by the Metformin treatment. This confirms earlier results on vitamin B_{12} absorption during biguanide treatment (3). The present results also showed that 2 months of Metformin treatment did not influence serum vitamin B_{12} levels. Most likely, this is due to the fact that the storage of vitamin B_{12} in the body is sufficient for several years even with a complete block in the B_{12} absorption (9, 11).

The observed reduced level of serum folic acid during Metformin treatment would suggest either an increased utilization or a decreased intake or intestinal absorption of folic acid (10). The absorption of dietary folic acid has recently (5) been demontrated to occur in duodenum and jejunum by an active process across the intestinal mucosal barrier. Furthermore, a deprivation of the dietary folic acid absorption by an inhibition of intestinal conjugase has been observed in patients receiving diphenylhydantoin or oral contraceptives (5). So far only two cases suspected of having folic acid deficiency anemia due to biguanide treatment have been reported (16).

In the light of knowledge gained in the present study on Metformin effects on intestinal enzymes, it is tempting to propose an influence by Metformin on intestinal conjugase also.

The relatively unchanged value of whole blood folic acid is taken as an indication that no significant depletion of folic acid stores has had time to occur during 2 months treatment with Metformin.

REFERENCES

1. Asp., N.-G. 1970. Rat small-intestinal β-galactosidase. Studies on the fractionation of 'acid' β-galactosidase with isoelectric focusing, gel filtration and ion exchange chromatography. *Biochem. J. 117*, 369-375.
2. Beckman, R. 1969. Resorption, Verteilung im Organismus und Ausscheidung von Metformin. *Diabetologia 5*, 318-324.
3. Berchtold, P., Bolli, P., Arbenz, U. & Keiser, G. 1969. Intestinale Absorptionsstörung infolge Metforminbehandlung (Zur Frage der Wirkungsweise der Biguanide). *Diabetologia 5*, 405-412.
4. Berg, N. O., Dahlqvist, A., Lindberg, T. & Nordén, A. 1971. In preparation.
5. Bernstein, L. H., Gutstein, S., Weiner, S. & Efron, G. 1970. The absorption and malabsorption of folic acid and its polyglutamates. *Amer. J. Med. 48*, 570-579.
6. Czyzyk, A. 1970. Effect of biguanides on the intestinal absorption of glucose and amino acids. Seventh Congress of the International Diabetes Federation. Excerpta med. Foundation, International Congress Series No. 209, p. 88, Amsterdam.
7. Dahlqvist, A. 1970. Assay of intestinal disaccharidases. *Enzym. biol. clin. 11*, 52-66.
8. Dahlqvist, A. & Telenius, U. 1969. Column chromatography of human small-intestinal maltase, isomaltase and invertase activities. *Biochem. J. 111*, 139-146.
9. Gräsbeck, R. 1960. Physiology and pathology of vitamin B_{12} absorption, distribution and excretion. *Advanc. clin. Chem. 3*, 299-366.
10. Hansen, H. A. 1964. On the diagnosis of folic acid deficiency. A methodological and clinical study with special reference to the incidence and etiology of megaloblastic anaemias in

pregnancy and puerperium. Diss. Göteborg, 1964.

11. Heyssel, R. M., Bozian, R. C., Darby, W. J. & Bell, M. C. 1966. Vitamin B_{12} turnover in man. *Amer. J. clin. Nutr. 18*, 176-184.
12. Hollobaugh, S. L., Rao, M. B. & Kruger, F. A. 1970. Studies on the site and mechanism of action of Phenformin. I. Evidence for significant 'nonperipheral' effects of phenformin on glucose metabolism in normal subjects. *Diabetes 19*, 45-49.
13. Hutner, S. H., Bach, M. K. & Ross, G. I. M. 1956. A sugar-containing basal medium for vitamin B_{12} assay with Euglena; application to body fluids. *J. Protozool. 3*, 101-112.
14. Kruger, F. A., Altschuld, R. A., Hollobaugh, S. L. & Jewett, B. 1970. Studies on the site and mechanism of action of Phenformin. II. Phenformin inhibition of glucose transport by rat intestine. *Diabetes 19*, 50-52.
15. Schilling, R. F. 1954. Recent studies of intrinsic factor and the utilization of radioactive vitamin B_{12}. *Fed. Proc. 13*, 769-775.
16. Stowers, J. M. & Bewsher, P. D. 1969. Studies on the mechanism of weight reduction by phenformin. *Postgrad. med. J.* Suppl. *45*, 13-19.
17. Tomkin, G. H., Hadden, D. R., Weaver, J. A. & Montgomery D. A. D. 1971. Vitamin-B_{12} status of patients on long-term Metformin therapy. *Brit. med. J. 2*, 685-687.
18. Willms, B. & Creutzfeldt, W. 1970. Contribution to the intestinal resorption of vitamin B_{12} (Schilling-Test) and of d-xylose in biguanide treatment. *Diabetologia 6*, 652 abstract.

Received 26 June 1971
Accepted 22 July 1971

Acta med. scand. Vol. 184, pp. 247–258, 1968

ORAL TREATMENT OF PERNICIOUS ANEMIA WITH HIGH DOSES OF VITAMIN B_{12} WITHOUT INTRINSIC FACTOR

Hans Berlin, Ragnar Berlin and Gunnar Brante

From AB Kabi, Stockholm, the Department of Internal Medicine, Region Hospital, Linköping, and the Department of Clinical Chemistry, Central County Hospital, Eskilstuna, Sweden

Abstract. The concluded results of an experimental study are reported, confirming that the absorption of vitamin B_{12} takes place in two ways: 1. through the mediation of intrinsic factor; 2. without mediation of intrinsic factor. The former mechanism allows a B_{12} absorption in normally IF-sensitive cases of pernicious anemia and in normal persons with an upper limit of about 2 μg. The latter mechanism allows an absorption of vitamin B_{12} which is roughly proportional to the oral dose administered, and amounts to about 1.2% of the dose, within a very wide dose range. Thus, it is possible to increase the uptake to any desired level. This direct uptake was found to be of the same magnitude, irrespective of whether the patient had a normal absorption, was suffering from pernicious anemia with or without resistance to intrinsic factor preparations, or had some other form of disturbed B_{12} absorption (idiopathic malabsorption, postoperative state after extensive gastric or intestinal resections, or ileitis). The result of a long-term clinical trial is also reported, in which a daily oral dose of 500–1000 μg of vitamin B_{12} without intrinsic factor was given to patients with pernicious anemia and other types of vitamin B_{12} deficiency. The material comprised 64 patients followed for up to more than 5 years. After the remission period very few serum B_{12} determinations showed borderline or subnormal values, and low levels were never of long duration. At the conclusion of the study the serum B_{12} values were well within the normal range in all cases. Also the individual mean values of the B_{12} determinations, made during the observation period from the second month of treatment, were normal in all cases. No neurological complications were observed. The blood values were normal in all but a few cases, in which concurrent diseases were present (malignancy, chronic infections, iron deficiency states). Experimental evidence obtained indicates that oral treatment with 500 μg of B_{12} daily has also resulted in replenishment of the B_{12} depots. The treatment with high oral doses of vitamin B_{12} thus constitutes a convenient and completely reliable maintenance therapy in pernicious anemia and other states of B_{12} deficiency, and is a fully acceptable alternative to the conventional method of vitamin B_{12} injections. This type of therapy (1000 μg B_{12} daily) was introduced into Sweden in 1964 and is now extensively used.

During recent years many reports have been published suggesting that high doses of vitamin B_{12} alone, administered orally, can produce a satisfactory effect on pernicious anemia (3, 4, 6, 8, 9, 10, 11, 12, 15, 16, 17, 18, 19, 23, 24, 25, 26, 27). However, other investigators have not studied quantitatively how pernicious anemia patients absorb vitamin B_{12} from the intestine without the mediation of intrinsic factor (IF), nor have they studied the absorption process in cases of pernicious anemia with an acquired resistance to IF preparations or in other types of B_{12} deficiency. Furthermore, most earlier clinical studies have been made with lower B_{12} doses which cannot be expected to give a safe long-term effect in all cases. In the present report an account is given of the quantitative *experimental studies*, presented in part in earlier papers (3, 4), which confirmed that absorption of vitamin B_{12} takes place in two ways:

1. *Absorption through the mediation of IF*

Quantitative absorption studies employing the radioactive isotope technique have shown that the absorption of a certain quantity of B_{12}, administered together with an IF preparation, increases when the IF dose is increased, but only up to a certain maximum (1, 4). When the dose of B_{12}, given in combination with an optimum amount of IF, is varied within the range of 1–100 μg, normally IF-sensitive patients with pernicious anemia absorb, on an average, a maximum of 0.5–2.2 μg of the given dose, or about the same quantity as the uptake in healthy subjects producing their own IF (3, 4, and present studies). Patients with partial resistance to IF preparations absorb less of the B_{12} dose, but can, in most

cases, be induced to absorb the same quantity of B_{12} if the IF dose is greatly increased (1).

2. *Absorption without mediation of IF*

The studies presented in this paper, which were briefly reported earlier, demonstrate that *the amount of vitamin B_{12} absorbed from the intestine, without the mediation of IF, corresponds to approx. 1.2% of the dose administered, within a very wide dose range.* (In a previous report (4) the uptake was calculated to be approx. 1.5%. The new average figure, 1.2%, is derived from a more comprehensive material and based on statistical analysis.)

Thus, by increasing the B_{12} dose sufficiently, it is possible to increase the uptake to any desired level, since a fairly constant ratio between dose and uptake has been demonstrated throughout the entire dose range from 1 μg to 100,000 μg (100 mg!). The mechanism is not known, but the demonstrated relationship between dose and uptake indicates the possibility of a passive diffusion process, for which we have used the term "direct uptake" to differentiate from uptake mediated by IF.

It is particularly noteworthy that the direct uptake was found, on an average, to be of the same magnitude, irrespective of whether the patient had completely normal absorption, was suffering from pernicious anemia with or without resistance to IF preparations, or had some other form of disturbed absorption due to idiopathic malabsorption, ileitis, total gastrectomy, ileoectomy or jejunectomy.

Finally, a summary is given of long-term *clinical trials* in pernicious anemia, up to more than 5 years, in which oral doses of 500–1000 μg vitamin B_{12} without IF were administered daily. A preliminary report has been presented earlier (4). The results have shown that the treatment is definitely safe and provides a fully acceptable alternative to injection therapy (Behepan tablets, 1 mg, KABI). It is effective both in pernicious anemia and in other forms of B_{12} malabsorption. Thus, older preparations containing IF together with vitamin B_{12}, which are less reliable, are, in our opinion, no longer justified (4, 5).

MATERIAL AND METHODS

In the experimental studies the oral B_{12} doses were tagged with B_{12}–Co^{60} or B_{12}–Co^{58} (Merck & Co.). In most experiments, where a very small fraction of the dose was expected to be absorbed, 0.75 μC was given as B_{12}–Co^{58}. Otherwise a radioactivity dose of 0.1–0.5 μC was administered.

The IF concentrate used was a dry commercial preparation from hog pylorus mucosa, with a B_{12}-binding capacity of approx. 0.2 μg of vitamin B_{12} per mg (2). Of this material 50 mg exert an optimal IF effect with 1 μg of vitamin B_{12} in ordinary pernicious anemia. The IF dose needed for maximal absorption increases up to about 500 mg when the B_{12} dose is increased to 10 μg or more (3).

The dose of tagged B_{12} was mixed with the IF concentrate (if any) in water prior to administration. The doses were given on an empty stomach, if not otherwise specified.

The meal, used in some tests, was standardized to contain bread, butter, cheese, milk, and coffee. In these tests the dose was given one-half hour after the meal.

An intramuscular flush dose of 1000 μg of non-radioactive cyanocobalamin was given 2 hours after the dose.

After each dose 0–24 h urine collections were made. Samples of the urines were evaporated to one-tenth of their original volume. The radioactivity of 5 ml portions of the concentrates was measured with a Tracerlab detector in an arrangement for spectrometric analysis of Co^{60} or Co^{58} peaks.

The uptake of vitamin B_{12} was calculated according to Callender and Evans (7) from the 24-hour urinary radioactivity excretion after conversion of radioactivity to μg of vitamin B_{12} and multiplication by 3. The value thus obtained is not exact, since it is based on measurement of excretion, not absorption. However, as discussed in this and earlier reports (3, 4), this method gives an approximate value of the amount of B_{12} absorbed which is sufficiently reliable for the validity of the practical conclusions drawn.

The diagnoses of the different B_{12} deficiency states included in the clinical study were established in each case by the use of a battery of examinations including ordinary hematological tests, bone marrow aspiration, determination of serum-B_{12}, analysis of gastric juice, Schilling tests (with few exceptions), and other tolerance and balance tests of gastrointestinal function. The serum-B_{12} determinations were made with the Euglena gracilis technique by Dr A. Killander, Institute of Medical Chemistry, University of Uppsala, Sweden.

EXPERIMENTAL STUDIES

Before clinical studies were started, extensive experiments were carried out to study the absorption of radioactive vitamin B_{12} given orally, with and without IF, at varying doses, and in various clinical conditions.

Vitamin B_{12} without IF in pernicious anemia

Tests were carried out with doses varying from 1 μg to 100,000 μg. Fig. 1 shows the calculated uptake in a double logarithmic diagram. For each dose level mean values of calculated uptake and standard deviation are plotted, and the number of patients tested is specified.

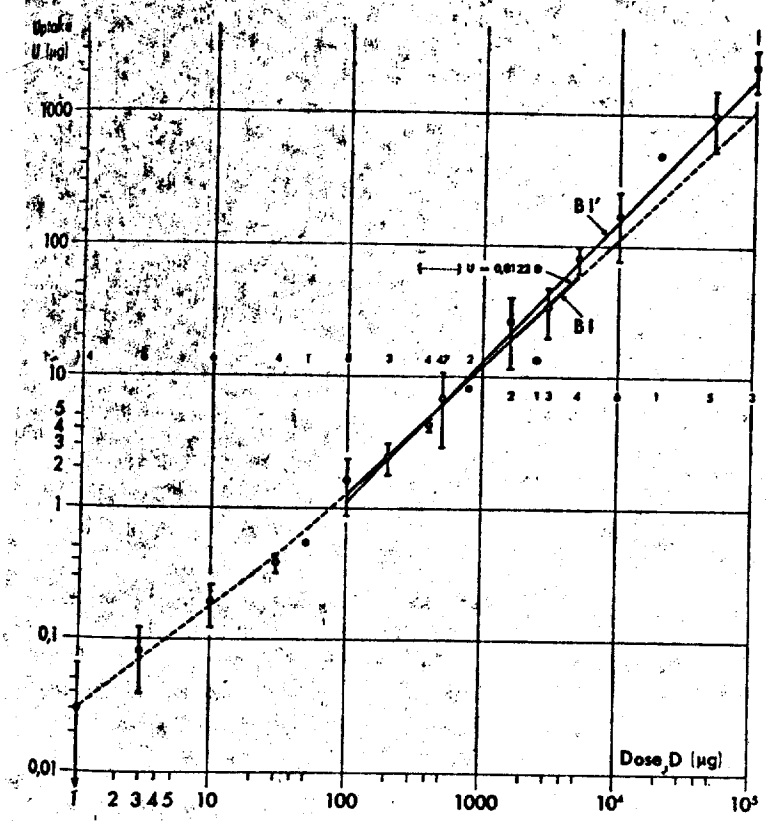


Fig. 1. Calculated uptake of vitamin B_{12}, given orally without intrinsic factor to pernicious anemia patients. Mean, standard deviation, and number of subjects are specified for every dose level. *BI'* is calculated regression line for interval 100–100,000 µg. *BI* is calculated regression line for interval 100–5000 µg; coincides closely with $U = 0.0122\ D$, i.e. uptake = 1.22% of dose.

BI' is regression line for the interval 100–100,000 µg, BI for the limited interval 100–5000 µg.

BI was calculated separately for two reasons:

1. this range has a greater interest for clinical practice;
2. at doses exceeding 5000 µg a significant urinary excretion is obtained also without flush; for this and other reasons (see below) the uptake as calculated may be slightly too high in this upper region.

Equation for regression line BI' (100–100,000 µg):

$$U = 0.00798 \cdot D^{1.071}.$$

Equation for regression line BI (100–5000 µg):

$$U \cong 0.0146 \cdot D^{0.973}.$$

It is interesting to note that the equation for BI, within its interval, coincides very closely with the simple equation $U = 0.0122 \cdot D$, i.e. the calculated uptake equals 1.22% of the dose.

Consequently, the average uptake of vitamin B_{12}, given orally without IF in pernicious anemia, is about 1.2% of the dose within a very wide dose range.

The regression lines do not comprise the range below 100 µg, since at low doses the average relative uptake is higher, as evident from Fig. 1. The natural explanation is the presence of some IF activity remaining in some patients, which will influence the total average uptake at low doses.

Vitamin B_{12} without IF in subjects with normal absorption

Tests were carried out with doses from 1 to 800 µg. Fig. 2, curve BII, shows the calculated uptake. Mean values, standard deviations, and number of subjects tested are specified as in Fig. 1.

The curve has been drawn by manual fitting. No regression line has been calculated, since it cannot be expected to be a straight line, and the material is not comprehensive enough to warrant, with any certainty, the calculation of a curve with an equation of a higher degree.

Regression line BI (see Fig. 1) is included for comparison.

The result shows that the average absorption at doses from 1 to 100 µg increases slowly from approx. 0.5 to 2 µg. As a consequence of the presence of IF in the subjects' gastric juice, this absorption is far above the absorption found in pernicious anemia (Fig. 1). At higher dosage the absorption curve rises more rapidly, as the direct,

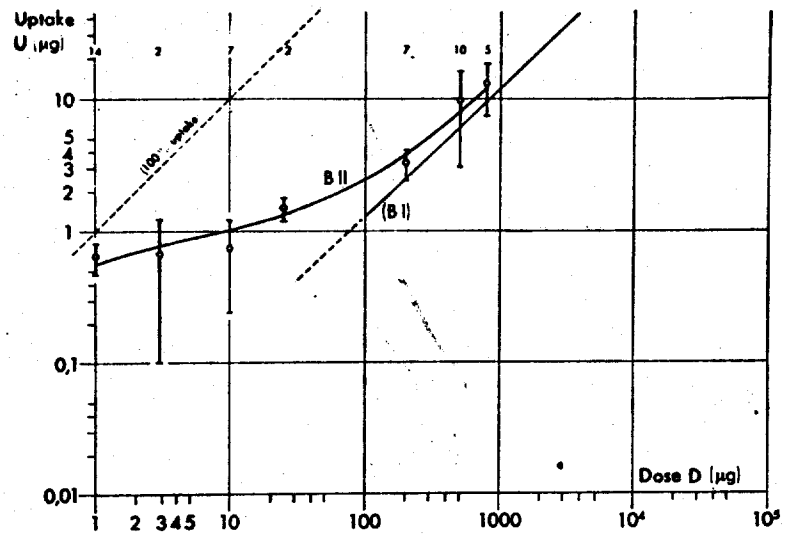


Fig. 2. Calculated uptake of vitamin B_{12}, given orally without intrinsic factor to test subjects with normal B_{12} absorption. Mean, standard deviation, and number of subjects are specified for every dose level. Curve BII is drawn by manual fitting. Regression line *BI* (Fig. 1) is included for comparison.

proportional uptake dominates over the IF-mediated absorption. The difference between healthy subjects and pernicious anemia patients becomes smaller, as the dose is increased, showing that the IF-mediated absorption is limited and of little importance for the total uptake at high B_{12} doses.

The B_{12} uptake in normal subjects has also been studied by Gaffney et al. (12), Heinrich et al.

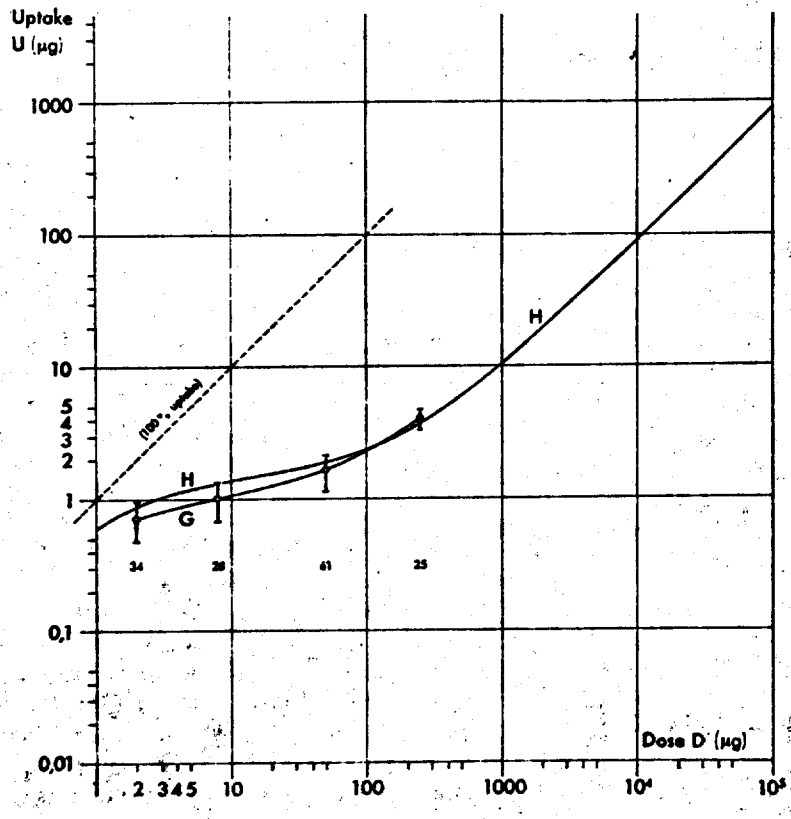


Fig. 3. Calculated uptake of vitamin B_{12}, given orally without intrinsic factor to normal subjects, according to other investigators. *G*, Uptake calculated from urinary excretion values found by Gaffney et al. (12). Mean, standard deviation, and number of subjects are specified for every dose level. The curve is drawn by manual fitting. *H*, Curve according to an equation for the relationship between dose and uptake, suggested by Heinrich et al. (15).

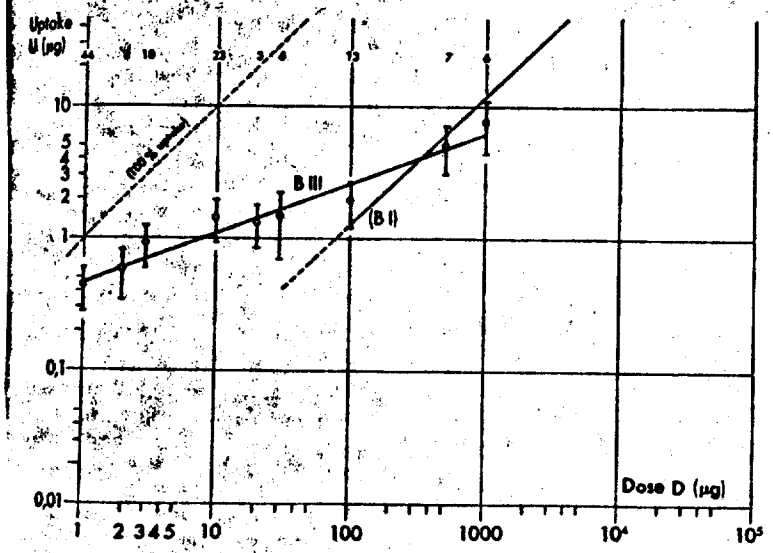


Fig. 4. Calculated uptake of vitamin B_{12}, given orally with an adequate dose of hog intrinsic factor to pernicious anemia patients. Mean, standard deviation, and number of subjects are specified for every dose level. *BIII* is calculated regression line. Regression line *BI* (Fig. 1) is included for comparison.

(15), and Heinrich and Wolfsteller (16). Their results are presented in Fig. 3, curves G and H, respectively.

The average absorption values and standard deviations plotted for curve G were calculated from urinary radioactivity excretion values presented by Gaffney et al. in the same way as the uptake was calculated in our own studies.

Curve H represents an equation suggested by Heinrich et al. for the relationship between dose and total uptake in healthy subjects:

$$U = \frac{1.5D}{D+1.5} + \left(1 - \frac{1.5}{D+1.5}\right) \cdot 0.009\,D.$$

This equation was based on values obtained from total body measurements of absorbed radioactivity after oral administration of radioactive B_{12}.

It is evident that curves G and H differ very little, and they are also in good agreement with our curve BII.

Since the B_{12} absorption ought to be about the same in pernicious anemia patients as in healthy subjects, when the doses are high (mainly direct absorption independent of IF), one might also expect good agreement between the upper part of curve H and our curve BI. Above a dose of 1000 µg Heinrich found in healthy subjects 0.9–1% absorption (total body measurement), while we found in pernicious anemia approx. 1.2% absorption between 100 and 5000 µg (calculated from urinary excretion). The agreement is fairly good, considering the very different techniques used.

Vitamin B_{12} with IF in pernicious anemia

Tests were carried out with doses from 1 to 1000 µg vitamin B_{12} together with an optimal amount of IF concentrate. The subjects were ordinary pernicious anemia patients, i.e. were known not to have developed refractoriness to hog IF. From earlier studies (1) we know that IF-refractory patients absorb much less B_{12} in this test, unless the IF dose is increased very considerably.

Fig. 4 shows the calculated uptake. Mean values, standard deviations, and number of subjects tested are specified as before.

Curve BIII is the regression line calculated from the values obtained.

Equation for regression line BIII:

$$U = 0.455 \cdot D^{0.374}.$$

Theoretically, the curve cannot be a straight line in this type of diagram, since it would cross the 100% absorption limit, if extended downwards. Within the interval, however, it ought to give an acceptable description of the relationship between dose and uptake.

As expected, the uptake is, within a wide range, approximately the same as that obtained by vitamin B_{12} alone in normal subjects (curve BII). At high B_{12} doses and correspondingly high IF doses, however, the uptake becomes lower than that obtained from B_{12} without IF in pernicious anemia patients or in normal subjects. Obviously the direct B_{12} uptake is hampered by IF and other B_{12}-binding material in the hog IF concentrate. At B_{12} doses from a few hundred µg and up, the IF dose was far higher than the amount normally present in the gastric juice of a healthy subject.

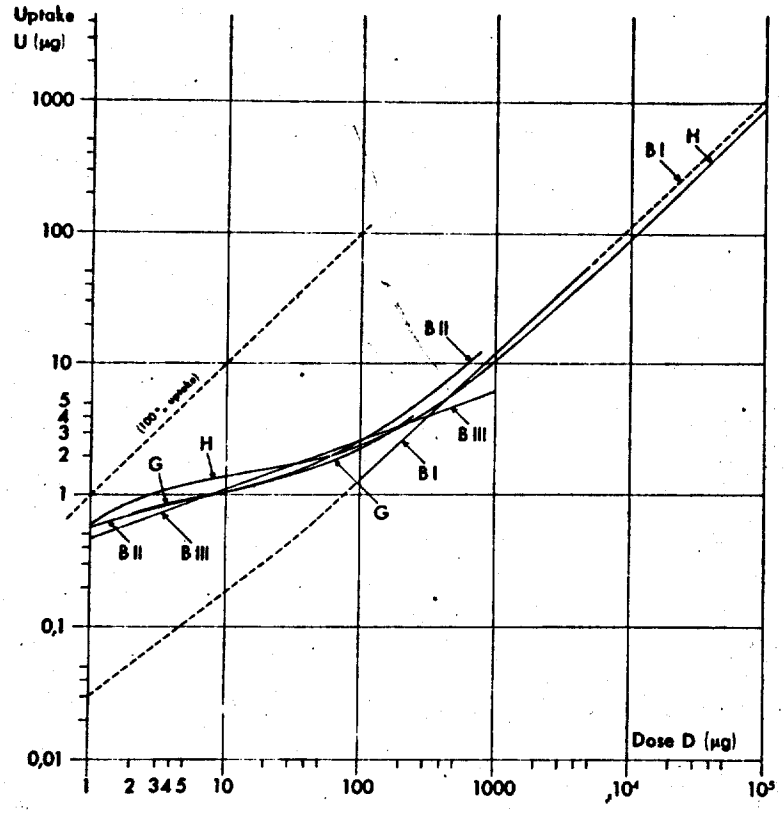


Fig. 5. Calculated uptake of vitamin B_{12}; curves presented in Figs. 1–4, combined for direct comparison. B_{12} without intrinsic factor in pernicious anemia: *BI*, Berlin et al., regression line for interval 100–5000 µg. B_{12} without intrinsic factor to normal subjects: *BII*, Berlin et al., curve by manual fitting; *G*, Gaffney et al. (12), curve by manual fitting; *H*, Heinrich et al. (15), according to equation. B_{12} with hog intrinsic factor in pernicious anemia: *BIII*, Berlin et al., regression line for interval 1–1000 µg.

In Fig. 5 our curves BI, BII and BIII are presented in one diagram together with curve G and curve H from Fig. 3. Here a direct comparison of the uptake under various conditions can be made, and also a comparison of our results with those of other investigators.

Vitamin B_{12} without IF in various conditions; individual variations

In addition to the experiments reported above a series of tests has also been made in totally gastrectomized patients, and in cases of other types of B_{12} malabsorption.

Table I shows the calculated uptake in different conditions at the 500 µg dose, the level for which the largest number of determinations is available.

It is evident that the uptake of a high dose of vitamin B_{12} alone is largely the same, regardless of the condition of the subject. However, the average uptake in pernicious anemia patients tends to be slightly lower than in normals, probably as a consequence of the lack of IF in their gastric juice. A small difference of this magnitude is to be expected. At this dose level the IF-mediated portion of the total uptake is low, compared to the direct uptake (cf. curves BI and BII). The direct uptake in pernicious anemia may also be expected to be about the same, whether the patients have developed refractoriness to hog IF or not.

Fig. 6 illustrates the variation at the 500 µg dose level in the entire material. Only 3 out of the 74 subjects showed an uptake below 3 µg (the lowest value is 1.8 µg), and 8 had values exceeding 15 µg or 3% of the dose. The majority (70%) showed an uptake within the interval 4–10 µg or 0.8–2% of the dose.

Effect of flush dose at high B_{12} doses

If the oral B_{12} dose is very high, the uptake approaches or exceeds 1000 µg, i.e. the size of the intramuscular flush dose. For this reason a significant excretion can be expected from high oral B_{12} doses also without flush.

A series of tests was carried out with and without flush, when B_{12} doses of 500 µg and more

Table I. *Calculated uptake of vitamin B_{12} (μg) in healthy subjects and in various pathological states after an oral dose of 500 μg of radioactive B_{12} on an empty stomach*

In brackets are given mean values after the omission of extreme values (< 3μg and > 15 μg)

Normal subjects (10 cases)	Pernicious anemia, IF-sensitive (37 cases)			Pernicious anemia, IF-refractory (9 cases)	Mal-absorption (13 cases)
3.5	2.6	5.6	7.2	1.8	3.3
5.4	2.7	5.6	7.4	3.5	4.5
5.6	3.6	5.7	8.3	4.1	4.5
6.3	3.8	5.9	8.4	4.2	4.8
7.5	3.9	5.9	8.4	5.6	5.1
7.7	4.4	5.9	9.0	6.2	6.3
8.7	4.8	5.9	9.6	6.3	6.8
11.7	4.8	6.3	13.4	9.6	7.7
14.0	5.0	6.5	13.8	14.0	12.5
26.3	5.0	6.6	16.5	Mean 6.1 μg	16.1
Mean 9.7 μg	5.0	6.8	21.9	(6.7 μg)	16.4
(7.8 μg)	5.1	6.8			22.1
	5.4	6.8			29.4
	Mean 7.0 μg				Mean 10.7 μg
	(6.6 μg)				(6.2 μg)

Total gastrectomy (3 cases)	Jejunectomy (1 case)	Ileitis (1 case)
5.5	9.3 μg	17.6 μg
7.4		
9.0		
Mean 7.3 μg		

Total material (74 cases): Mean 8.1 μg (6.8 μg).

were given. The results are shown in Table II. Doses of 500 μg still need the intramuscular flush dose for a significant urinary excretion, whereas at 10,000 μg the excretion without flush approaches the excretion after flush. After B_{12} doses of 50,000–100,000 μg the oral B_{12} dose apparently acts as a full flush dose in itself, since the radioactivity excreted is about the same with and without flush.

Since, after very high oral B_{12} doses, the relationship between oral absorption and 24-hour urinary excretion has not been determined, we do not know if the factor 3, used by us and found by Callender and Evans (7) at lower doses, is reliable. Nevertheless, the absorption must be at least as high as or higher than the amount actually found in urine during 48 hours (cf. 3). Table II shows that the amount absorbed is of a magnitude of about 1% of the dose or more, also when the highest doses are given. Even if the ratio, absorption to 24-hour excretion, may thus differ from 3 at high doses (cf. 15 and Fig. 5 curve H), this uncertainty has no decisive influence on the conclusions drawn from the studies presented here.

Influence of food intake on B_{12} absorption

Finally, a study was made to find out if the B_{12} absorption of a dose given after a meal was different from the absorption of a dose taken on an empty stomach. Table III gives the result of measurements in ten test subjects at a dose of 500 μg.

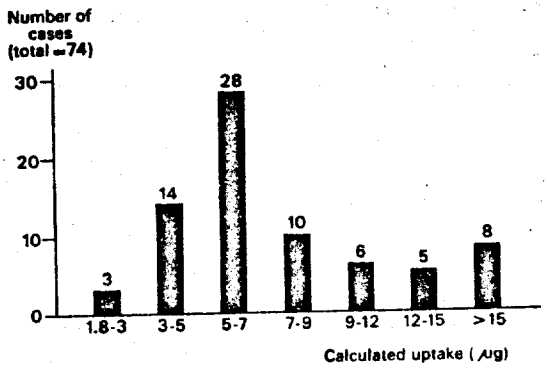


Fig. 6. Calculated uptake of vitamin B_{12} following an oral dose of 500 μg radioactive B_{12}, taken on an empty stomach. Distribution of the entire material (74 subjects), comprising normal persons and patients with pernicious anemia or other gastrointestinal absorption disturbances.

Table II. *Effect of intramuscular flush dose on urinary B_{12} excretion after high oral doses of vitamin B_{12}*

Oral dose of radioactive B_{12} (μg)	Per cent of radioactivity recovered in urine			
	With flush[a]		Without flush	
	0–24 h	0–48 h	0–24 h	0–48 h
500	0.55	0.65	0.03	0.05
500	0.48	0.57	0.01	0.02
500	0.43	0.52	0.02	—
500	0.60	0.75	0.09	0.10
500	0.92	1.38	0.05	0.11
500	0.30	0.42	0.00	0.02
500	0.89	1.29	0.01	0.02
500	0.70	1.18	0.05	0.10
5,000	0.55	—	0.03	0.05
5,000	0.71	1.10	0.24	0.27
5,000	0.43	0.52	0.06	0.19
10,000	0.67	0.85	0.12	0.25
10,000	0.79	0.98	0.69	0.80
10,000	0.97	1.08	0.51	0.54
25,000	0.28	0.57	0.18	0.19
50,000	0.35	0.62	0.94	1.08
50,000	0.69	0.91	0.75	0.83
50,000	0.65	1.00	0.57	0.67
50,000	—	—	0.53	0.77
100,000	0.48	0.70	0.41	0.58
100,000	0.97	1.16	0.88	1.09

[a] The intramuscular flush dose of 1000 μg of non-radioactive B_{12} was given 2 h after the oral dose, and repeated 24 h after the oral dose.

It is obvious that the uptake is moderately higher when the dose is taken on an empty stomach. The dose should, therefore, preferably be taken before or between meals, although this does not seem to be a critical point.

CLINICAL STUDIES

Several authors have attempted to determine the daily requirement of vitamin B_{12} in order to obtain a criterion for establishing the necessary daily uptake in the treatment of pernicious anemia.

Table III. *Calculated uptake (μg) of vitamin B_{12} on an empty stomach and after a standard breakfast*

Oral dose 500 μg radioactive B_{12}. Mean values and range for ten patients with pernicious anemia

	Fasting	After meal
Calculated uptake (μg)	7.8 (2.8–13.4)	4.8 (1.8–7.5)

Table IV. *Observation period for long-term oral treatment of pernicious anemia with 500–1000 μg of vitamin B_{12} (64 cases)*

All six patients treated for three years or less, and a few of the others, died during the observation period, for reasons not connected with the disease studied

Time (mo.)	No. of cases	Time (mo.)	No. of cases
70	2	35–39	3
65–69	4	30–34	—
60–64	11	25–29	1
55–59	17	20–24	1
50–54	8	15–19	—
45–49	5	10–14	1
40–44	11		

It may be assumed that about 2 μg constitutes the daily turnover rate and an amount sufficient to prevent relapse in pernicious anemia (14, 20, 21). Even though other studies (13, 20, 22) have indicated a higher requirement, a quantity of about 2 μg corresponds fairly well to practical experience.

As demonstrated above, an oral dose of 500–1000 μg of vitamin B_{12} gives, on an average, an absorption of 6–12 μg. Even when individual variations are taken into account, a daily dose of this magnitude can be expected to give a safe maintenance effect.

Altogether, the *clinical material* comprises 64 established B_{12} deficiency cases from three Swedish medical centers (Eskilstuna, Falköping and Linköping), all examined and supervised personally by one of the authors (R. Berlin or G. Brante). The patients were started on 500 or 1000 μg of B_{12} daily; during the last three years of the study 1000 μg daily were given throughout. Sixty-one of the patients have been under treatment more than 36 months, 44 more than 48 months, and 17 during 60 months or more. A more detailed review of the observation period of the entire material is shown in Table IV. The clinical diagnoses are given in Table V.

The *clinical results*, which are presented in Table VI, Fig. 7 and Fig. 8, are more positive throughout, to our knowledge, than any results reported earlier in oral treatment of pernicious anemia. It should be noted that in this study a strict evaluation of the effect was made, including regular determination of serum B_{12} for all patients. The patients were encouraged to adhere strictly to

Table V. *Case material in long-term clinical trials with 500 μg or 1000 μg of vitamin B_{12} administered daily in tablet form*

	Pernicious anemia		Malabsorption			
	Ordinary	Resistant[a]	After ileal resections	Cause not established	Total gastrectomy	Total
No. of cases	18	37	4	4	1	64

[a] Demonstrated resistance of various degrees to preparations containing hog intrinsic factor plus vitamin B_{12} or liver. Criteria: falling values for Hb and red blood cells and/or low values for B_{12} in blood serum during treatment with hog IF preparations, subnormal response in the Schilling test with B_{12} + hog intrinsic factor.

Table VI. *Results of oral long-term therapy with 500–1000 μg of vitamin B_{12} daily in tablet form*

Sixty-four cases of pernicious anemia and other types of vitamin B_{12} deficiency

Hb and RBC	Satisfactory values 55 cases	Subnormal values 9 cases[a]	Total 64 cases
Serum-B_{12}	Normal 64 cases	Subnormal 0 cases	

[a] One gastric carcinoma; one chronic pyelonephritis, postoperative abscess of left thigh; one metastasing urinary bladder carcinoma; one chronic uremia; one gastric carcinoma; one menorrhagia + iron deficiency anemia; one ileitis with bleedings; one iron deficiency after total gastrectomy; one idiopathic malabsorption.

the medication and were interviewed at each visit for checking of their medication habits. They were also instructed to discontinue the treatment for three days before the blood sample was to be taken, in order to avoid temporary peaks in the serum B_{12} level.

The therapy was discontinued for six patients in order to study the time needed for reappearance of low serum B_{12} values. The patients were kept under strict clinical control. Table VII shows that the serum B_{12} values stayed well within normal limits in all the patients for about 4 months. Three patients reached the lower normal limit of 150 pg/ml after about 5 months and one patient after 7

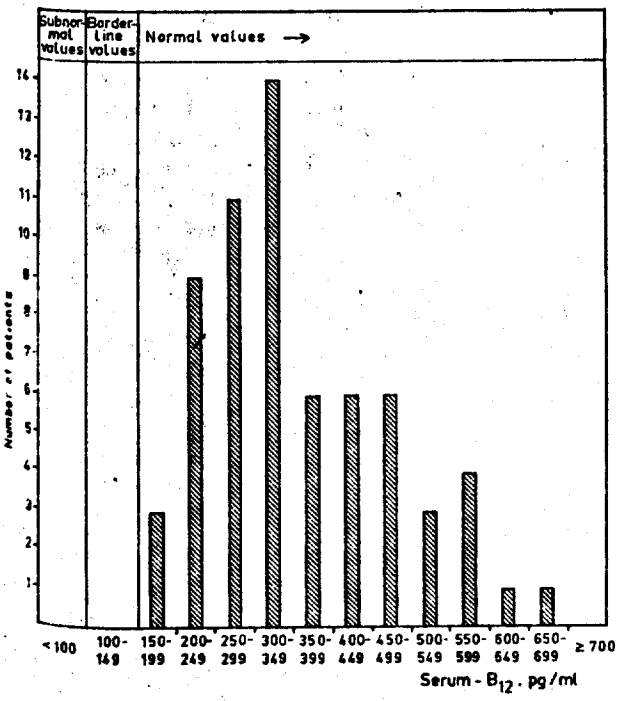


Fig. 7. Results of oral long-term therapy with 500–1000 μg vitamin B_{12} daily in tablet form: mean values of all serum-B_{12} determinations made from the second month of treatment. Sixty-four cases of pernicious anemia and other types of vitamin B_{12} deficiency.

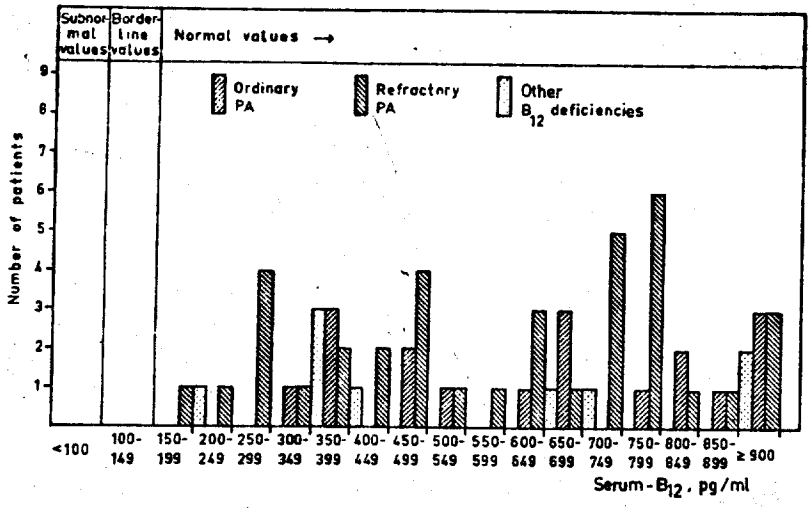


Fig. 8. Results of oral long-term therapy with 500–1000 μg vitamin B_{12} daily in tablet form: serum-B_{12} values at the end of the follow-up period. The material (64 cases) is divided into groups according to type of B_{12} deficiency.

months. Two patients still had values within the normal range after 8 1/2 and 7 months, respectively. In a further case (B_{12} deficiency after total gastrectomy) all B_{12} therapy was interrupted by inadvertence after 44 months of oral treatment with 500 μg of B_{12} daily. After 1 1/2 years his serum B_{12} value had decreased from 570 to 350 pg/ml. In no case did the values for hemoglobin, red cells, serum iron, iron-binding capacity, serum lactic acid dehydrogenase, or serum glutamic acid-oxalacetic acid transaminase show any notable changes during the observation periods. The results indicate that *oral treatment with 500 μg of B_{12} daily had also replenished the B_{12} depots.*

Fig. 7 shows the mean values of all serum B_{12} determinations made for each case after the initial two months of therapy. Fig. 8 presents the last serum B_{12} values determined in the follow-up study. In this figure the material is divided according to type of B_{12} deficiency. Of all serum B_{12} determinations made after 2 months of treatment or more, comprising 562 values, only 40 values were below 150 pg/ml and 6 values below 100 pg/ml. Generally, these low values were obtained during the early months of treatment of patients in definite relapse. Repeated low values on several consecutive occasions were found only in exceptional cases, where 500 μg daily was given; they could easily be corrected by increasing the dose to 1000 μg daily. Thus, the long-term treatment with high doses of vitamin B_{12} alone has resulted in serum B_{12} levels well within the normal range in all cases.

It should also be noted that no negative reactions were recorded that could be attributed to the medication. No observations were made of

Table VII. *Serum B_{12} values after discontinuance of oral B_{12} therapy, 500 μg daily (after the time for last value reported the treatment was resumed)*

Patient and type of B_{12} malabsorption	Duration of treatment when discontinued (mo.)	Serum B_{12} (pg/ml), time after discontinuance: Days 3	Days 10	Months 1	2	3	4	5	6	7	8
1. Ordinary PA[a]	19	440	200	205	190		190	150		130	
2. Ileal resection	28	515	460		210	210		110	140		
3. IF refractory	33	315	470		170	210		145	170		
4. Idiopathic malabsorption	33	390	400		255	290		210		135	
5. Ileal resection	33	265	300		220		230	235			205
6. IF refractory PA	33	570	510		235	265		260		270	

[a] PA = pernicious anemia.

3. — The absorption of IF-bound and free B_{12} in various clinical conditions. Paper read at the 2nd European Symposium on vitamin B_{12} and intrinsic factor, Hamburg 1961.
4. Berlin, H., Berlin, R. & Brante, G.: Peroral behandling av perniciös anemi med höga doser vitamin B_{12} utan intrinsic factor. Läk.-Tidn. 62: 773, 1965.
5. — Crude or refined intrinsic factor in preparations for the oral treatment of pernicious anaemia. Scand. J. Haemat. 3: 236, 1966.
6. Brody, E. A., Estren, S. & Wasserman, L. R.: Treatment of pernicious anemia by oral administration of vitamin B_{12} without added intrinsic factor. New Engl. J. Med. 260: 361, 1959.
7. Callender, S. T. & Evans, J. R.: The urinary excretion of labelled vitamin B_{12}. Clin. Sci. 14: 295, 1955.
8. Chalmers, J. N. M. & Hall, Z. M.: Treatment of pernicious anaemia with oral vitamin B_{12} without known source of intrinsic factor. Brit. med. J. 1: 1179, 1954.
9. Chalmers, J. N. M. & Shinton, N. K.: Absorption of orally administered vitamin B_{12} in pernicious anemia. Lancet 2: 1298, 1958.
10. Conley, C. L. & Krevans, J. R.: New developments in the diagnosis and treatment of pernicious anemia. Ann. intern. Med. 43: 758, 1955.
11. Doscherholmen, A. & Hagen, P. S.: A dual mechanism of vitamin B_{12} plasma absorption. J. clin. Invest. 36: 1551, 1957.
12. Gaffney, G. W., Watkin, D. M. & Chow, B. F.: Vitamin B_{12} absorption: relationship between oral administration and urinary excretion of cobalt^{60} labeled cyanocobalamin following a parenteral dose. J. Lab., clin. Med 53: 525, 1959.
13. Gräsbeck, R.: Calculations on vitamin B_{12} turnover in man with a note on the maintenance treatment in pernicious anemia and the radiation dose received by patients ingesting radiovitamin B_{12}. Scand. J. clin. Lab. Invest. 11: 250, 1959.
14. Heinrich, H. C. & Pfau, A. A.: Der Einsatz von Gesamtkörper-Radioaktivitäts-Detektoren mit flüssigen organischen Szintillatoren in der klinischen Radioisotopen-Diagnostik und Forschung. Atomkernenergie 6: 463, 1961.
15. Heinrich, H. C., Gabbe, E. E., Whang, D. H. & Wolfsteller, E.: Eine für die Berechnung der intestinalen Vitamin B_{12}-Resorption beim Menschen sowohl im physiologischen, Intrinsic Factor-abhängigen als auch im unphysiologischen hohen, diffusionsbedingten Dosisbereich allgemein gültige Formel. Z. Naturforsch. 20 b: 1067, 1965.
16. Heinrich, H. C. & Wolfsteller, E.: Hochdosierte orale Vitamin-B_{12}-Therapie. Med. Klin. 61: 756, 1966.
17. McIntyre, P. A., Hahn, R., Masters, J. M. & Krevans, J. R.: Treatment of pernicious anemia with orally administered cyanocobalamin (vitamin B_{12}). Arch. intern. Med. 106: 280, 1960.
18. Meyer, L. M., Sawitsky, A., Cohen, B. S., Krim, M. & Fadem, R.: Oral treatment of pernicious anemia with vitamin B_{12}. Amer. J. med. Sci. 220: 604, 1950.
19. Reisner, E. H., Weiner, L., Schittene, M. T. & Henck, E. A.: Oral treatment of pernicious anemia with vitamin B_{12} without intrinsic factor. New Engl. J. Med. 253: 502, 1955.
20. Reizenstein, P. G.: Vitamin B_{12} metabolism. Acta med. scand. Suppl. 347, 1959.
21. — Excretion of non-labeled vitamin B_{12} in man. Acta med. scand. 165: 313, 1959.
22. — Body distribution, turnover rate, and radiation dose after the parenteral administration of radiovitamin B_{12}. Acta med. scand. 165: 467, 1959.
23. Ross, G. I. M., Mollin, D. L., Cox, E. V. & Ungley, C. C.: Hematologic responses and concentration of vitamin B_{12} in serum and urine following oral administration of vitamin B_{12} without intrinsic factor. Blood 9: 473, 1954.
24. Shinton, N. K.: Oral treatment of pernicious anemia with vitamin-B_{12}-peptide. Brit. med. J. 1: 1579, 1961.
25. Spies, T. D., Stone, R. E., Lopez, G. G., Milanes, F., Toca, R. L. & Aramburu, T.: Vitamin B_{12} by mouth in pernicious and nutritional macrocytic anaemia and sprue. Lancet 2: 454, 1949.
26. Unglaub, W. G., Rosenthal, H. L. & Goldsmith, G. A.: Studies of vitamin B_{12} in serum and urine following oral and parenteral administration. J. Lab. clin. Med. 43: 143, 1954.
27. Waife, S. O., Jansen, C. J., Jr., Crabtree, R. E., Grinnan, E. L. & Fouts, P. J.: Oral vitamin B_{12} without intrinsic factor in the treatment of pernicious anemia. Ann. intern. Med. 58: 810, 1963.

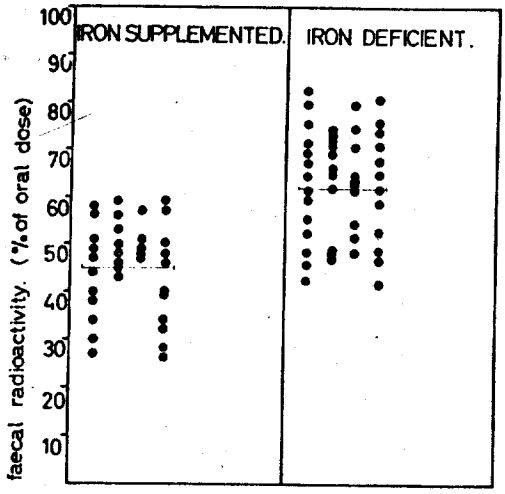


FIG. 1. Excretion of radioactivity by iron deficient and iron-supplemented rats after administration of 5 mμg. ^{58}Co-vitamin B_{12} (7 days' collection of urine and feces).

Normal gastric juice was obtained from rats fed standard rat cake. Each animal was fasted overnight and juice was obtained by gastric intubation after histamine stimulation. This juice was centrifuged at low speed to remove debris and neutralized to pH 7, and either used immediately, or stored at −20° C. until used or concentrated by dialysis against 50 per cent gum acacia, stored at −20° C. and diluted to its original volume just before use, the pH being adjusted to 7. One milliliter of normal juice was given with the dose of ^{58}Co-vitamin B_{12} to iron deficient rats which had failed to absorb a normal fraction of the vitamin when it was administered by itself.

Gastric juice was also obtained from the iron deficient animals and their controls, and these specimens were tested for intrinsic factor activity in rats which had been subjected to total gastrectomy but were otherwise in good health and were maintained on a diet of standard rat cake. Excreta were collected for 7 days after alimentary administration of the mixture of labeled vitamin B_{12} and gastric juice.

Results

The pattern of excretion of radioactivity by the rat after an alimentary dose of labeled vitamin B_{12} has been studied.[5] A large fraction of the radioactivity which appeared in the excreta 6 days and more from the time of administration of the dose represents material which has been absorbed and reexcreted into the gastrointestinal tract, as well as into the urine, so that in the normal rat a 5-day collection of feces provides the best index of nonabsorbed radioactivity. In the iron deficient rat the pattern is more variable, so that large amounts of radioactivity, sometimes as much as 15 per cent of the dose, may appear in the feces on the sixth and seventh days. Therefore, a collection period of 7 days was used for both normal and iron deficient rats. The results are shown in figure 1 for control and iron deficient rats. Radioactivity was estimated in combined 7 days' collections of feces and urine. A highly significant difference was observed between the two groups, and further experiments were performed in an attempt to elucidate the cause of this difference.

When feces and urine were collected separately for the 7-day period, very much more radioactivity appeared in the feces of deficient animals as compared with their controls, whereas there was less activity in the urine of the deficient animals (table 1). Further evidence that the excess radioactivity in the feces of the deficient animals represents unabsorbed activity as opposed to activity reexcreted into the gastrointestinal tract is provided by the results in figure 2. In this experiment fecal and urinary excretion of radioactivity after injection of 5-mμg. doses of labeled vitamin B_{12} into the spleen in 2 iron-supplemented and 2 iron deficient animals was measured. This route of injection was chosen because vita-

TABLE 1

Excretion of radioactivity in feces and urine of iron-supplemented and deficient Rats for 7 days after 5 mμg. ^{58}Co-vitamin B_{12}

	Feces		Urine	
	Dose	S.E.	Dose	S.E.
	%		%	
Iron-supplemented (7)	42	0.85	7	0.38
Iron deficient (12)	64	2.2	4	0.6

neurological disturbances indicating development of subacute combined degeneration of the cord. Absent ankle tendon reflexes, recorded in a few cases, were connected with concomitant diseases, such as diabetes, advanced arteriosclerosis or a history of sciatica. Absence of vibratory sensibility in the feet was reported in one case, an old woman with senile dementia.

Many patients spontaneously expressed satisfaction with the discontinuance of injections. At a special interview some patients declared that they did not care whether they received injections or tablets. No patients had any objection to oral treatment.

Since the uptake is proportional to the dose, it has been possible to supply the entire daily requirement by one small tablet daily. Only very few patients tended to neglect the medication. In all but one, a mentally deficient patient, negligence could be corrected by information.

With regard to the daily standard dose—500 μg or 1000 μg—the higher dose was chosen. Although it was found, both theoretically and through experience, that 500 μg per day gave satisfactory results, the higher dose offers better protection in the event of temporary negligence in medication. Even at the higher dose, the treatment is simple and inexpensive, and no report has been received of any side reactions or other unfavourable effects from long-term therapy.

The investigation has also demonstrated that it is possible to give oral treatment with high doses of vitamin B_{12}, not only as maintenance therapy but also as initial treatment during relapse, without any untoward effects. For investigational reasons, the maintenance dose has been used also initially throughout this study. In routine use we recommend, however, initial oral treatment of 2000 μg twice daily during the first month, or injection therapy in order to fill the body stores as rapidly as possible.

Our clinical experience has provided complete confirmation of the experimental results. It is now definitely established that the treatment of pernicious anemia with high oral doses of vitamin B_{12} without IF is a fully satisfactory alternative to injection therapy, not only in cases of ordinary pernicious anemia but also in those showing IF refractoriness, and in other cases of B_{12} malabsorption.

This treatment, although the cost of the tablets is somewhat higher than the cost of injectable B_{12}, is economically quite reasonable, because it saves personnel and allows less frequent visits to the doctor. The tablets were introduced in Sweden in 1964 and are now in widespread use. No negative experiences have been reported in connection with their practical routine use.

ADDENDUM

In a review of his experimental work on oral B_{12} without IF, Heinrich (Ergebn. inn. Med. Kinderheilk. 25: 1, 1967) discusses the suitable daily maintenance dose and finds 300 μg daily to be adequate.

Statistically this is correct, because this dose results in an absorbed and retained average quantity of approx. 3 μg, or slightly more than the normal daily turnover. However, considering the variations in absorption, a wider safety margin seems to be appropriate in order to provide a safe standard therapy. Heinrich's figures, as well as ours, show some absorption values far below average, and in our experience low values are found rather constantly in certain individuals. Heinrich refers to clinical trials, published by others, indicating that 300 μg daily might be sufficient. Our more comprehensive material, followed up for a much longer period of time, showed that 500 μg daily, although in general adequate, may give borderline serum B_{12} values in a few cases. In these patients normal values were rapidly restored by 1000 μg daily.

Considering that some patients may have a higher B_{12} requirement or a lower uptake than average and, further, that (*a*) the absorption is slightly lower if the dose is taken with a meal; (*b*) a reasonable allowance for occasional negligence should be taken into account in this type of self-medication; (*c*) a dose of 1000 μg daily has not caused any untoward reactions during 5 years' study; and, (*d*) the difference has little economic significance, we have preferred to recommend 1000 μg as daily maintenance dose.

Heinrich also discusses much higher oral doses at correspondingly longer intervals. We have seriously considered such alternatives, since it is no doubt possible to work out other dosage regimens which are equally effective. In our opinion, however, it is necessary that an important selfmedication like this can be included in a simple daily routine. The possible cost reduction gained by higher doses at longer intervals would most probably not be large enough to justify the increased risk of the patient's forgetting his tablet and the more serious consequences if a dose is neglected.

REFERENCES

1. Berlin, R., Berlin, H., Brante, G. & Sjöberg, S. G.: Refractoriness to intrinsic factor-B_{12} preparations abolished by massive doses of intrinsic factor. Acta med. scand. 162: 317, 1958.
2. — Studies on intrinsic factor and pernicious anemia. I. Oral uptake of vitamin B_{12} in pernicious anemia with increasing doses of an intrinsic factor concentrate. Scand. J. clin. Lab. Invest. 10: 278, 1958.

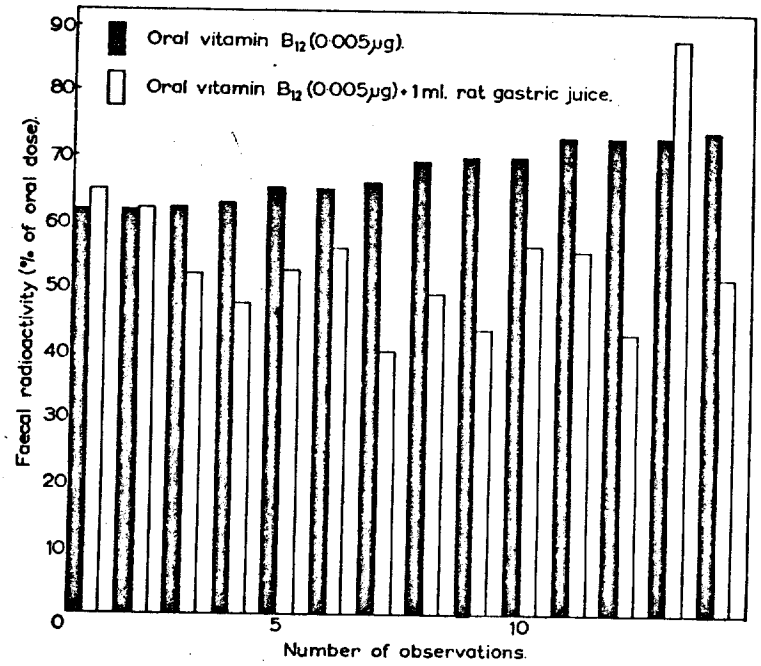


FIG. 4. Effects of gastric juice from normal rats on the excretion in combined urine and feces of radioactivity by iron deficient rats following alimentary ^{58}Co-vitamin B_{12}.

TABLE 2

Excretion of vitamin B_{12} by rats maintained on an iron-supplemented and iron deficient diet for 28 days after weaning

Status of Animal	Hemoglobin	Percentage of Radioactivity		
		Intestinal contents	Feces	Total
	gm./100 ml.			
Iron-supplemented....	16.4	1.4	43.8	45.2
Iron deficient.....	8.0	1.7	67.0	68.7

TABLE 3

Effect of iron-supplementation on vitamin B_{12} excretion (urine and feces)

Rat No.	June 19, 1961		August 3, 1961		September 20, 1961	
	Hb	^{58}Co-B_{12}	Hb	^{58}Co-B_{12}	Hb*	^{58}Co-B_{12}
	gm./100 ml.	%	*gm./100 ml.*	%	*gm./100 ml.*	%
108	3.7	69	17.8	61		53
111	4.3	76	16.4	63		42
112	6.6	71	15.7	71		48
116	5.1	69	16.8	50		43
117	5.7	69	16.1	56		44
118	5.6	65	17.5	55		37
119	5.8	66	16.9	61		42
120	5.4	64	16.9	50		44

* Presumed within normal range because animals were still maintained on supplemented diet.

two subsequent tests of absorption of vitamin B_{12} were carried out in each animal; the tests were separated by 48 days. A delayed but complete return to values within the normal range was observed in each case (table 3).

Possible Effect of Changes in the Intestine

One of the most dramatic changes observed in iron deficient animals is the marked enlargement of the cecum.[3] Such enlargement might constitute a blind loop, although it would be unlikely to interfere with absorption of vitamin B_{12} because the absorption of this vitamin occurs in the rat predominantly in the third quarter of the small intestine.[7, 8] Treatment of iron deficient and

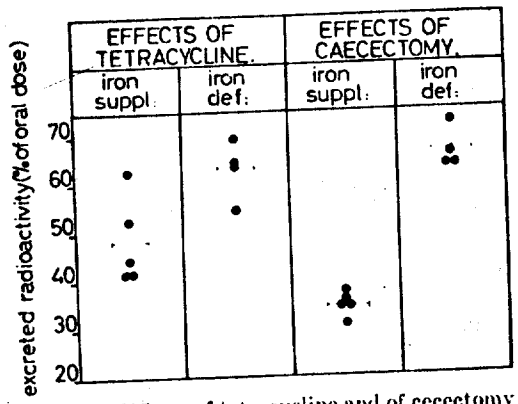


FIG. 5. Effects of tetracycline and of cecectomy on excretion in combined urine and feces of radioactivity by iron-supplemented and iron deficient rats given alimentary ^{58}Co-vitamin B_{12}.

iron-supplemented rats with tetracycline added to their diets (5.0 gm. per kg. of diet) produced diarrhea in all of the iron deficient animals but did not significantly affect the excretion of radioactivity after test doses of labeled vitamin B_{12} given 5 days after the animals had started on the antibiotic (fig. 5).

The ceca were removed from 6 iron deficient and 6 iron-supplemented animals. The operation was followed by severe diarrhea, which subsequently improved to the extent that the animals had bulky, semi-formed stools. They remained in apparently good health and continued to gain in weight. The results of vitamin B_{12} absorption studies are shown in figure 5. Cecectomy resulted in no changes in the percentage of excretion of radioactivity in either iron deficient or iron-supplemented animals and the pattern of excretion also was not changed.

Discussion

Iron deficiency in the rat results in a reduction of secretion of gastric intrinsic factor and consequent impairment of absorption of vitamin B_{12}. The period of deficiency necessary for this defect to evolve is not greater than 4 weeks in the young, rapidly growing animal; we have no observations of this sort in mature animals owing to the greater difficulty of inducing in them a state of iron deficiency. Administration of iron leads to the recovery of normal capacity to absorb vitamin B_{12}, although this response is not immediate and seems to require between 4 and 6 weeks in the young, adult rat.

It is still not evident whether or not there is any superadded disturbance of absorption of the vitamin because of changes in the small intestine. The methods used would have revealed any gross disturbance of the small intestine, but a minor disturbance might possibly have been overlooked. The predominant cause of malabsorption of vitamin B_{12} seems to be a deficient production of intrinsic factor.

The defect in absorption of vitamin B_{12} is not necessarily associated with severe reduction of other gastric secretions. Studies[3] of gastric pepsinogen and of hydrochloric acid production were carried out in many of the animals included in figure 1, many of which showed impaired absorption of vitamin B_{12}. There was no fall in secretion of pepsin and although there was a significant fall in secretion of acid in the iron deficient group, there was not a total loss of secretion of acid, such as occurs in humans with pernicious anemia. In this respect the iron deficient rat is more closely akin to those relatives of patients with pernicious anemia studied by McIntyre *et al*,[9] in whom defective production of intrinsic factor was not associated with loss of capacity to secrete hydrochloric acid. The gastric mucosae of all iron deficient rats which have been subjected to histologic examination show no definite structural changes suggestive of atrophic gastritis or atrophy.

Cox *et al.*[10] have described 13 patients with a variety of diseases who had low levels of serum vitamin B_{12} activity and who were iron deficient. The concentration of serum vitamin B_{12} gradually returned to within the normal range on treatment with iron. These authors postulated that the iron therapy may have corrected a defect in secretion of intrinsic factor. In the pilot series and in the second group of iron deficient rats we have estimated the level of vitamin B_{12} activity in the serum. There was no significant difference between the serum levels of iron deficient and iron-supplemented animals, in spite of impaired absorption in the former. However, all of the diets used have contained large excesses of the vitamin which would

0277

ensure the absorption of adequate amounts even in the presence of defective production of intrinsic factor. This supplementation was accomplished intentionally, so that none of the changes occurring in iron deficient rats could be ascribed to an induced deficiency of vitamin B_{12}.

Summary

Iron deficient rats excrete a significantly higher percentage of radioactivity than iron-supplemented controls after an alimentary dose of ^{58}Co-vitamin B_{12}.

The gastric juice of iron deficient animals has little or no effect on the excretion of labeled vitamin B_{12} by gastrectomized rats, whereas the juice of iron-supplemented controls reduces excretion of the vitamin. This suggests that iron deficiency in rats impairs the production of intrinsic factor and this is supported by the finding that juice from normal animals tends to correct the defect of absorption of vitamin B_{12}.

Impaired secretion of intrinsic factor occurs within 28 days of placing weanling rats on an iron deficient diet.

Iron supplementation of iron deficient animals results in recovery of the capacity to absorb vitamin B_{12} after 10 weeks or more.

There was no evidence of a disturbance of function in the small intestine of the iron deficient animals and neither broad spectrum antibiotics nor cecectomy improved the absorption of vitamin B_{12}.

The relevance of these findings to iron deficiency in man is discussed.

Acknowledgments. We wish to thank Dr. G. H. Spray for estimating vitamin B_{12} activity in sera, Miss B. J. Mallett for performing hematologic tests, and Mr. John Charles and Mr. John Halfacree and the technical staff of the department for their assistance. The work was supported by a grant to L. J. W. from the Medical Research Council, and J. C. B. was aided by a grant from the Wellcome Trust.

REFERENCES

1. Coghill, N. F. The significance of gastritis. Postgrad. Med. J., **36:** 733, 1960.
2. Taylor, K. B., and Witts, L. J. The effects of chronic iron deficiency in the rat. Quart. J. Med., **27:** 565, 1958.
3. Valberg, L. S., Taylor, K. B., Witts, L. J., and Richards, W. C. The effect of iron deficiency on the stomach of the rat. Brit. J. Nutr., **15:** 473, 1961.
4. Taylor, K. B., Mallett, B. J., Witts, L. J., and Taylor, W. H. Observations on vitamin B_{12} absorption in the rat. Brit. J. Haemat., **4:** 63, 1958.
5. Biggs, J. C., and Witts, L. J. Vitamin B_{12} absorption and excretion in the rat. Brit. J. Haemat., **8:** 241, 1962.
6. Taylor, K. B., and French, J. E. The role of the lymphatics in the intestinal absorption of vitamin B_{12} in the rat. Quart. J. Exp. Physiol., **45:** 72, 1960.
7. Booth, C. C., Chanarin, I., Anderson, B. B., and Mollin, D. L. The site of absorption and tissue distribution of orally administered ^{58}Co-labelled vitamin B_{12} in the rat. Brit. J. Haemat., **3:** 253, 1957.
8. Reynell, P. C., Spray, G. H., and Taylor, K. B. The site of absorption of vitamin B_{12} in the rat. Clin. Sci., **16:** 663, 1957.
9. McIntyre, P. A., Hahn, R., Conley, C. L., and Glass, B. Genetic factors in predisposition to pernicious anemia. Bull. Johns Hopkins Hosp., **104:** 309, 1959.
10. Cox, E. V., Meynell, M. J., Gaddie, R., and Cooke, W. T. Interrelation of vitamin B_{12} and iron. Lancet, **2:** 998, 1959.

Experientia 25(3): 294-296 1969

Uptake of Labelled Vitamin B_{12} and 4-Iodophenylalanine in some Tumours of Mice

During the last years there has been a rapid improvement of the equipment for external scintillation counting, a method of increasing importance in tumour diagnosis[1]. There is, however, a demand for gamma-emitting substances with selective affinity for malignant tumours. Using whole body autoradiography[2] we have obtained some results which seem to be of interest in this respect. Whole body autoradiography has the advantage, in screening substances for tumour diagnosis, that the distribution within the tumour can be studied and that the tumour uptake can be compared with the uptake in all normal tissues.

Material and methods. Labelled vitamin B_{12} (^{57}Co, ^{60}Co) was administered i.v. to 4 groups of mice with different kinds of tumours, which had been transplanted s.c. into the neck region and grew as solid masses: ^{90}Sr induced soft fibroblastic osteosarcoma, spontaneous mammary carcinoma, Moloney-virus induced tumour, and Ehrlich

[1] F. J. Bonte, T. S. Curry III., E. Russel, B. S. Oelze and A. J. Greenberg, Am. J. Roentg. *100*, 801 (1967).
[2] S. Ullberg, Acta radiol., suppl. *118* (1954).

ascites cell tumour. The animals were killed by ether anaesthesia at intervals from 15 min to 4 days after injection, and then immersed in a mixture of hexane and solid carbon dioxide (−70 °C). Sagittal 20 μ sections through the frozen animals were cut and dried at −10 °C. Autoradiographic exposure was obtained by apposition against Gevaert Structurix X-ray film.

^{125}I-labelled DL-4-iodophenylalanine was given i.v. to 3 mice with the following transplanted tumours: ^{90}Sr induced soft fibroblastic osteosarcoma, Ehrlich ascites cell tumour, and lymphatic leukemia. The animals were killed 15 min after injection and subjected to the same procedure as cited above.

The whole body autoradiography technique has been described in detail previously[2].

Results and discussion. Vitamin B_{12}. The ^{90}Sr induced soft fibroblastic osteosarcoma, which was the most rapidly growing tumour, showed the greatest uptake of radio-B_{12}, the concentration being higher than in any normal tissue (Figure 1). The other tumours also accumulated the substance although less extensively. In all tumours the radioactivity was localized to the peripheral actively growing parts.

The results suggest that B_{12} is needed for rapid cell multiplication. This may be related to the findings of several authors claiming that B_{12} participates in the synthesis of nucleic acids (e.g.[3]).

[3] R. L. BLAKLEY, J. biol. Chem. *240*, 2173 (1965).

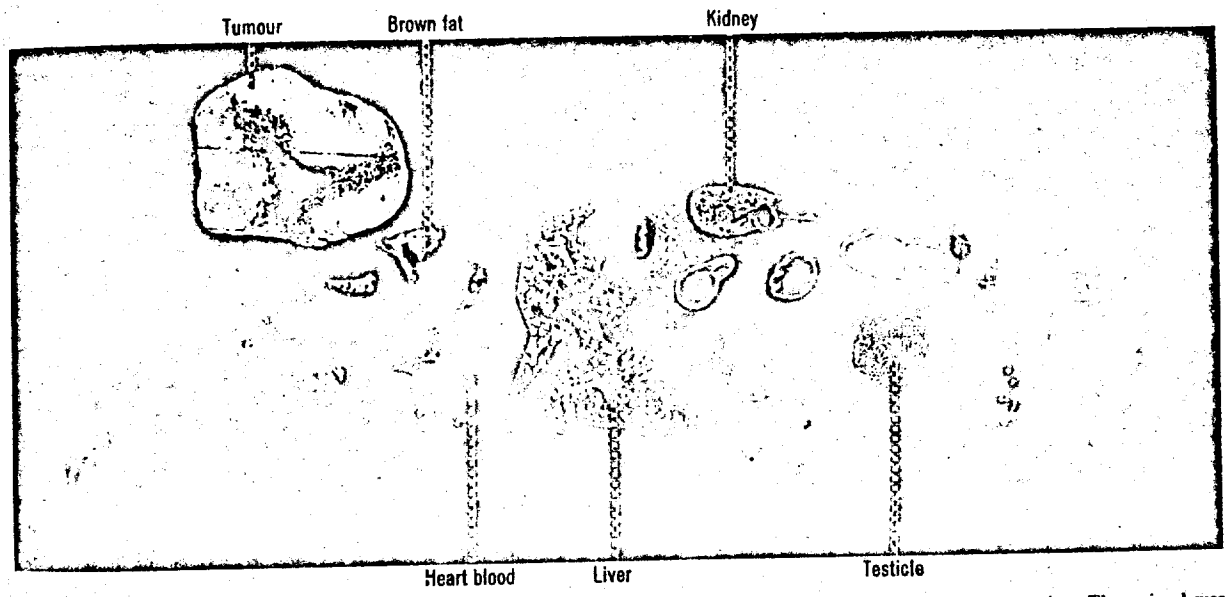


Fig. 1. Autoradiogram of mouse carrying a transplanted ^{90}Sr-induced soft fibroblastic osteosarcoma in the neck region. The animal was sacrificed 4 days after i.v. injection of ^{60}Co-vitamin B_{12}. Note high accumulation of radioactivity in the actively growing peripheral parts of the tumour. Accumulation is also seen in the gastric and intestinal mucosa, renal cortex, testis, liver and brown fat.

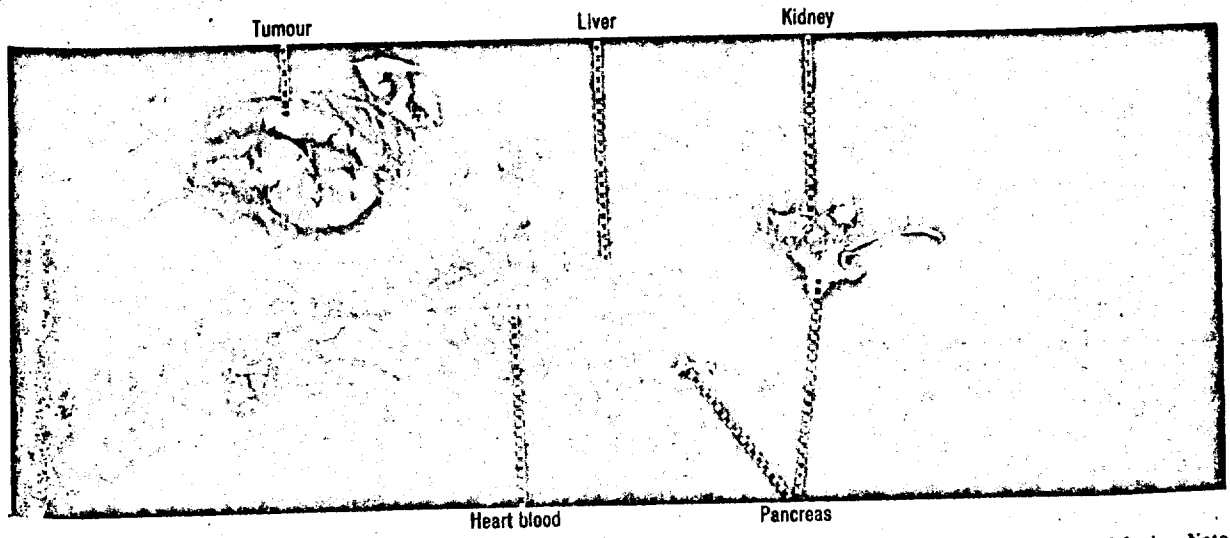


Fig. 2. Autoradiogram of mouse with a transplanted Ehrlich ascites cell tumour 15 min after i.v. injection of ^{125}I-4-iodo-phenylalanine. Note high uptake in the growing parts of the tumour. In addition to the tumour, the substance is mainly concentrated in the pancreas and kidney.

It has been claimed that patients with neoplasms have an increased requirement for vitamin B_{12}. Carcinoma cells, like normal cells, need B_{12} and the infiltrative growth of the carcinomas could possibly increase the demand for B_{12}[4]. Following injection of vitamin B^{12} increased growth has been reported in some sarcomas[5].

4-Iodophenylalanine. This amino acid has earlier been found to localize almost selectively in the exocrine pancreas of normal mice[6]. The substance appeared to be transported across the pancreatic cell membrane similarly to an 'ordinary' amino acid but not to be accepted in the protein synthesis and therefore rapidly returned to the blood. In the rest of the body the distribution was very even, almost all tissues having lower concentration than the blood.

In the present investigation, a concentration of 4-iodophenylalanine estimated to approximately 3 times that of blood was seen in the actively growing parts of the Ehrlich ascites cell tumour (Figure 2). The lymphatic leukemia showed somewhat higher concentration than the blood, while the soft fibroblastic osteosarcoma did not take up the drug.

Unlike vitamin B_{12} 4-iodophenylalanine did not show an uptake which correlated with the growth rate of the tumour, since the soft fibroblastic osteosarcoma grew faster than the Ehrlich ascites cell tumour but did not concentrate the drug. Rather, it would seem that the Ehrlich ascites cells and to a less extent the lymphatic leukemic cells have a membrane transport system which can accept 4-iodophenylalanine, which is not the case for the cells of the soft fibroblastic osteosarcoma or the cells of most normal tissues. This may be of interest with regard to the possibility of slightly modifying naturally occurring compounds, such as amino acids, sugars, nucleosides, vitamins etc., to obtain selective localization in tumours. Another non natural amino acid which has been found to accumulate in certain tumours without being incorporated into proteins is 1-aminocyclopentane carboxylic acid[7].

One practical consequence from our findings might be the use of gamma-emitting vitamin B_{12} or 4-iodophenylalanine for clinical localization of tumours. However, these compounds do not accumulate equally in all tumours and a comprehensive mapping of their uptake in different neoplasms in various species must be done before their possible use as tumour diagnostic agents can be appraised[8].

Zusammenfassung. Die Verteilung von radioaktivem Vitamin B_{12} und 4-Jodphenylalanin in tumortragenden Mäusen wurde ganztierautoradiographisch untersucht. Die grösste Aufnahme von B_{12} wurde im weichen fibroblastischen Osteosarkom gefunden, während 4-Jodphenylalanin in einem Ehrlich-Ascites-Tumor aufgenommen wurde.

L. Blomquist, H. Flodh and S. Ullberg

Department of Pharmacology, Royal Veterinary College, Stockholm 50 (Sweden), 23 September 1968.

[4] K.-U. Blum and H. C. Heinrich, Vitamins Horm. 7, 486 (1957).
[5] J. J. Oleson and P. A. Little, Proc. Soc. exp. Biol. Med. 71, 226 (1949).
[6] S. Ullberg and L. Blomquist, Acta pharm. suec. 5, 45 (1968).
[7] L. Berlinguet, N. Bégin and L. M. Babineau, Can. J. Biochem. Physiol. 40, 1111 (1962).
[8] Acknowledgments. This work has been supported by research grants from the Swedish Cancer Society (No. 68:37) and from Söderbergs Stiftelse.

THE AMERICAN JOURNAL OF CLINICAL NUTRITION
Vol. 21, No. 6, June, 1968, pp. 657-664
Printed in U.S.A.

Excretion of Cobalamins and Coenzyme B_{12} following Massive Parenteral Doses[1,2]

KEITH BODDY AND J. F. ADAMS

CONFLICTING VIEWS on the kinetics of tracer doses of vitamin B_{12} have led to considerable confusion and doubt about the significance and value of many published radiobiological studies. In essence, the conflict is whether a tracer dose effectively equilibrates metabolically with the body stores of natural B_{12}. A detailed examination of the literature on the subject led us to the conclusion that the evidence against equilibration within a finite time was less convincing than the evidence in favor of equilibration within a short time (5). The study reported here was designed to elaborate the point in a practical fashion.

Subjects with vitamin B_{12} deficiency to the point of megaloblastosis are known to have minute body stores of microbiologically active material as judged by assays of liver obtained by needle biopsy or at postmortem. If such patients, who are incapable of absorbing cobalamins for their diet, were given a large parenteral dose, or doses, of radioactive cobalamin then, for practical purposes, their entire body stores would be the fraction of dose retained after the initial urinary loss. If, after the initial perturbation caused by such dosing, a steady state were established within a finite time, and the subsequent daily loss (percent per day) could be adequately described by a single exponential term, it would be reasonable to assume that the kinetics were then those of a single pool system for which the assumption of metabolic equilibration is justified rather than those of a multipool system for which the assumption is not justified. Supporting evidence for the single pool concept would be obtained if B_{12}-repleted subjects or partially B_{12}-repleted subjects given similar doses were found to have similar patterns.

MATERIALS AND METHODS

Fourteen subjects were studied. One was a healthy volunteer who was not anemic, had a normal serum vitamin B_{12} level, and ate a normal diet. Thirteen were initially B_{12} deficient, all having subnormal serum B_{12} levels and megaloblastic erythropoiesis on marrow biopsy smears: some had presented with signs and symptoms of anemia and others, who were not anemic, or were just mildly anemic, had presented with symptoms suggesting B_{12} deficiency such as glossitis. Additional diagnostic investigations led to the diagnosis of postgastrectomy megaloblastic anemia in one case (JMK) and Addisonian pernicious anemia in the remainder. The pretreatment hematological data are summarized in Table 1 together with treatment schedules.

Whole-body monitoring was performed with the monitor described in detail previously (4), measurements being made with the subject in the prone and supine positions when this was practicable. In some subjects the initial count was made within an hour after dosing but this was not practicable in all cases, as treatment had begun before the study was established. The normal subject was studied in more detail by whole-body monitoring and by measurement of urinary loss in order to examine variations in whole-body counting rates due to redistribution

[1] From Scottish Research Reactor Centre, East Kilbride, and Southern General Hospital, Glasgow, Scotland.

[2] Supported by a grant from the Secretary of State on the advice of the Advisory Committee on Medical Research.

TABLE I

Pretreatment hematological data and treatment schedules

Subject	Sex	Age, Years	Hemoglobin, g/100 ml	Serum B_{12} μμg/ml	Erythropoiesis[a]	Treatment Schedules
JMK	M	71	10.1	58	M	5,000 μg ^{57}Co-OH B_{12} iv
MB	F	34	6.4	25	M	5,000 μg ^{57}Co-OH B_{12} iv
AB	F	62	5.1	25	M	5,000 μg ^{57}Co-OH B_{12} iv
MO	M	51	7.9	26	M	5,000 μg ^{57}Co-OH B_{12} iv
JP	M	57	8.5	25	M	5,000 μg ^{57}Co-OH B_{12} iv
HM	F	66	10.7	59	M	5,000 μg ^{57}Co-OH B_{12} iv
TM	M	74	5.7	25	M	5,000 μg ^{57}Co-CN B_{12} iv
AM	F	41	11.1	25	M	5,000 μg ^{57}Co-CoE B_{12} iv then 8/12 later 5,000 μg ^{58}Co-CN B_{12} iv
JH	M	47	10.1	25	M	10 × 1,000 μg ^{57}Co-OH B_{12} im
JK	F	69	5.6	25	M	5,000 μg ^{58}Co-CN B_{12} iv then 1/52 later 5,000 μg ^{57}Co-OH B_{12} iv
ML	F	51	12.0	25	M	5,000 μg ^{58}Co-CN B_{12} iv then 1/52 later 5,000 μg ^{57}Co-OH B_{12} iv
RA	M	45	5.1	44	M	5,000 μg ^{58}Co-CN B_{12} iv then 2/52 later 5,000 μg ^{57}Co-OH B_{12} iv
JM	F	70	6.9	25	M	10 × 1,000 μg ^{58}Co-CN B_{12} im then 11/12 later 5,000 μg ^{57}Co-CN B_{12} iv
JA	M	42	14.8	35		5,000 μg ^{57}Co-OH B_{12} iv

OH B_{12} = hydroxocobalamin.
CN B_{12} = cyanocobalamin.
CoE = coenzyme.
[a] M = megaloblastic.
8/12 = 8 months.
11/12 = 11 months.
1/52 = 1 week.
2/52 = 2 weeks.

in the body of the injected dose. In evaluating results obtained from the subjects who were given both ^{58}Co-cyanocobalamin and ^{57}Co-hydroxocobalamin, allowance was made for the contribution of the Compton region of the ^{58}Co gamma-ray spectrum to the photopeak of the ^{57}Co spectrum. The subject was monitored immediately prior to the injection of ^{57}Co-cobalamin and the fraction (count rate in ^{57}Co photopeak region ÷ count rate ^{58}Co photopeak region) evaluated from the ^{58}Co whole-body spectrum. Monitoring was repeated shortly after the injection of ^{57}Co-cobalamin and this and subsequent counts were corrected for the ^{58}Co contribution by subtracting the product of the ^{58}Co photopeak count rate and the fraction obtained previously.

For each subject the regression coefficient (rate of loss per day) and its standard error were computed by the method of least squares. A single exponential term adequately described the data after about 30 days postinjection.

RESULTS

These are given in full in Tables II–VI and illustratively in Fig. 1. *Day 0* is the day of the first injection of cobalamin and is the zero reference for the time scale thereafter.

The largest variations in counting rate due to distribution of injected B_{12} may be expected within a few hours of administration. In the normal subject the maximum variation in the period 0–4½ hr was about 2% and the standard error 1% (Table II). These values are much less than the variation of about 40% reported by Heinrich (9) and over 20% by Reizenstein et al. (11) after oral dosing. Consequently, greater confidence can be afforded to the "100% value" and to measurements made shortly after administration.

The results for the individual patients

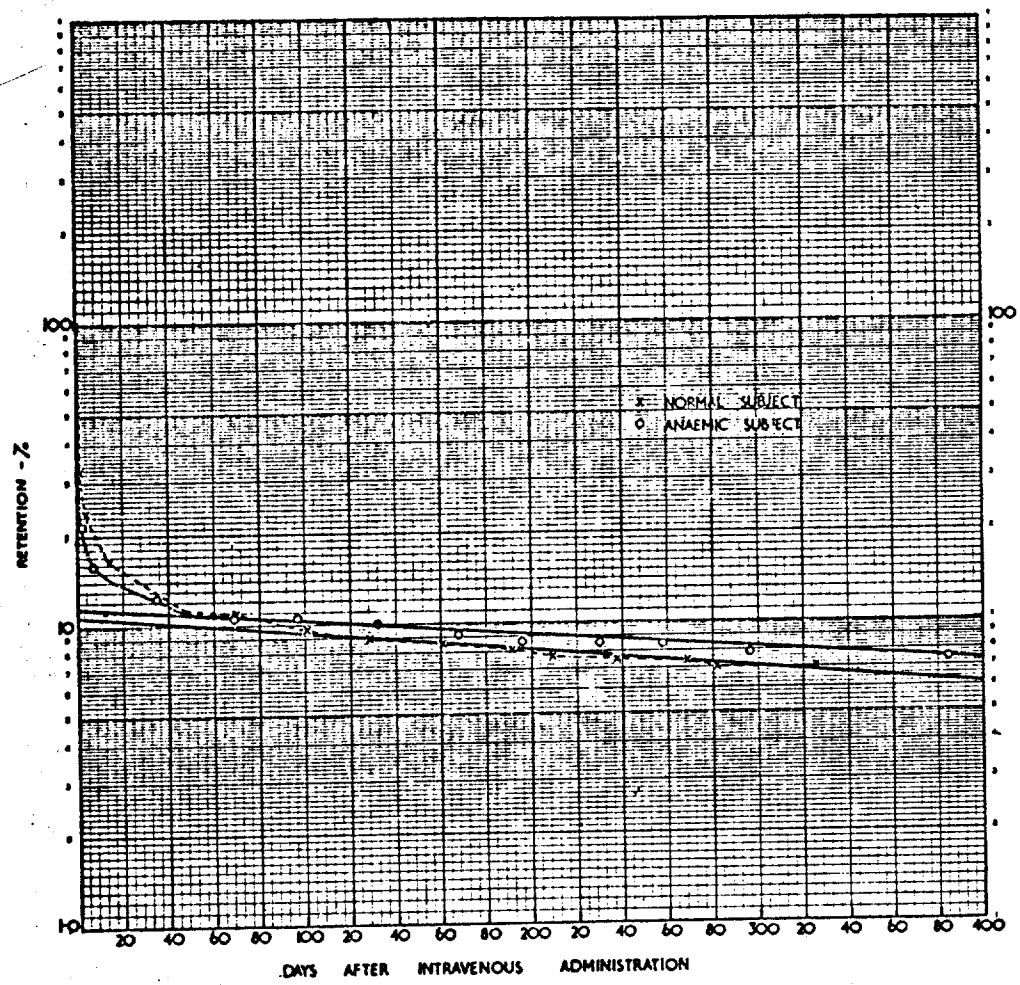


FIG. 1. Percentage retention of labeled hydroxo-vitamin B_{12} in a normal subject and an anemic subject.

TABLE II

Variation of whole-body counting shortly after intravenous administration of ^{57}Co-hydroxocobalamin and comparison with urinary loss

Time after Initial Whole-Body Count	Whole Body, Supine and Prone, cpm	Loss, % Total Injected	Collection Period	Urine Loss, % Total Injected	Cumulative Urine Loss, % Total Injected
0 hr	20,507				
2½ hr	20,915	σ = 2.3%			
4½ hr	20,902				
6 hr	9,594	53.8	0–6 hr	46.4	46.4
24 hr	6,719	67.7	6–30 hr	13.8	60.2
			30–48 hr	3.3	63.5
3 days	4,920	76.3	48–72 hr	2.6	66.1

TABLE III

Retention in anemic subjects following a single intravenous dose of 5,000 μg labeled vitamin B_{12}

Days	cpm	% Retained	Days	cpm	% Retained	Days	cpm	% Retained	Days	cpm	% Retained	Days	cpm	% Retained
Subject JMK (HM)			*Subject AB (HF)*			*Subject MB (HF)*			*Subject AM (EF)*			*Subject JH (HM)*		
0	5,774	100	14	2,770	100	9	2,595	100	8	1,141	100	73	1,405	[illegible]
2	1,321	22.9	35	2,400	88.8	27	2,270	87.4	22	697	61.1	114	1,394	[illegible]
7	915	15.8	88	2,000	74.0	56	2,109	81.2	50	516	45.2	135	1,347	[illegible]
35	717	12.4	116	1,958	72.5	105	1,905	73.5	85	510	44.7	170	1,204	[illegible]
69	612	10.6	137	1,890	70.0	133	1,845	71.1	113	390	34.2	206	1,204	85
97	615	10.6	165	1,780	65.9	168	1,743	67.2	153	330	28.9	234	1,197	[illegible]
132	577	10.0	193	1,711	63.4	236	1,648	63.5	176	338	29.6	268	1,153	[illegible]
168	527	9.1	221	1,730	64.1	253	1,681	64.9	212	319	27.9	296	1,115	[illegible]
196	495	8.6	263	1,571	58.2	302	1,524	58.7	225	306	26.8	334	1,109	[illegible]
230	491	8.5	292	1,523	56.4	330	1,426	55.0	253	310	27.1	394	1,123	[illegible]
258	484	8.4	320	1,499	55.5	358	1,445	55.7	281	307	26.9	432	954	67.9
296	435	7.9	354	1,454	53.8	386	1,392	53.7	309	301	26.4	488	1,031	73.3
385	436	7.5	376	1,472	54.5				337	302	26.4			
413	433	7.5												
441	445	7.7												
Subject HM (CF)			*Subject MO (HF)*			*Subject JP (HM)*			*Subject TM (CM)*					
7	467	100	7	2,730	100	7	2,820	100	61	784	100			
35	455	97.4	35	2,281	83.6	35	2,310	81.9	89	712	90.9			
66	426	91.2	63	2,140	78.4	63	2,070	73.4	117	765	97.5			
84	393	84.1	91	1,938	71.0	91	2,000	70.9	158	623	79.5			
112	401	85.9	112	1,948	71.4	112	1,872	66.4	179	634	80.9			
143	381	81.5	147	1,878	69.1	147	1,782	63.2	197	616	78.6			
156	365	78.1	176	1,770	64.8	176	1,758	62.4	225	623	79.5			
199	359	76.8	203	1,773	65.0	203	1,719	60.9	253	606	77.4			
227	378	81.0	218	1,690	61.9	218	1,662	58.9						
283	382	81.7	260	1,675	61.4	260	1,580	56.1						
304	334	71.5	281	1,673	61.3	309	1,498	53.1						
325	313	67.0	309	1,582	58.0	337	1,474	52.3						
			337	1,573	57.6	365	1,477	52.4						
			365	1,535	56.2									

H = hydroxocobalamin. *C* = cyanocobalamin. *E* = coenzyme. *M* = male. *F* = female.

are given in Tables III–V. The computed rates of loss per day and their standard errors and the biological half-life from about *day 30* onwards are summarized in Table VI. An overall range of 0.09–0.19%/day was obtained with a mean of 0.14%/day. No significant difference was observed between the excretion rate of the anemia subjects and the normal subject, and the form of the administered vitamin did not apparently influence the excretion rate. However, Table V shows significantly greater retention of hydroxocobalamin than of cyanocobalamin.

DISCUSSION

Our main interest in this study lay in the late excretion pattern: that is, the loss after the initial perturbation caused by the injection. It is apparent from the results shown in Table VI and Fig. 1 that a single exponential term adequately de-

TABLE IV

Retention in a normal subject (J.I(HM)[a]) following a single intravenous dose of 5,000 μg vitamin B_{12}

Days	cpm	% Retained
0	20,774	100
1	6,714	32.4
3	4,920	23.7
14	3,409	16.4
48	2,342	11.3
70	2,234	10.8
101	1,982	9.6
129	1,845	8.9
161	1,769	8.5
192	1,689	8.1
210	1,609	7.7
238	1,557	7.5
269	1,528	7.3
282	1,459	7.0
325	1,457	7.0
381	1,304	6.3
402	1,363	6.6
430	1,256	6.0

[a] HM = hydroxocobalamin, male subject.

scribes the late excretion pattern in the B_{12}-deficient subjects and in the normal subject. The mean standard error of the excretion rate is only about ±14% which would seem to be an adequate level of accuracy for most practical purposes. Consequently the loss at this time can be from only a single compartment. In the B_{12}-deficient subjects given a single dose, the entire stores would for practical purposes be the retained radioactive material and this would not be diluted by absorption of nonradioactive cobalamin from the diet. In the normal subject the retained dose would be only a fraction of the stores and subject to continual dilution by absorbed cobalamins. The fact that the results in these subjects are closely similar and also closely similar to those reported by Bozian et al. (6) for patients given tracer doses, constitutes good practical evidence that equilibration was established within about 1 month after injection. A more complete comparison of the results obtained by us (1), by Bozian et al. (6), and by other workers is to be published elsewhere. At the tracer dose level, compared with the large doses administered in the present study, it may be possible to demonstrate effective equilibration at even shorter times after injection.

The isolation by Barker (3) of coenzyme B_{12} from human liver led to two possibilities: first, that injected cyanocobalamin and hydroxocobalamin were converted to coenzyme form in vivo and utilized in coenzyme form, and second, that they were not converted but were utilized in their original form. Work on the in vivo interconversion of hydroxocobalamin and cyanocobalamin by Rosenblum (12) favored the first possibility, and recent studies on the in vivo conversion of injected cyanocobalamin and hydroxocobalamin to coenzyme form by Uchino et al. (13) provide good evidence that this is the case. One would therefore expect that the turnover of retained radioactive material, presumably coenzyme, after parenteral radioactive cyanocobalamin, hydroxocobalamin, and coenzyme would be identical. Our studies on this point are not complete but the results to date from patients given one of these substances and from a small number of patients given two of these substances justify preliminary comment. In the single patient given coenzyme B_{12} the late rate loss was 0.19% per day, a value which does not differ significantly from the rate losses of 0.09–0.18% per day in patients given cyanocobalamin or hydroxocobalamin. The late rate losses in patients given cyanocobalamin and in the patients given hydroxocobalamin do not differ significantly, and in the four patients given both hydroxocobalamin and cyanocobalamin it is clear that the utilization of the retained material is not different. These results,

TABLE VI

Excretion rates and their standard errors

Subject	Time Period after Administration	Biological $T_{1/2}$ Days	Rate of Loss, % per Day	Standard Error, % of Rate of Loss
Anemic				
Single isotope studies				
JMK (H)	35-441	630	0.11	±8.7
AB (H)	35-376	495	0.14	±7.4
MB (H)	27-386	533	0.13	±11.2
HM (C)	66-325	693	0.10	±13.8
AM (E)	50-337	365	0.19	±18.3
MO (H)	35-365	630	0.11	±8.2
JP (H)	35-365	533	0.13	±6.8
TM (C)	61-253	495	0.14	±20.8
JH (H)	73-488	770	0.09	±9.5
Anemic				
Double isotope studies				
JK (C)	28-154	495	0.14	±12.1
JK (H)	28-119	433	0.16	±15.6
ML (C)	42-154	433	0.16	±14.8
ML (H)	42-147	385	0.18	±22.9
RA (C)	43-245	408	0.17	±26.1
RA (H)	56-202	533	0.13	±14.4
JM (C)	59-283	630	0.11	±27.0
JM (H)	70-183	495	0.14	±10.5
Normal				
JA (H)	70-430	495	0.14	±7.4

C = cyanocobalamin.
H = hydroxocobalamin.
E = coenzyme.

four patients given both cyanocobalamin and hydroxocobalamin is that they form a group lying between the normal and initially deficient subjects. In the case of the patients given the two doses at short intervals the body stores at the time of the second injection were about 10% of the previous dose, that is about 500 μg. This value is greater than that found in depleted subjects. The fact that the late rate losses of the material retained from the second injection are not significantly different from those after the first, or from those found in the normal subject or initially deficient subjects given a single dose, constitutes additional evidence for the concept of equilibration of the retained material with the native stores within a finite time period.

A systematic study of the relative retention of cyanocobalamin and hydroxocobalamin was not attempted but the results for the patients given both cobalamins confirm previous reports that a greater proportion of a dose of hydroxocobalamin is retained than of a dose of cyanocobalamin (Glass et al. (7), Killander and Schilling (10), and Adams and Kennedy (2)).

Postinjection loss is usually measured by monitoring of urine passed within 3 days of injection. It is obvious from the results that with the dosage used this gives a false impression of postinjection loss and that there is a considerable loss between the 3 days after injection until a steady state is established. In terms of daily loss in urine it is small and unlikely to be detectable by monitoring of daily urine collections. The

value of a whole-body monitor in measuring the loss in this period is thus clear.

SUMMARY AND CONCLUSIONS

Whole-body monitoring of patients with vitamin B_{12}-deficiency states and a normal subject given parenteral doses, usually 5,000 μg of hydroxocobalamin or coenzyme B_{12}, was carried out for periods of up to 488 days after injection.

The excretion rate of the retained material was constant after an interval of 4–8 weeks after dosing and the late excretion pattern was adequately described by a single exponential term in all cases studied. This implies that metabolic equilibration had been established. No significant differences were found in the late excretion rates for the normal subject, initially B_{12}-deficient patients, or previously treated initially B_{12}-deficient subjects.

The late excretion rates for material retained after dosing with hydroxocobalamin, cyanocobalamin, and coenzyme B_{12} did not differ significantly.

A considerable loss of injected material occurred during the period from 3 days after injection until the steady state was attained 1–2 months after injection. This loss was only detectable by whole-body monitoring. The value of whole-body monitoring in studies of cobalamin kinetics is demonstrated.

The grant from the Secretary of State and the excellent technical assistance of Misses P. King and B. Holmes and of Mr. D. Dunn is greatly appreciated. The authors wish to thank Professor H. W. Wilson, Director of the Reactor Centre, for his interest and encouragement.

REFERENCES

1. Adams, J. F., and K. Boddy. Metabolic equilibrium of tracer and natural vitamin B_{12}—an experimental study. *J. Lab. Clin. Med.* In press.
2. Adams, J. F., and E. H. Kennedy. Hydroxocobalamin: excretion and retention after parenteral doses in anemic and nonanemic subjects, with reference to the treatment of vitamin B_{12} deficiency states. *J. Lab. Clin. Med.* 65: 4[illegible]0, 1965.
3. Barker, H. A. *Chemistry and Biology of the Vitamin B_{12} Coenzymes in Vitamin B_{12} and Intrinsic Factor*, edited by H. C. Heinrich. Stuttgart: Enke, 1962, pp. 82–105.
4. Boddy, K. The development and performance of a prototype shadow-shield whole body monitor. *Phys. Med. Biol.* 12: 43, 1967.
5. Boddy, K., and J. F. Adams. The validity of the assumption of tracer equilibrium with respect to the excretion of vitamin B_{12}. *Phys. Med. Biol.* 13: 55, 1968.
6. Bozian, R. C., J. L. Ferguson, R. M. Heyssel, G. R. Meneely and W. J. Darby. Evidence concerning the human requirement for vitamin B_{12}. *Am. J. Clin. Nutr.* 12: 117, 1963.
7. Glass, G. B. J., H. R. Skeggs, D. H. Lee, E. L. Jones and W. W. Hardy. Hydroxocobalamin. I. Blood levels and urinary excretion of vitamin B_{12} in man after a single parenteral dose of aqueous hydroxocobalamin, aqueous cyanocobalamin, and cyanocobalamin zinc-tannate complex. *Blood* 18: 511, 1961.
8. Glass, G. B. J., and D. H. Lee. Biological half-life of hydroxocobalamin compared with that of cyanocobalamin in the human liver. *Blood* 27: 227, 1966.
9. Heinrich, H. C. Metabolic basis of the diagnosis and therapy of vitamin B_{12} deficiency. *Seminars Hematol.* 1: 199, 1964.
10. Killander, A., and R. F. Schilling. Studies on hydroxocobalamin. I. Excretion and retention of massive doses in control subjects. *J. Lab. Clin. Med.* 57: 553, 1961.
11. Reizenstein, P. G., E. P. Cronkite and S. N. Cohn. Measurement of absorption of vitamin B_{12} by whole-body gamma spectrometry. *Blood* 18: 1, 95, 1961.
12. Rosenblum, C. Stability of cyanocobalamin in living systems. *Scand. J. Haematol. Ser. Haematol.* 3: 48, 1965.
13. Uchino, H., Y. Yagiri, T. Yoshimo, M. Kondo and G. Wakisaka. Conversion of cyano- and hydroxo-cobalamin in vivo into coenzyme form of vitamin B_{12} in the rat. *Nature* 205: 176, 1965.

A double-tracer test of the oral absorption of ^{57}Co- and ^{58}Co-vitamin B_{12} using a whole-body monitor: clinical experience and technical factors[1]

Keith Boddy,[2] B.Sc., M.Sc., Ph.D., F. Inst.P., Maureen E. Mahaffy,[3] B.Sc., and George Will,[4] B.Sc., M.B., Ch.B., F.R.C.P.G., M.R.C.P.E.

Vitamin B_{12} labeled with ^{57}Co has been considered unsuitable for tracer studies in man using whole-body monitors because the low energy (122 kev) gamma rays are considerably attenuated in the human body. It had been anticipated that variations in the degree of attenuation, which might result from tracer redistribution within the body, would lead to marked fluctuation in the whole-body counting rate. Experience with ^{57}Co-labeled vitamin B_{12} in whole-body monitoring studies has shown, however, that the problems are less formidable than anticipated (1) and a double-tracer test of vitamin B_{12} absorption has been described using ^{57}Co and ^{58}Co-labeled preparations (2).

We report here further clinical experience with the double-tracer test of oral absorption of vitamin B_{12} together with a detailed evaluation of the methodology of the test.

Materials and methods

Clinical experience with 132 subjects (71 controls and 61 patients with Addisonian pernicious anemia) is described: methodological studies were carried out in 29 control subjects. The controls were patients whose peripheral blood findings were within the accepted normal range in respect to hemoglobin level, packed cell volume, and mean corpuscular hemoglobin concentration, and who were also suffering from conditions such as psychoneurosis, obesity, mild hypertension, or chronic bronchitis, which are not known to influence the alimentary absorption of vitamin B_{12}. The criteria used for the diagnosis of Addisonian pernicious anemia were the peripheral blood findings, megaloblastic change in the sternal marrow, achlorhydria on maximum histamine stimulation, and a satisfactory response to treatment with hydroxocobalamin.

The Merlin static (3) and mobile (4) whole-body monitors were used. Each monitor utilized a NaI detector, 29.2 cm diameter by 10.2 cm deep, in a lead shadow shield 10.2 to 15.2 cm thick, in a scanning-bed geometry. All subjects were monitored in both supine and prone positions. An initial measurement of natural body radioactivity was made for each subject before the administration of the radioactive vitamin B_{12}. Subsequent counting rates were corrected for natural body radioactivity, background counting rates, and radioactive decay.

All subjects fasted overnight and following the initial whole body measurements each subject received orally 0.5 μg vitamin B_{12} labeled with 0.5 μCi ^{57}Co or ^{58}Co as indicated. The dose was given in 50 to 100 ml water and no food was taken for 2 hr after the dose was administered.

In the double-tracer test, ^{57}Co-vitamin B_{12} (free vitamin B_{12}) was administered and approximately 30 min later a whole-body measurement was made giving the counting-rate representing 100% retention. The patient then drank several glasses of water to assist in flushing the free vitamin B_{12} through the stomach and 20 to 30 min later, ^{58}Co-labeled vitamin B_{12}, together with 60 mg potent porcine intrinsic factor (bound vitamin B_{12}), was administered. Thirty minutes later a further whole-body measurement was made giving the counting-rate for 100% retention of ^{58}Co-vitamin with intrinsic factor (IF). Counting-rates in the ^{57}Co range were corrected for the contribution of ^{58}Co gamma rays. Seven days later a single whole-body measurement gave simultaneously the absorption of free and bound vitamin B_{12}.

Methodological studies

Four subjects received doses of ^{57}Co-vitamin B_{12} simultaneously and were then monitored in rotation for up to 6 hr postadministration to study the overall time sequence of changes in counting-rate on the day of administration. Four other subjects were given ^{58}Co-vitamin B_{12} and each was monitored 5 to 10 min after administration of the doses and at the same specific time intervals thereafter to determine if an optimum time could be

[1] Supported in part by a grant from the Scottish Hospital Endowments Research Trust.

[2] Senior Lecturer (Health Physics and Nuclear Medicine), Scottish (Universities) Research Reactor Centre, East Kilbride, Glasgow, Scotland. [3] Research Assistant. [4] Consultant Physician, Gateside Hospital, Greenock, Scotland.

The American Journal of Clinical Nutrition 25: JULY 1972, pp. 703–708. Printed in U.S.A.

found at which to measure the counting-rate corresponding to 100% retention. All eight subjects were re-monitored 24 hr after administration; urine and fecal collections were made during this 24-hr period and accidental losses of samples were noted. The samples were assayed in a fixed position in the whole-body monitor and the counting-rates were compared with those from standards of similar geometry counted in the same position. Four additional subjects received ^{57}Co-vitamin B_{12} and were monitored at intervals on the day of administration to examine the variation of the counting-rates in the ^{57}Co photopeak and in the energy range used for ^{57}Co. Seventeen subjects were given ^{57}Co-vitamin B_{12} and monitored approximately 30 to 60 min after dose administration as part of an absorption test. These subjects were re-monitored 7 days later, and the ratio of the counting-rate in the ^{57}Co energy band to that in the ^{57}Co photopeak was compared with the ratio found on the day of administration.

Results

Clinical experience with the double-tracer test is summarized in Table 1. The range of absorption of free vitamin B_{12} in control subjects did not overlap the range in patients with pernicious anemia. When the vitamin was administered with intrinsic factor, absorption was improved by a factor of more than two in patients with pernicious anemia and consistently less than two in controls.

For the subjects who were given ^{57}Co-vitamin B_{12} doses simultaneously and were subsequently monitored in rotation, the whole-body counting-rates, expressed as a percentage of the post 0.5-hr value, are summarized in Table 2. In these subjects, the greatest individual variation (maximum/minimum counting-rates) ranged from 1.06 to 1.11. The result obtained within 1 hr of administration was lower in each subject than the result after 3 hr. There was no other consistent and obvious trend. Assuming the variations are random, the standard deviation as a percentage of the post 30-min value in individuals ranged from 2.6 to 4.1% with a mean of 3.4%. The loss of ^{57}Co-vitamin B_{12} estimated by whole-body counting and by assay of feces and urine is shown in Table 3. The results are in fair agreement.

The findings in four subjects given ^{57}Co-vitamin B_{12} and subsequently counted at the same time intervals after administration are summarized in Table 4 and are normalized

TABLE 1
Summary of clinical experience in measuring oral absorption of free and bound vitamin B_{12}

Clinical condition	No. of subjects	Range of absorption, %		Range of ratio, bound/free
		Free B_{12}	Bound B_{12}	
Controls	71[a]/25[b]	19.6–84.9	27.1–73.6	0.44–1.91
Pernicious anemia	48[a]/60[b]	0.3–16.2	9.7–73.2	2.32–15.2

[a] Free B_{12} absorption measured. [b] Bound B_{12} absorption measured.

TABLE 2
Variation of whole-body counting-rate following oral administration of ^{57}Co-vitamin B_{12} given simultaneously to four subjects

Case 1		Case 2		Case 3		Case 4	
Time after dose, hr	WBM,[a] %	Time after dose, hr	WBM, %	Time after dose, hr	WBM, %	Time after dose, hr	WBM, %
0.08	90.3	0.42	94.6	0.58	100	0.92	100
3.17	100	3.67	100	3.92	108.7	4.17	104.1
4.58	97.5	4.83	99.8	5.08	108.7	5.33	102.8
5.75	95.6	6.00	100.8	6.25	101.9	6.50	106.3
σ = 4.1		σ = 2.8		σ = 4.1		σ = 2.6	

[a] WBM represents whole-body monitoring result.

to the counting-rate at 30 min after administration. The greatest individual variation ranged from 1.11 to 1.22 and the standard deviations as percentages of the 30-min value ranged from 3.6 to 7.0% with a mean of 5.7%. The variation in the counting-rate in this group was greater than in the previous group. A comparison of the loss of ^{57}Co-vitamin B_{12} estimated by whole-body counting and excreta assay is given also in Table 4. The results are in fair agreement with the exception of subject 6 who did not cooperate satisfactorily with collection of excreta.

The variation in whole-body counting-rates in the four subjects given ^{58}Co-vitamin B_{12} and then monitored at specific intervals on the day of administration is shown in Table 5. Results normalized to the initial value are given for the ^{58}Co photopeak range and for the energy band used for ^{57}Co. The maximum variation for individual subjects ranged from 1.04 to 1.12 with a mean of 1.07 and the standard deviations ranged from 1.98 to 5.12% with a mean of 3.54% in the ^{58}Co-photopeak. Corresponding values for the ^{57}Co energy band were 1.06 to 1.08% (mean 1.07%) and 2.50 to 3.66% (mean 3.08%). The ratio of the counting-rate in the ^{57}Co energy band and that in the ^{58}Co photopeak was calculated for each result and in individual subjects the standard error of the ratio ranged from 0.83 to 3.17% with a mean of 2.33%.

In 17 subjects to whom ^{58}Co vitamin B_{12} had been administered, the ratio of the counting-rates in the ^{57}Co and ^{58}Co energy bands was calculated on the day of administration and 7 days later. The difference between these values was expressed as the percentage of the ratio on the day of administration. The mean difference was −2.3% (SEM ± 3.4%), which was not significantly different from the "expected" value of zero. The difference ranged from 0 to 10% in ten subjects, from 10 to 20% in six, and a difference of 42.2% was found in one subject.

Discussion

Measurement of the oral absorption of vitamin B_{12} given without and with intrinsic factor is a definitive test in the diagnosis of pernicious anemia. The customary Schilling test (5) and the modified double-tracer test using ^{57}Co and ^{60}Co (6) or ^{57}Co and ^{58}Co (7, 8) measure absorption indirectly and involve the administration of a large flushing dose of the vitamin, which complicates sub-

TABLE 3
Whole-body retention from monitoring and excreta assay on 1 and 2 days postadministration, %

Case 1		Case 2		Case 3		Case 4	
Excreta	WBM	Excreta	WBM	Excreta	WBM	Excreta	WBM
59.5	65.0	93.3	98.7	99.5	104.4	99.5	113.1
56.5	63.6	59.6	56.9	98.4	103.4	92.5	103.4

TABLE 4
Variation in whole-body counting-rate following oral administration of ^{57}Co-vitamin B_{12} to four subjects monitored at the same time intervals

Case no.	Time after administration, hr								SD	24-hr retention, %	
	0.08–0.17	0.5	1	1.5	2	3	4	6	%	Excreta	WBM[a]
5	95.5	100	103.2	106.0	101.1	99.0	97.5	105.0	3.7	68.8	69.8
6	96.8	100	86.0	91.7	103.2	93.7	90.6	91.4	7.0	82.0[b]	69.4
7	83.6	100	102.0	94.5	97.5	99.6	101.3	100.0	6.2	96.8	95.6
8	104.3	100	107.0	118.0	107.0	109.2	111.8	116.7	5.8	81.7	87.5

Whole-body retention at the 24th hr measured by whole-body monitoring and excreta assay.
[a] WBM represents whole-body monitoring result. [b] Excreta collection incomplete.

TABLE 5

Variation of whole-body counting-rate following oral administration of ^{58}Co-vitamin B_{12}, in the ^{58}Co, ^{57}Co energy ranges and the ratio of the counting-rates in these ranges

Case no.	Whole-body counting-rate, %					SD, %
	^{58}Co energy range					
9	100	109.0	108.7	100.8		4.89
10	100	98.6	98.9	89.0		5.12
11	100	100.1	103.4	104.3	103.0	1.98
12	100	100.5	97.6	95.9		2.15
	^{57}Co energy range					
9	100	105.8	104.6	100.9		2.50
10	100	101.7	101.1	93.8		3.66
11	100	99.6	102.9	106.8	104.0	2.89
12	100	106.0	102.9	102.3		3.25
	Ratio: ^{57}Co energy range/^{58}Co energy range					
9	0.47	0.45	0.45	0.47		2.34
10	0.44	0.46	0.45	0.47		2.96
11	0.48	0.48	0.48	0.49	0.48	0.83
12	0.41	0.43	0.43	0.44		3.17

sequent hematological investigations. The tests are unreliable in the presence of concomitant conditions, such as renal disease.

These difficulties and the problems associated with the collection and assay of urine or feces are eliminated by the use of whole-body radioactivity monitors, which directly measure the absorption of the labeled vitamin B_{12}. Whole-body monitor tests can be performed as outpatient procedures thus effecting considerable economy in hospital costs. A period of approximately 7 days is required between administration of the tracer dose and the measurement of absorption. Consequently, a sequential test of absorption of vitamin B_{12} given initially by itself and subsequently administered together with porcine intrinsic factor will take a minimum of 14 days. A double-tracer test using a whole-body monitor was reported (9) in which the labeling isotopes were ^{58}Co and ^{60}Co. Unfortunately, ^{60}Co has a long half-life (5.26 years) and the radiation dose per μCi to the patient is comparatively large, approximately 26 times that of the alternative isotope ^{57}Co. The suitability of ^{57}Co for simultaneous use with ^{58}Co in a modified Schilling test was demonstrated (7, 8). The gamma rays emitted by ^{57}Co are comparatively low in energy with relatively greater absorption in the body, and so it appeared likely that the isotope might be unsuitable for in vivo whole-body monitoring (10). However, double-tracer studies of vitamin B_{12} turnover (1, 11) demonstrated the feasibility of the simultaneous use of ^{57}Co and ^{58}Co in vivo using whole-body monitoring techniques. Subsequently, in vivo tests of oral vitamin B_{12} absorption using ^{57}Co have been reported using ^{57}Co-vitamin B_{12} alone (12), and using a double isotope test with ^{57}Co and ^{58}Co-labeled vitamin B_{12} (2). The additional experience with the double-tracer absorption test reported here has permitted a fuller evaluation of the clinical usefulness and the factors relevant to the optimum use of the test.

Following oral administration, vitamin B_{12} is redistributed within the body causing a change in counting geometry and in the whole-body counting-rate. Up to approximately 7 hr after administration, the labeled vitamin varies in depth in the body as it progresses along the gastrointestinal tract at which time it is primarily in the small intestine with a small fraction reaching the liver. At 24 hr after administration the proportion in other organs, particularly the liver, may be collectively four times that in the small intestine with the liver fraction approaching its maximum and persistent level (13). Thereafter, there is little further change in the distribution of the vitamin within the body organs and the pattern of distribution at 7 days, when excretion of the oral dose is virtually complete, remains much the same as that at 24 hr. In the clinical use of the double-tracer test, the whole-body counting-rate representing "100% absorption" was measured approximately, but not sooner than, 30 min after administration of each dose. This time interval was selected initially on the basis of convenience. More detailed examination was required to determine if there was an optimum time interval. The sequential counting studies reported here indicate that a time interval of 30 min is satisfactory for measuring 100% absorption for both ^{57}Co and ^{58}Co-labeled vitamin B_{12}.

Additional factors influencing the accuracy of the absorption measurements are the var-

iation of the ^{57}Co and also the ^{58}Co whole-body counting-rates on the day of administration. A third factor, which affects only the double-tracer test, is the variation of the fraction of ^{58}Co gamma rays detected in the ^{57}Co energy range on the day of administration and 7 days later when the absorption measurement is made. Following oral administration of ^{57}Co-vitamin B_{12}, there was no consistent trend in the whole-body counting-rates apart from finding (in those patients who were given doses simultaneously) that the 3-hr values were higher than readings in the 1st hr. The variations in whole-body counting-rate following oral administration of ^{58}Co-vitamin B_{12} failed to show any consistent trend in either the ^{58}Co energy range, in the energy range used for ^{57}Co, or in the fraction of ^{58}Co gamma rays detected in the ^{57}Co energy band. As the variations are evidently random, the mean standard deviations were used for convenience and comparison. They were 4.6% for ^{57}Co vitamin B_{12}, 3.5% for ^{58}Co vitamin B_{12}, and 2.3% for the fraction of ^{58}Co gamma rays in the ^{57}Co energy band. As might be expected, the standard deviation is smaller using ^{58}Co presumably because the higher energy gamma rays are attenuated less in the body than those of ^{57}Co. The uncertainty in the counting rates representing 100% absorption in the double-tracer test is represented by these standard deviations.

In the 17 subjects in whom the fractional contribution of ^{58}Co to the ^{57}Co energy band was measured on the day of administration and again at 7 days after administration, the mean change found was −2.3%, which is similar to the uncertainties in the 100% absorption value. However, in the double isotope test of absorption, the uncertainty due to this factor will be even less because the dose (1 μCi) of ^{57}Co vitamin B_{12} used is two to four times greater than that of ^{58}Co vitamin (0.25 to 0.50 μCi). There will be a further diminution in patients with Addisonian pernicious anemia who absorb significantly more of the ^{57}Co-vitamin B_{12} given with intrinsic factor than of the ^{58}Co-vitamin given alone. The subject acts as his own control in determining the fractional contribution of ^{58}Co to the ^{57}Co energy band in this test and so variation of the fraction with body habitus is eliminated.

The present variation of the counting rate in the ^{58}Co energy range on the day of administration of ^{58}Co-vitamin B_{12} is similar to that reported in single isotope studies using whole-body monitors with scanning-bed geometries (14, 15), less than that using a stretcher-geometry (16), and less than in a double-tracer study using ^{58}Co and ^{60}Co with a tilting-chair monitor (9). The variation with ^{57}Co-vitamin B_{12} was similar to that found by Naversten et al. (12) who also used a scanning-bed technique. The present findings confirm and amplify an earlier study, which demonstrated the feasibility of the double-tracer absorption test (2).

To date, clinical experience with the test has shown clear differentiation between control subjects and patients with pernicious anemia (Table 1). The range of absorption of free vitamin B_{12} found in all control subjects was 20 to 85% and in no individual was the absorption ratio (vitamin B_{12} + IF)/(vitamin B_{12} alone) greater than 2.0 (range 0.4 to 1.9). In pernicious anemia, the range of absorption was 0.3 to 16% and the absorption ratio was always greater (range 2.3 to 152) than 2.0. Similar findings have been reported with the double-tracer modification of the Schilling test (6–8). Thus, the percentage absorption of free vitamin B_{12} and the absorption ratio taken together will differentiate these conditions diagnostically.

The present range of absorption of free vitamin B_{12} is similar to that found by other workers using whole-body monitors (9, 12, 16–18). The area of discrepancy is the absorption range of free vitamin B_{12} in pernicious anemia in which two groups, using the same whole-body monitor, found values that overlapped the absorption range in control subjects (17, 18). This has not yet been the experience of other workers nor of ourselves. As suggested retrospectively by Irvine et al. (18), and as we have shown both previously (2) and in the present study, the double-tracer test provides criteria that are more definitive than the single isotope method.

In clinical practice, the test has proved entirely reliable. It is also highly convenient

for the clinician and the patient as only two outpatient visits are required.

Summary

Clinical experience with a double-tracer test of the oral absorption of ^{57}Co-vitamin B_{12} (free vitamin B_{12}) and of ^{57}Co-vitamin B_{12} with intrinsic factor (bound vitamin B_{12}) is reported. Absorption of the free and bound vitamin was measured simultaneously using a whole-body monitor. Control subjects absorbed 19.6 to 84.9% of free vitamin B_{12} and the absorption ratio of bound/free in any subject did not exceed 2. In patients with pernicious anemia, the absorption of free vitamin B_{12} ranged from 0.3 to 16.2% and the absorption ratio in all patients was greater than 2. Technical sources of error are considered and examined; the errors are shown to be acceptable and the advantages of the test are outlined.

We wish to thank Professor H. W. Wilson for his interest and encouragement and Dr. J. F. Adams for his kind permission to include results for patients under his care.

References

1. Boddy, K., and J. F. Adams. Excretion of cobalamins and coenzyme B_{12} following massive parenteral doses. *Am. J. Clin. Nutr.* 21: 657, 1968.
2. Boddy, K., G. Will and B. M. Holmes. An evaluation of ^{57}Co-labelled vitamin B_{12} in a double tracer test of absorption using a whole-body monitor. *Phys. Med. Biol.* 14: 455, 1969.
3. Boddy, K. The development and performance of a prototype shadow-shield whole body monitor. *Phys. Med. Biol.* 12: 43, 1967.
4. Boddy, K. A high sensitivity shadow-shield whole body monitor with scanning-bed and tilting chair geometries, incorporated in a mobile laboratory. *Brit. J. Rediol.* 40: 631, 1967.
5. Schilling, R. F. Intrinsic factor studies. II. The effect of gastric juice on the urinary excretion of radioactivity after the oral administration of radioactive vitamin B_{12}. *J. Lab. Clin. Med.* 42: 860, 1953.
6. Katz, J. H., J. Dimase and R. M. Donaldson. Simultaneous administration of gastric juice-bound and free radioactive cyanocobalamin: rapid procedure for differentiating between intrinsic factor deficiency and other causes of vitamin B_{12} malabsorption. *J. Lab. Clin. Med.* 61: 266, 1963.
7. Bell, T. K., J. M. Bridges and M. G. Nelson. Simultaneous free and bound radioactive vitamin B_{12} urinary excretion test. *J. Clin. Pathol.* 18: 611, 1965.
8. Bell, T. K., and D. Lee. Evaluation of a duel radioisotope urinary excretion test in the diagnosis of pernicious anaemia. *Acta Haematol.* 42: 183, 1969.
9. Reizenstein, P. G., E. P. Cronkite and S. H. Cohn. Measurement of absorption of vitamin B_{12} by whole-body gamma spectrometry. *Blood* 18: 95, 1961.
10. Mollins, D. L., and A. H. Waters. The study of vitamin B_{12} absorption using labelled cobalamins. *Med. Monograph 6.* Amersham: The Radiochemical Centre, 1968.
11. Boddy, K., P. C. King, L. Mervyn, A. MacLeod and J. F. Adams. Retention of cyanocobalamin, hydroxocobalamin and coenzyme B_{12} after parenteral administration. *Lancet* 2: 710, 1968.
12. Naversten, Y., K. Liden, K. G. Stahlberg and A. Norden. The study of ^{57}Co-vitamin B_{12} absorption using a whole-body counter. *Phys. Med. Biol.* 14: 441, 1969.
13. Dorcherholmen, A., and P. S. Hagen. Kinetics of ingested radiocyanocobalamin in man. In: *Vitamin B_{12} and Intrinsic Factor*, edited by H. C. Heinrich. Stuttgart: Ferdinand Enke, 1962, p. 381.
14. Warner, G. T., and R. Oliver. A whole-body counter of clinical measurements utilizing the shadow-shield technique. *Phys. Med. Biol.* 11: 83, 1966.
15. Simpson, J. D., and D. J. C. Shearman. A whole-body counter for determination of the absorption of ^{58}Co-vitamin B_{12}. *Phys. Med. Biol.* 13: 61, 1968.
16. Tappin, J. W., I. A. Rahman and K. Rogers. A study of absorption of vitamin B_{12} using a whole-body spectrometer. *Brit. J. Radiol.* 39: 295, 1966.
17. Finlayson, N. D. C., J. D. Simpson, P. Tothill, R. R. Sampson, R. H. Girdwood and D. J. C. Shearman. Application of whole body counting to the measurement of vitamin B_{12} absorption with reference to achlorhydria. *Scand. J. Gastroenterol.* 4: 397, 1969.
18. Irvine, W. J., D. R. Cullen, L. Scarth, J. D. Simpson and S. H. Davies. Total body counting in the assessment of vitamin B_{12} absorption in patients with pernicious anemia, achlorhydria without pernicious anemia and in acid secretors. *Blood* 36: 20, 1970.

nt. J. Cancer: 2, 13-19 (1967)

/ITAMIN B_{12} LEVELS IN WHOLE BLOOD, PLASMA, AND BLOOD CELLS IN TRANSPLANTABLE RAT LEUKAEMIAS

by

M. BOSTELLI and L. M. RINALDINI [1]
Institute of Cellular Biology, University of Córdoba, Casilla Postal 362, Córdoba, Argentina

Vitamin B_{12} levels in whole blood, plasma, and red and white cells were determined by microbiological assay in normal and leukaemic A - C inbred rats. The concentration of B_{12} in white cells was 200 to 300 times higher than in red cells in both groups.

A marked increase in the B_{12} content of whole blood was observed in the leukaemic animals which was of the same order of magnitude as the increase in the number of white cells.

The concentration of B_{12} in red cells was the same in normal and leukaemic blood. That in plasma and white cells was only slightly increased in leukaemia. Therefore, the overall increase in blood B_{12} is attributable to the increase in the number of white cells in the leukaemic animals.

The fact that the ability to synthesize an essential metabolite may be lost in the course of differentiation (Fabro and Rinaldini, 1965) suggests that dedifferentiating malignant cells might perhaps acquire, or revert to, the ability to synthesize an essential metabolite. The idea that malignancy may involve prototrophic traits was first proposed by Lederberg (1946) by analogy with back mutations in leucineless neurospora. This possibility has also been discussed by Woolley (1957) who reported some inconclusive, but hitherto unchallenged evidence for the synthesis of vitamin B_{12} by spontaneous mouse mammary carcinomas (Woolley, 1953, 1955). The well-known increase in the levels of B_{12} found in chronic myelocytic leukaemia in human patients led us to examine the B_{12} content of blood fractions in experimental rat leukaemias under well-controlled conditions, as a first approximation to an inquiry into the possible synthesis of B_{12} by leukaemic cells.

Our results showed an approximately ten-fold increase in the B_{12} content of whole blood roughly correlated with the white cell count, but only a 30% increase in the B_{12} concentration per white cell, and thus tend to discourage the hypothesis of endogenous synthesis in leukaemic cells.

We do not propose to do further work on this problem and we present here our preliminary findings in the hope that they may be of some use to active workers in this field. This work features the use of inbred rats, a source of transplantable leukaemias hitherto little exploited.

Received: 3 January, 1966.
Approved: 4 October, 1966.

[1] Present address: Department of Microbiology, New York University School of Medicine, 550 First Avenue, New York, N.Y., USA.

equal volume of distilled water. Heparinized blood was prepared with 200 units of heparin (Abbot, Teterboro, N.J., USA) for each 6-8 ml of blood. Plasma was obtained by centrifuging heparinized blood at $900 \times g$ for 30 min.

Red blood cells were obtained by centrifugation from heparinized blood at $84 \times g$ for 45 to 60 min, the upper layers contaminated with leucocytes being discarded. The cells were then suspended in phosphate-buffered saline, pH 7.4.

We collected normal white cells after sedimenting erythrocytes at $10 \times g$ from a mixture of one part blood and two parts 3% dextran solution in buffered saline, recentrifuging the supernatant at $84 \times g$ and resuspending the pellet in buffered saline. Cell counts for estimating the B_{12} content per cell were done in buffered saline.

Good separation of leukaemic white cells from red cells was obtained without the addition of dextran; this was due to the marked leucocytosis accompanied by anaemia found in the leukaemic animals.

Contamination of leucocyte samples with red cells was of the order of 0.11 to 1.83 (average 0.73) erythrocytes per white cell in leukaemic blood and from 2.47 to 7.52 (average 5.19) in normal blood. Since the concentration of B_{12} is from 200 to 300 times higher in white than in red cells the error due to contamination with erythrocytes was ignored.

The collection of representative samples of red cells presented the problem of contamination with white cells in leukaemic blood. To cancel out this source of error relative white counts were made in samples of different purity, ranging from 0.04 to 0.12 white cells per 100 red cells in normal blood and from 0.08 to 0.54 white cells per 100 red cells in leukaemic blood. Once the content of B_{12} per white cell was known the content of red cell samples was corrected accordingly.

Vitamin assay

Vitamin B_{12} was assayed in whole haemolysed blood, whole heparinized blood, heparinized plasma and red and white blood cells. A hundred and one animals, 48 males and 53 females, were used.

The microbiological method of Hutner, Bach and Ross (1956) was used, employing *Euglena gracilis*, strain " Z ", as test organism.

Standard solutions were prepared with Merck, Sharp and Dohme (Philadelphia, Pa., USA) crystalline B_{12} and titrated spectrophotometrically according to The United States Pharmacopeia (1955). Stock solutions were prepared from these in 25% ethanol. Aliquots containing 40 pg/ml in distilled water were used for the standard curves.

B_{12} was liberated from biological samples by autoclaving in 5 to 10 volumes of transaconitic buffer pH 4.5 with 0.05% sodium metabisulfite (Baker *et al.*, 1959). The clear supernatant was collected after centrifuging at $680 \times g$. Assays were performed in squat conical flasks of 25 ml capacity with loose glass caps. In these flasks the cultures can spread over the bottom as a thin layer, and give more reproducible standard curves than in test tubes.

Each assay comprised 72 tubes, 18 containing increasing quantities of pure B_{12} (standard curve), and 54 containing different concentrations of the test samples. All flasks contained the same final volume (5 ml) and they were all incubated at $23 \pm 1°$ C with evenly distributed illumination from four 40 watt fluorescent tubes placed at a distance of 80 cm from the cultures. Nephelometric readings were made at 670 mμ and corrected for zero blanks to cancel traces of B_{12} carried over by the inoculum.

Each sample was assayed at three levels in duplicate and therefore each result represents an average of 6 determinations.

RESULTS

Whole blood

Table II shows the vitamin B_{12} concentration in whole blood.

The increase of B_{12} concentration in leukaemic blood with respect to normal controls is about six-fold in haemolysed samples and ten-fold in heparinized samples.

It may be noted that both the control and test values are mucher higher in haemolysed than in heparinized blood. The explanation of this finding is not clear. It is certainly not due to an inhibiting effect of heparin on the growth of *Euglena*, since addition of heparin alone in concentrations 10 times higher that those present in our samples had no effect at different levels of B_{12}. The decrease after heparinization is more marked in normal than in leukaemic blood.

Relative distribution of B_{12} between cells and plasma

The content per ml is significantly lower in plasma than in whole blood, particularly in the leukaemic animals (Table II). This indicates that a considerable proportion of the vitamin is present in the cells. Moreover, the increase in B_{12} concentration found in leukaemic animals is much smaller in plasma than in whole blood; about two-fold for LR III and 30% for LR XII. Three parallel estimations in serum after slow clotting at 4° C gave equivalent results to those obtained in the plasma of the same animals.

Haematocrit values for each animal were taken, and the vitamin content per cm^3 of packed cells was calculated after deduction of

TABLE II

B_{12} CONTENT IN WHOLE BLOOD
(pg/ml)

	Controls	Leukaemias	
		LR III	LR XII
Haemolysed blood	441 ± 30 [1]	2,145 ± 248 (P < 0.01)	2,965 ± 385 (P < 0.01)
Heparinized blood	81 ± 6	725 ± 84 (P < 0.01)	931 ± 107 (P < 0.01)

[1] Standard error.

TABLE III

DISTRIBUTION OF B_{12} IN PLASMA AND CELLS

	Controls	Leukaemias	
		LR III	LR XII
Plasma (pg/ml)	70.9 ± 5.9 [1]	168 ± 14 (0.01 < P < 0.02)	101 ± 6 (0.01 < P < 0.02)
Packed cells (pg/cm^3)	91.5 ± 12.0	1,434 ± 187 (P < 0.01)	1,879 ± 195 (P < 0.01)
Per cent in plasma	48.8	16.3	8.8
Per cent in cells	51.2	83.7	91.2

[1] Standard error.

TABLE IV

B_{12} CONCENTRATION IN BLOOD CELLS
(pg/10^7 cells)

	Control	Leukaemia LR III
White cells	44.1 ± 3.3	66.9 ± 6.8 (P < 0.01)
Red cells	0.22 ± 0.037	0.21 ± 0.044 (0.8 < P < 0.9)

the plasma content (Table III). It is immediately apparent from these figures that the bulk of the increase in B_{12} can be ascribed to the cellular fraction. Table III also shows that the relative proportion of B_{12} present in the cellular as compared to the acellular fraction is much higher in leukaemic than in normal blood. To ascertain whether the high content of the cellular fraction in leukaemic samples is due to an increased intracellular concentration in one or both cell types or simply to the higher proportion of white cells in leukaemic blood, the B_{12} concentration in red and white cells was determined (Table IV).

The content of B_{12} per cell is from 200 to 300 times greater in white than in red cells in both groups of animals. No significant difference in B_{12} concentration was found in erythrocytes from normal and leukaemic animals.

The content per white cell was only 30% higher in leukaemia. The statistical probability was lower than 0.01, but there was overlapping between both groups and the scatter was larger in leukaemic cells (from 34 to 133.3 pg/10^7 cells) than in normal leucocytes (from 17.8 to 72.3 pg/10^7 cells).

Since the vitamin content per cell as well as the concentration in plasma are only slightly higher than in normal blood, it must be concluded that the marked increase in blood levels of B_{12} observed in these experimental leukaemias is almost entirely due to the increase in the number of white cells.

DISCUSSION

The small increase in B_{12} concentrations found in leukaemic plasma and white cells from heparinized blood samples cannot account for the ten-fold increase found in heparinized whole blood. The bulk of the vitamin is present in the leucocytes, and since the increase in the number of white cells in LR III leukaemia was approximately sixteen-fold at the time of sampling it appears that in our system the increment in blood B_{12} is mainly attributable to the increase in the white cell population. The small increase in the plasma levels could be due to extrusion from circulating or tissue cells and/or to a higher binding capacity of leukaemic plasma. The high level of serum B_{12} found in human myelocytic leukaemias (Beard *et al.*, 1954; Rachmilevitz *et al.*, 1957; Mendelsohn and Watkin, 1958; Hall, 1961) has been ascribed to a higher B_{12} binding capacity of seromucoid or globulin fractions (Weinstein *et al.*, 1959; Miller and Sullivan, 1959; Weinstein and Watkin, 1960; Hall and Finkler, 1965). The situation in human leukaemias, however, may be more complicated than in our experimental system. Beard *et al.* (1954) found some correlation between the B_{12} level in serum and the white cell count in chronic myeloid leukaemias, while Rachmilevitz *et al.* (1957) and Kidd and Thomas (1962) found no consistent correlation.

Since in A × C rats 1 cm^3 of packed cells contains 9 to 18 times more B_{12} than an equal volume of plasma, and most of it is in the white cells, it may be inferred that the latter can store B_{12} by some mechanism of active transport and/or intracellular binding against the osmotic gradient. Meyer *et al.* (1961) found that the B_{12} binding capacity of serum in chronic myeloid leukaemic patients was correlated with the number of circulating myelocytes but they did not determine the B_{12} content of these cells. In a later work, however, Meyer *et al.* (1962) found that mature polymorphonuclear leucocytes take up B_{12} *in vitro* and have a high binding capacity while the immature ones and blast forms have little or none. They conclude that disintegration products from mature leucocytes may be largely responsible for the increased B_{12} binding capacity of serum in myeloproliferative diseases. Thomas and Anderson (1956), on the other hand, had previously found that myelocytic leucocytes from normal and leukaemic subjects have the same B_{12} content and binding capacity. Kidd and Thomas (1962) found that the content of white cells was low in myeloid leukaemias, normal in lymphoid leukaemias and high in acute blast leukaemias.

The view that the serum binding capacity may be due to cell disintegration products is in keeping with the observation of Doctor *et al.* (1959) that in myeloid human leukaemias treated with X-rays, myleran or 6-mercaptopurine, the reduction in the leucocyte count is followed after a lag period by a reduction in the serum B_{12} level. This, however, could also be explained by extrusion of the vitamin from intact leukaemic cells.

The very low concentration of B_{12} found in rat erythrocytes is in agreement with the finding of Bertcher *et al.* (1962) that intact human erythrocytes do not take up the vitamin *in vitro*.

Our figures for the B_{12} content of normal rat leucocytes (4.41 pg/10^6 cells) agree well with those of Kidd and Thomas (1962) for human leucocytes (3.84 pg/10^6 cells). The small increase in the content per cell observed by us in leukaemic white cells could be a metabolic characteristic of these cells, such as increased synthesis of binder (Simons, 1964), or it could simply be due to the larger volume of the immature elements. This small statistical increase in intracellular concentration may have no biological significance, however, since the difference in the number of white cells in our experimental leukaemias is more than sufficient to account for the overall difference in B_{12} blood levels between leukaemic and normal rats.

For the same reason, our results do not encourage the hypothesis of net B_{12} synthesis by leukaemic cells unless an increased turnover is also postulated. Further biochemical work is still required to ascertain whether there is an essential difference in B_{12} metabolism between the normal and the malignant white cell.

ACKNOWLEDGEMENT

This work was supported by USPHS grant GM08899 and by a small grant from Fundalei

CONCENTRATION DE LA VITAMINE B_{12} DANS LE SANG TOTAL, LE PLASMA ET LES CELLULES SANGUINES CHEZ DES RATS ATTEINTS DE LEUCÉMIE TRANSPLANTABLE

On a déterminé la concentration de vitamine B_{12} dans le sang total, le plasma, les hématies et les leucocytes par dosage microbiologique chez des rats endogames $A \times C$, les uns normaux, les autres leucémiques. Pour les deux groupes de rats, cette concentration était de 200 à 300 fois plus grande dans les leucocytes que dans les hématies.

On a observé chez les animaux leucémiques un accroissement marqué de la teneur du sang total en vitamine B_{12} ; cet accroissement était du même ordre de grandeur que celui du nombre des leucocytes.

La concentration de vitamine B_{12} dans les hématies était semblable dans le sang normal et dans le sang leucémique. Dans le plasma et les leucocytes, elle n'était que légèrement augmentée chez les animaux leucémiques. Les auteurs concluent que l'élévation globale de la vitamine B_{12} dans le sang résulte de l'augmentation du nombre des leucocytes chez les animaux leucémiques.

REFERENCES

Baker, H., Pasher, I., Frank, O., Hutner, S. H., Aaronson, S., and Sobotka, H., Assay of thiamine in biologic fluids. *Clin. Chem.*, **5**, 13-17 (1959).

Beard, M. F., Pitney, W. R., and Sanneman, E. H., Serum concentrations of vitamin B_{12} in patients suffering from leukemia. *Blood*, **9**, 789-794 (1954).

Bertcher, R. W., Meyer, L. M., and Schneider, M., Characteristics of vitamin B_{12} binding by erythrocytes and their components. *Acta Hematol.*, **27** (6), 354-358 (1962).

Doctor, V. M., Bergsagel, D. E., and Shullenberger, C. C., Effect of myleran and 6-mercaptopurine (6-MP) on the serum level of vitamin B_{12} in chronic myelocytic leukemia. *Blood*, **14**, 274-278 (1959).

Fabro, S. P. and Rinaldini, L. M. Loss of ascorbic acid synthesis in embryonic development.. *Devel. Biol.*, **11**, 468-488 (1965).

Hall, C. A., The plasma disappearance of radioactive vitamin B_{12} in myeloproliferative diseases and other blood disorders. *Blood*, **18**, 717-726 (1961).

Hall, C. A., and Finkler, A. E., The dynamics of transcobalamin II. A vitamin B_{12} binding substance in plasma. *J. lab. clin. Med.*, **65**, 459-468 (1965).

Hutner, S. H., Bach, M. K., and Ross, G. I. M., A sugar-containing basal medium for vitamin B_{12}-assay with Euglena. Application to body fluids. *J. Protozool.*, **3**, 101-112 (1956).

Kidd, H. M., and Thomas, J. W., The level of vitamin B_{12} in circulating leukaemic leucocytes. *Brit. J. Haematol.*, **8**, 64-76 (1962).

LEDERBERG, J., A nutritional concept of cancer. *Science*, **104**, 428-429 (1946).

MENDELSOHN, R. S., and WATKIN, D. M., Serum vitamin B_{12} concentrations determined by *L. leichmanii* assay in patients with neoplastic diseases. *J. lab. clin. Med.*, **51**, 860-866 (1958).

MEYER, L. M., BERTCHER, R. W., CRONKITE, E. P., SUAREZ, R. M., MILLER, I. F., MULZAC, C. W., and OLIVARRETA, S. T., Vitamin B_{12} binding capacity of serum in persons with hematologic disorders, various medical diseases, and neoplasms. *Acta med. Scand.*, **169**, 557-575 (1961).

MEYER, L. M., CRONKITE, E. P., MILLER, I. F., MULZAK, C. W., and JONES, I., Co^{60} vitamin B_{12} binding capacity of human leukocytes. *Blood*, **19**, 229-235 (1962).

MILLER, A., and SULLIVAN, J. F., Electrophoretic studies of the vitamin B_{12} binding protein of normal and chronic myelogenous leukemia serum. *J. clin. Invest.*, **38**, 2135-2143 (1959).

RACHMILEWITZ, M., IZAK, G., HOCHMAN, A., ARONOVITCH, J., and GROSSOWICZ, N., Serum vitamin B_{12} in leukemias and malignant lymphomas. *Blood*, **12**, 804-813 (1957).

SIMONS, K., Vitamin B_{12} binders in human body fluids and blood cells. *Soc. Sci. Fennica, Comm. Biol.*, **27**, fasc. 5 (1964).

The Pharmacopeia of the United States of America, 15th Edition, Spanish Translation, p. 158, Mack Printing Co., Easton, Pa. (1955).

THOMAS, J. W., and ANDERSON, B. B., Vitamin B_{12} content of normal and leukemic leukocytes. *Brit. J. Haematol.*, **2**, 41-43 (1956).

WEINSTEIN, I. B., and WATKIN, D. M., Further purification of the plasma vitamin B_{12} binding protein by fractionation of seromucoid on anion exchange cellulose column. *Clin. Res.*, **8**, 219 (1960).

WEINSTEIN, I. B., WEISSMAN, S. M., and WATKIN, D. M., The plasma vitamin B_{12} binding substance: I. Its detection in the seromucoid fraction of plasma from normal subjects and patients with chronic myelocytic leukemia. *J. clin. Invest.*, **38**, 1904-1914 (1959).

WOOLLEY, D. W., Evidence for the synthesis of vitamin B_{12} by spontaneous tumors. *Proc. nat. Acad. Sci. (Wash.)*, **39**, 6-18 (1953).

WOOLLEY, D. W., A further study of the apparent synthesis of vitamin B_{12} by mammary cancers of mice. *Proc. nat. Acad. Sci. (Wash.)*, **41**, 111-118 (1955).

WOOLLEY, D. W., Discussion to Lwoff's paper in Proc. Decennial Review Conf. on tissue culture. *J. nat. Cancer Inst.*, **19**, 529-531 (1957).

The American Journal of Clinical Nutrition
Vol. 20, No. 5, May, 1967, pp. 443–445
Printed in U.S.A.

Failure of Long-Term Oral Vitamin B_{12} Therapy to Replenish Body B_{12} Stores[1,2]

Eugene A. Brody, M.D.

It has been previously reported that patients with pernicious anemia in relapse can be treated and then maintained by the oral administration of vitamin B_{12} (50 μg three times/day) without added intrinsic factor (1). Such patients are clinically well, with normal peripheral blood and bone marrow findings and do not develop neurologic syndromes associated with pernicious anemia. However, the total serum vitamin B_{12} levels[3] remain subnormal or in the low normal range. The implication drawn was that the supply of B_{12} was sufficient for bone marrow and nervous system needs but did not fully replenish the tissue storage depots (1).

The purpose of the present paper is to report low stores of vitamin B_{12} in the liver of a patient who was treated for 10 years by oral B_{12} alone, and who had maintained a consistently low normal serum vitamin B_{12} level during this period.

CASE REPORT

The patient is a 68-year-old white female who first developed symptoms of pernicious anemia in 1950 (age 52). Clinical remission followed treatment with liver extract injections for 2 years, but symptoms and partial relapse reappeared when the injections were discontinued. In 1956 she had a macrocytic anemia with a hemoglobin of 10.7 g/100 ml, a mean corpuscular volume of 112 μ^3, and an intermediate megaloblastic bone marrow. The serum vitamin B_{12} level was 32 pg/ml[3]. The hepatic uptake (Glass (3)) test of B_{12} absorption revealed 0.3 net counts/sec when ^{60}Co B_{12} was administered alone and 18.6 net counts/sec when ^{60}Co B_{12} with intrinsic factor concentrate was given; these results are typical of pernicious anemia.

Treatment with oral vitamin B_{12}, 50 μg of cyanocobalamin three times/day, was begun in March 1956 and continued until April 1966. Maximum reticulocytosis to 8.2% occurred 13 days after the onset of therapy. Clinical and hematological remission was complete in 4 months and was maintained for 10 years. The serum vitamin B_{12} level rose to 212 pg/ml in 13 months and remained in the low normal range (140–270 pg/ml) for the following 9 years. The average of 43 determinations of the serum vitamin B_{12} level over this 9-year period was 203 pg/ml.

In April 1966 the patient was hospitalized for resection of a gastric carcinoma found on routine upper gastrointestinal X-ray examination. At the time of admission the hemoglobin was 13.6 g/100 ml, the mean corpuscular volume was 91 μ^3, the serum vitamin B_{12} level was 198 pg/ml, and the serum folate was 17 ng/ml. Antibody to intrinsic factor was not present in the serum (5).

Examination of the surgical specimen was reported as, "ulcerated, infiltrating adenocarcinoma of stomach. Sections of fundus showing marked atrophy. . . ." Coated charcoal immunoassay (5) of a saline extract of the gastric mucosa revealed that it contained no intrinsic factor activity.

The liver appeared normal at surgery and a small section of it was removed for assay. The vitamin B_{12} and folate concentrations were determined using *Euglena gracilis* (2) and *Lactobacillus casei* (4) as the test organisms. The B_{12} con-

[1] From the Department of Hematology, The Mount Sinai Hospital, New York City.

[2] This work was supported by Public Health Service Grants AM09564 and AM09062 from the Institute of Arthritis and Metabolic Diseases and by the Albert A. List, Frederick Machlin, and Anna Ruth Lowenberg Funds.

[3] *Euglena gracilis* microbiologic assay (2).

TABLE I

Vitamin B_{12} Concentration in Normal Liver (micrograms/gram liver tissue)

	Range	Mean	Test Organism
Pitney and Onesti (6)	0.61–1.02	0.78	*Euglena gracilis*
Joske (7)	0.39–1.33	0.87	*Euglena gracilis*
Nelson and Doctor (8)	1.41–2.58	1.94	*Lactobacillus leichmannii*
Halsted et al. (9)	0.24–1.41	0.614	*Lactobacillus leichmannii*
Swendseid et al. (10)	0.17–1.4	0.72	*Lactobacillus leichmannii*
Baker et al. (11)	0.8 –3.0	1.7	
Girdwood (12)	0.5 –1.5	0.8	*Lactobacillus leichmannii*

TABLE II

Folate Concentration in Normal Liver (micrograms/gram liver tissue)

	Range	Mean	Test Organism
Pitney and Onesti (6)	0.69–1.6		*Streptococcus lactis*
Baker et al. (11)	4.5 –20	11.2	*Lactobacillus casei*
Girdwood (13)	2.5 –5.36[a]		*Streptococcus faecalis*
Herbert (14)	3.57–7.14[a]	5.36	*Lactobacillus casei*

[a] Assuming an average liver weight of 1,400 g.

centration was 0.29 μg/g liver tissue (low) and the folate concentration was 9.0 μg/g liver tissue (normal).

DISCUSSION

Mean normal human liver stores of vitamin B_{12} have been variously reported (in micrograms/gram liver) as 0.78 (6), 0.87 (7), 1.94 (8), 0.614 (9), 0.72 (10), 1.7 (11), and 0.8 (12) (Table I). The reduced concentration of vitamin B_{12} in the liver of our patient (0.29 μg/g) indicates that the oral therapy of 50 μg B_{12} t.i.d. for 10 years did not supply sufficient B_{12} to normalize the depleted liver stores. In contrast, Nelson and Doctor (8) investigated the storage of vitamin B_{12} in the liver of pernicious anemia patients treated with parenteral B_{12} and concluded that with this mode of administration, the liver vitamin B_{12} reaches normal values in 6–7 months. Thus, the retained B_{12} following oral therapy (estimated at between 1 and 2 μg/day (1)) was sufficient to supply the daily needs of the hematopoietic and nervous systems but was insufficient to fully replenish liver stores.

The lower tissue store was evidenced by decreased concentration of B_{12} in both the liver and the serum. There is good correlation in this patient between the low normal serum B_{12} level and the low liver B_{12} concentration. It should be noted that in liver disease the liver B_{12} content is not related to serum B_{12} levels (7).

Mean normal concentration of folate in human liver has been reported to be 0.69–20.0 μg/g liver (Table II) in which range our patient fell. It is interesting that a low normal range was found consistently in subjects studied in Perth, Australia. This may be due to differences in the extraction procedure (6) performed prior to assay or more probably, to extensive cooking in Perth of all vegetables, removing a large part of the folate content.

SUMMARY

A patient with pernicious anemia in relapse was treated with orally administered vitamin B_{12}, 50 μg t.i.d. for 10 years. Clinical, hematological, and neurological remission was maintained during this period, but serum vitamin B_{12} levels never rose above low normal values. A section of normal liver removed at surgery after the 10-year period had a low B_{12} concentration.

This suggests that oral treatment of pernicious anemia in these doses is sufficient to correct the clinical and hematological abnormalities but is not sufficient to increase the body stores of vitamin B_{12} to normal in 10 years.

I am indebted to Miss Leona Bandel and Mr Alan Kelly for performing the assays and to Dr Victor Herbert for aid in preparation of the study.

REFERENCES

1. Brody, E. A., S. Estren and L. R. Wasserman. Treatment of pernicious anemia by oral administration of vitamin B_{12} without added intrinsic factor. *New Engl. J. Med.* 260: 361, 1959.
2. Ross, G. I. M. Vitamin B_{12} assay in body fluids using *Euglena gracilis*. *J. Clin. Pathol.* 5: 250, 1952.
3. Glass, G. B. J., L. J. Boyd, G. A. Gellin and L. Stephanson. Uptake of radioactive vitamin B_{12} by liver in human: test for measurement of intestinal absorption of vitamin B_{12} and intrinsic factor activity. *Arch. Biochem.* 51: 251, 1954.
4. Herbert, V. Aseptic addition method for *Lactobacillus casei* assay of folate activity in human serum. *J. Clin. Pathol.* 19: 12, 1966.
5. Gottlieb, C., K.-S. Lau, L. R. Wasserman and V. Herbert. Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated B_{12} binding capacity, antibody to IF, and serum unsaturated B_{12} binding capacity. *Blood* 25: 875, 1965.
6. Pitney, W. R., and P. Onesti. Vitamin B_{12} and folic acid concentrations of human liver with reference to the assay of needle biopsy material. *Australian J. Exptl. Biol. Med. Sci.* 39: 1, 1961.
7. Joske, R. A. The vitamin B_{12} content of human liver tissue obtained by aspiration biopsy. *Gut* 4: 231, 1963.
8. Nelson, R. S., and V. M. Doctor. The vitamin B_{12} content of human liver as determined by bio-assay of needle biopsy material. *Ann. Internal Med.* 49: 1361, 1958.
9. Halsted, J. A., J. Carroll and S. Rubert. Serum and tissue concentration of vitamin B_{12} in certain pathologic states. *New Engl. J. Med.* 260: 575, 1959.
10. Swendseid, M. E., E. Hvollboll, G. Schick and J. A. Halsted. Vitamin B_{12} content of human liver and its nutritional significance: comparison study of various age groups. *Blood* 12: 24, 1957.
11. Baker, H., O. Frank, H. Ziffer, S. Goldfarb, C. Leevy and H. Sobotke. Effect of hepatic disease on liver B-complex vitamin titers. *Am. J. Clin. Nutr.* 14: 1, 1964.
12. Girdwood, R. H. Some aspects of the metabolism of antimegaloblastic substances in man. *Blood* 8: 469, 1953.
13. Girdwood, R. H. Folic acid, its analogs and antagonists. *Advan. Clin. Chem.* 3: 235, 1960.
14. Herbert, V. Minimal daily adult folate requirement. *Arch. Internal Med.* 110: 649, 1962.

Nonleukemic elevation of serum vitamin B_{12} and B_{12}-binding capacity levels resembling that in chronic myelogenous leukemia

RALPH CARMEL AND CHARLES A. COLTMAN, JR.
Lackland AFB, Texas

In vitro serum vitamin B_{12} abnormalities characteristic of chronic myelogenous leukemia were found in 5 patients without leukemia. All had leukocytosis, and 3 transiently had immature myeloid cells in peripheral blood. The B_{12} abnormalities were: (1) marked elevation of alpha-globulin B_{12}-binding capacity in all subjects and (2) high serum B_{12} levels in 3 patients with high-normal levels in 2. Beta-globulin binding was normal in 2 but elevated in the others. Electrophoretic and reticulocyte uptake studies also demonstrated the findings seen in chronic myelogenous leukemia. Of 3 patients studied at different stages, one's abnormalities disappeared as leukocytosis subsided, one's improved similarly but quickly rebounded as leukocytosis returned, and the third remained abnormal as leukocytosis persisted. A patient recovering from agranulocytosis due to chemotherapy was studied serially. The B_{12} abnormalities paralleled the recovery phase to the exact day, returning to normal as leukocytosis subsided. High alpha-globulin B_{12} binding and, often, serum B_{12} levels resembling those in chronic myelogenous leukemia, thus, are not infrequently found in nonleukemic leukocytosis. The exact mechanisms responsible remain unknown. However, it is apparent that the abnormality is not an expression of the leukemic process itself and is not diagnostic for the disease.

Abnormally elevated alpha-globulin binding of vitamin B_{12} is a well-recognized, consistent phenomenon in chronic myelogenous leukemia (CML).[1-7] Elevation of the binding capacity and, secondarily, of the serum vitamin B_{12}

From the Aerospace Medical Laboratory (Clinical) and the Hematology-Oncology Service, Wilford Hall USAF Medical Center.

This study was supported by United States Air Force funds made available through AFR No.169-6.

Received for publication Oct. 14, 1970.

Accepted for publication March 31, 1971.

Reprint requests: Dr. Ralph Carmel, MRC Experimental Haematology Unit, St. Mary's Hospital Medical School, London W. 2, England.

level are often used diagnostically, occurring even when the Philadelphia chromosome is absent.[7]

This report describes such in vitro B_{12}-binding abnormalities in 5 subjects without leukemia; all had leukocytosis. In some patients, the binder abnormalities were transient, paralleling the white cell count. This finding is of interest as indirect evidence suggests that mature granulocytes are the source of the alpha-globulin B_{12}-binding protein (transcobalamin I [TC I]).[2, 8-10] However, it indicates that elevated alpha-globulin binding and serum B_{12} levels are not confined to CML, and the abnormality is not diagnostic for leukemia.

Materials and methods

Serum vitamin B_{12} level[11] and unsaturated B_{12}-binding capacity (UBBC)[12] were determined by coated charcoal radioassay. B_{12}-binding protein was fractionated by batch separation with diethylaminoethyl (DEAE) cellulose.[13]

In several instances, fractionation was also done by: (1) cellulose acetate electrophoresis of serum (preincubated with a slight excess of $^{57}CoB_{12}$) for 20 minutes at 250 v. in 0.075 ionic strength barbital buffer (pH 8.6) (The unstained strips were exposed to Cronex 4* x-ray film for 8 weeks, and the autoradiograph was compared to that of ^{59}Fe-labeled serum.) and (2) vertical electrophoresis of serum (0.02 ml.) in sucrose on 10 per cent acrylamide: 0.3 per cent bisacrylamide gel for 16 hours at 2 mamp. per sample in Tris-HCl buffer (pH 8.6). (The sera were previously incubated with a slight excess of $^{57}CoB_{12}$ and treated with hemoglobin-coated charcoal pellets to remove unbound vitamin. The gel was cut in 0.6 mm. sections, the radioactivity of which was counted.)

Serum-mediated uptake of $^{57}CoB_{12}$ by reticulocytes was determined by the method of Retief and associates.[14]

All serum was studied fresh or after storage at −20° C. $^{57}CoB_{12}$ (10 to 15 μCi per microgram specific activity) was used in all studies except determination of UBBC and DEAE cellulose fractionation in which $^{57}CoB_{12}$ (1 μCi per microgram specific activity) was used. Samples were counted in a well-type scintillation counter, usually to 10,000 counts. All studies were done in duplicate and often repeated on different days. A 10 per cent variability in serum B_{12} and UBBC levels generally occurs in our laboratory.

Fig. 1 is a conceptualization of the serum B_{12}-binding pool with its various compartments—the alpha- and beta-globulin (or transcobalamin I [TC I] and transcobalamin II [TC II], respectively) unsaturated and total B_{12}-binding capacities.

Case reports

F. C., a 33-year-old woman, had undergone radical mastectomy and irradiation for adenocarcinoma of the breast 2 years before and oophorectomy one year ago. Due to subsequent lymphatic and liver involvement, combination chemotherapy (cyclophosphamide, vincristine, amethopterin, 5-fluorouracil, and prednisone) was begun. Severe granulocytopenia, with fever, chills, and diarrhea, necessitated hospitalization 3 days after stopping oral chemotherapy (and 8 days after intravenous chemotherapy). Abnormal physical findings were fever, 90/0 blood pressure, alopecia, left axillary adenopathy, and an enlarged nodular liver.

On admission, blood count showed 44 white cells per cubic millimeter, 180,000 platelets per cubic millimeter, and 41 per cent hematocrit. Partial thromboplastin and prothrombin times were abnormal, and fibrinogen level was low. Blood cultures revealed *S. aureus*. She was treated with antibiotics, heparin, aramine, and dexamethasone with good clinical response and return of coagulation studies to normal. Her white cell count returned to normal (Fig. 2). Leukocyte alkaline phosphatase score at peak leukocytosis was 178 (normal = 40 to 100). Other laboratory studies were normal except for mild liver function abnormalities

*E. I. DuPont de Nemours Inc., Wilmington, Del.

363

O. Acb. Clin. Path. 78:289-291 1971 U45

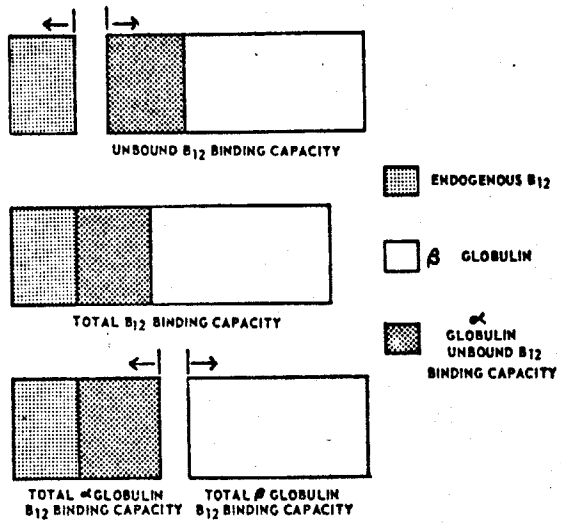


Fig. 1. The serum B_{12}-binding protein pool. The center figure depicts the total pool. The upper separates UBBC and endogenous vitamin B_{12}, and the lower alpha- and beta-globulin total B_{12}-binding capacities. (This conceptualization is patterned after that of Dr. V. Herbert.)

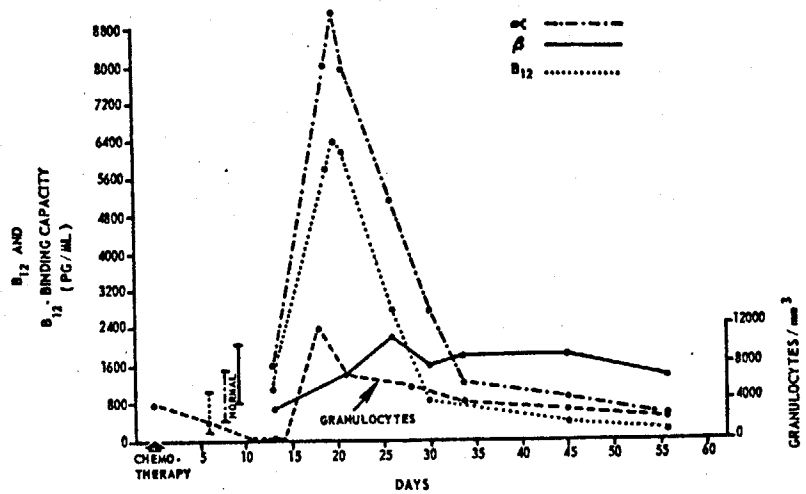


Fig. 2. Serial data on patient F. C. (B_{12}) Serum B_{12} level; (α and β) these denote the respective total B_{12}-binding capacities. Normal ± 2 S.D. is given in the lower left-hand corner.

and abnormal stool fat excretion. The patient is well one year later, currently on a modified chemotherapeutic regimen, without recurrence of agranulocytosis or leukocytosis.

M. S., a 56-year-old man who underwent lung resection for carcinoma 7 years before, was admitted for cough, dyspnea, pleuritic pain, and weight loss of 3 months' duration. Abnormal physical findings were fever, diffuse pulmonary rhonchi, hepatomegaly, pulmonary osteoarthropathy, and adenopathy. Hemoglobin was 8.7 Gm. per cent, and his white blood

Table I. Summary of B_{12} and leukocyte data on 5 subjects

Patient	*Day No.*	*B_{12} (pg./ml.)*	*UBBC (pg./ml.)*	*UBBC (%α-globulin)*
F. C.	20	6,393	4,124	65
	45	404	2,272	22
M. S.		1,495	7,804	60
R. W.	3/4	4,044	4,701	38
	3/13†	1,774	2,832	41
	3/24	1,849	5,240	34
D. B.	4/7	617	4,864	77
	5/5	392	1,665	23
H. S.	12/12	770	4,430	44
	12/29	516	7,000	42
Normal	± 2 S.D.	465 ± 348	1,759 ± 538	25 ± 6
CML	± 2 S.D.‡	2,892 ± 2,168	4,808 ± 6,216	58 ± 36

*Total B_{12}-binding capacity.
†3 Days after first course of chemotherapy.
‡CML = chronic myelogenous leukemia.

cell count was 24,000 per cubic millimeter with 92 per cent neutrophils. Abnormal laboratory results included hypoxia, abnormal liver function tests, guaiac-positive stools, and a weakly reactive VDRL. The patient's leukocyte alkaline phosphatase score was 263. Fever continued, with appearance of pleural fluid and a progressive increase in white cell count. The patient died one day after the white cell count reached 140,000 per cubic millimeter, and metamyelocytes and nucleated red blood cells appeared in peripheral blood. Necropsy revealed epidermoid carcinoma of the lung with widespread metastases. The spleen weighed 100 Gm. with normal architecture. The bone marrow was normocellular and without tumor invasion.

E. W., a 48-year-old man, was admitted with a 3 month history of abdominal pain, weight loss, and adenopathy. Abnormal physical findings were mild fever, marked hepatomegaly, spider angiomas, tender nodules on the skull, Virchow's node, and a soft apical systolic murmur. Hemoglobin was 12.6 Gm. per cent, white cell count was 16,500 per cubic millimeter with 84 per cent neutrophils, and platelet count was 128,000 per cubic millimeter. Laboratory studies were normal except for glucose tolerance and liver function test abnormalities. There was initial subjective improvement with chemotherapy (cyclophosphamide, vincristine, amethopterin, and 5-fluorouracil), but the patient died with progressive anemia and hepatic failure during the second course. Necropsy showed carcinoma of the pancreas with widespread metastases, subacute bacterial endocarditis, pneumonia, and pulmonary emboli. The spleen showed passive congestion and weighed 120 Gm. The marrow was involved by metastases.

D. B., a 19-year-old man, was admitted with 20 per cent surface area burns. He had previously been in good health. Fever and jaundice appeared shortly after admission. His white cell count was 41,500 per cubic millimeter with 80 per cent neutrophils, including 2 per cent metamyelocytes, and hematocrit was 33 per cent. Laboratory results included hypoalbuminemia, blood urea nitrogen (BUN) of 37 mgm. per cent, and abnormal liver function tests. The patient has recovered fully with supportive care.

H. S., a 62-year-old man, was admitted with fever, weight loss, and a neck mass 4 years after a goiter was first noted. Physical examination showed fever and a neck mass, with a mild superior vena cava syndrome. Hemoglobin was 9.2 Gm. per cent, white cell count was 46,400 per cubic millimeter, and platelet count was 800,000 per cubic millimeter. Abnormal laboratory findings were hypocalcemia, mild liver function abnormalities, hypoal-

364
0451

TBBC(pg./ml.)*		White blood cells/mm.³	Neutrophils (%)	
α-Globulin	β-Globulin			
9,074	1,443	13,000	90	1% Myelocytes 14% metamyelocytes
904	1,772	5,000	61	
6,177	3,122	140,000	94	4% Metamyelocytes
5,830	2,915	19,300	90	
2,938	1,671	4,200	68	
3,630	3,458	24,200	83	
4,362	1,119	41,500	86	2% Metamyelocytes
775	1,282	10,200	78	
2,719	2,481	46,400	91	
3,456	4,060	71,400	96	
963 ± 562	1,260 ± 510			
6,109 ± 2,522	1,592 ± 349			

buminemia, elevated beta (1.4 Gm. per cent) and gamma globulin (1.9 Gm. per cent) levels, and BUN of 23 mgm. per cent. Biopsy of the mass showed a sterile abscess within carcinoma. The patient was treated with emetine and antibiotics without response. Necropsy showed carcinoma of the thyroid with widespread metastases and pneumonia. The bone marrow was hypercellular with marked granulocytosis and contained metastases. The spleen was involved by metastases, weighed 320 Gm., and showed acute splenitis.

Results

All 5 subjects had very high UBBC, with elevated alpha-globulin fractions, at peak leukocytosis. Three had elevated beta-globulin fractions, whereas 2 were normal. Three had abnormally high serum B_{12} levels, the others having high-normal values.

Table I summarizes the data. Of 4 patients studied more than once, 2 showed normal levels after leukocytosis subsided. One (RW) had lower values after chemotherapy lowered the white cell count, but rebounded as leukocytosis returned. One (HS) showed progressive increase of B_{12}–binding capacity as leukocytosis progressed.

Patient FC was studied serially during recovery from agranulocytosis induced by chemotherapy. Fig. 2 illustrates the remarkable rise in alpha-globulin binding and serum B_{12} level paralleling the change in circulating granulocytes. Beta-globulin binding remained relatively normal. Within 2 weeks, the B_{12}–binding pattern became completely normal as leukocyte counts leveled off. As shown in Fig. 3, our subjects' B_{12} data at peak leukocytosis closely resembled those of chronic myelogenous leukemia.

Autoradiography confirmed the predominant alpha-globulin B_{12} binding in Patient FC, resembling chronic myelogenous leukemia, and the normal beta-globulin predominance subsequently. Acrylamide-gel electrophoresis also demon-

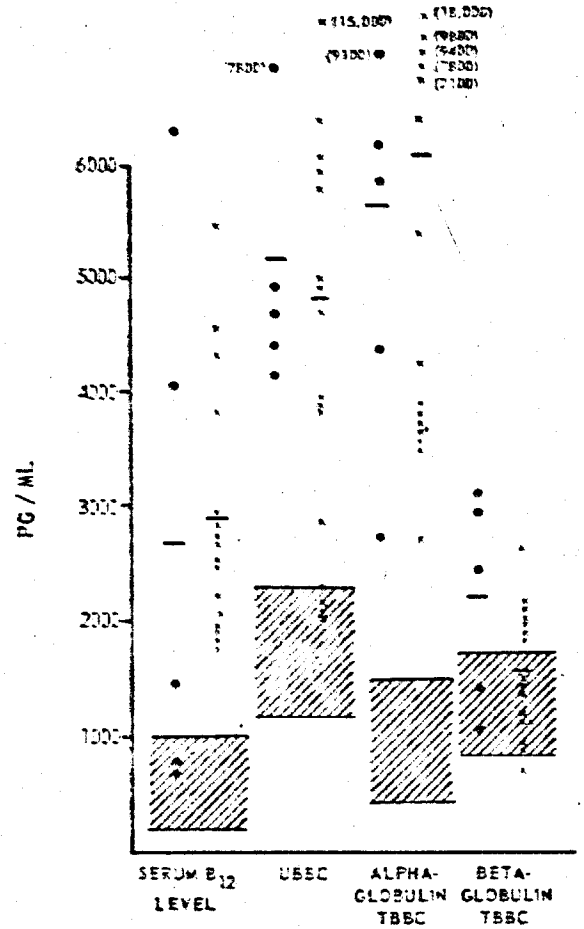


Fig. 3. Comparison of patients at peak leukocytosis (●) with chronic myelogenous leukemia patients (x). (Except for 3 who were partially treated, all the leukemic patients were untreated; none were in blastic crisis.) Mean values are shown by lines. The shaded areas represent normal ± 2 S.D. *TBBC* = total B_{12}-binding capacity.

strated this in Patient FC as well as alpha-globulin binding predominance in Patient MS.

Serum from Patient HS was tested for mediation of $^{57}CoB_{12}$ uptake by a reticulocyte-rich red cell suspension. It transferred only 39 per cent as much vitamin as did 2 normal sera, one of which had a comparable endogenous vitamin B_{12} level. This compares with 10 and 47 per cent transfer by 2 chronic myelogenous leukemia sera. FC serum at peak leukocytosis produced similar results. When leukocytosis subsided, however, 122 per cent transfer occurred.

Discussion

The evidence that serum alpha-globulin binder of vitamin B_{12} arises from mature granulocytes, the richest cellular source of the protein yet found,[5, 15]

includes the close resemblance of leukocytic binder to it, including immunologic cross-reactivity, and a demonstration of probable synthesis of the protein by leukocytes.[9] Most striking has been the very high alpha-globulin UBBC invariably seen in chronic myelogenous leukemia.[1-7] As this protein binds vitamin B_{12} strongly and does not release it readily,[14] high serum B_{12} levels result. These features are so characteristic as to be virtually diagnostic of the disease. Beta-globulin UBBC has been found to be lower than normal in CML by Hall and Finkler.[6] Other studies indicate that although the per cent beta-globulin UBBC is lower, the beta-globulin total B_{12} binding capacity (TBBC) is normal or elevated.[3, 7] Low, normal, and elevated beta-globulin TBBC have been seen in CML patients in our laboratory (Fig. 3).

However, the data in leukocytosis not due to myeloproliferative disease are not so clear-cut. Beard and associates[16] mentioned, without specific data, finding increased serum B_{12} in leukocytosis of infection, with return to normal when leukocytosis subsided. Mollin and Ross[2] reported B_{12} levels of 1,680 and 950 pg. per milliliter in 2 subjects with leukemoid reaction. Others, however, reported low-normal[17] and normal[7, 18] results.

Similarly, UBBC has varied in leukocytosis. Normal[19] and high[7] levels have been reported. In the latter report, however, the elevation was of beta-globulin, with little rise in alpha-globulin binding. Mikkonen and associates[20] studied transcobalamin I:II ratios in 5 patients with leukocytosis. Elevated ratios were found in only 2, both having epidermoid carcinoma. In one of these, coexisting leukemia could not be ruled out. The other, interestingly, was studied during recovery from bone marrow depression by chemotherapy. However, neither had a ratio above 0.5, which corresponds to 33 per cent alpha-globulin UBBC. Actual UBBC and B_{12} levels were not given. The most detailed study to date[3] reported high UBBC in 7 of 8 patients with leukocytosis. Only 4, however, had levels resembling that in CML. Of these, 2 had polycythemia vera and myeloid metaplasia, conditions themselves associated with binding abnormalities.[7, 19, 21] Only one patient, suffering from widespread epidermoid carcinoma, had high serum B_{12} levels as well.

The finding in our study of CML-like alpha-globulin TBBC abnormality in 5 subjects indicates the phenomenon is not rare (although the majority of our patients with leukocytosis have shown normal serum B_{12} levels and high UBBC without alpha-globulin predominance[22]). It should be noted, however, that 2 of the subjects (MS and RW) had beta-globulin TBBC elevations higher than those found in leukemic patients in our laboratory. Two others (DB and HS) had high-normal serum vitamin B_{12} levels, another feature not typical of our CML patients.

None of the 5 had CML, either by clinical criteria or necropsy findings. Four had underlying metastatic malignancy, but of different organs and histologic types. In a review of 71 patients with cancer, only 3 had elevated UBBC, while 6 had low UBBC.[19] The finding of the abnormal binding in our patient DB further indicates that tumor itself is not responsible.

Two subjects showed complete, and one partial, correction of B_{12}-binding abnormalities when leukocytosis subsided. One whose leukocytosis worsened

showed progression of abnormality. Especially interesting was Patient FC. The massive rise in serum B_{12} level and alpha-globulin binding accompanying recovery from agranulocytosis due to chemotherapy was striking. While we have noted marked UBBC rise in all patients recovering from marrow suppression after chemotherapy,[22, 23] none had a rise in serum B_{12} level or the predominance of alpha-globulin binding which this patient manifested. The difference may be that none had such severe granulocytopenia. Furthermore, she, like 2 other subjects in the present report, had some immature myeloid cells in peripheral blood.

The fact that her peak white cell count was only 13,000 per cubic millimeter emphasizes that the actual peripheral leukocyte count need not correlate with any random B_{12}-binding parameter.[16, 18, 24] However, it is evident in serial studies[23] that the 2 fluctuate in a parallel and simultaneous fashion, as they did, to the day, in Patient FC.

While virtually all evidence implicates the granulocyte, the exact factors responsible for B_{12}-binding abnormalities remain obscure. Whether the granulocytes (or possibly even other cells) simply synthesize more, release more, or alter B_{12}-binding protein is not known. Fever or liver abnormality, present in all our patients, or possibly interaction with other proteins elaborated, may also play a role. Fever and liver disease, however, have been present in other patients without such B_{12} abnormalities.[22]

It is clear that markedly elevated alpha-globulin B_{12}-binding is not limited to CML and myeloproliferative disease. Although the highest values are generally seen in untreated, florid CML, it is obvious that similar findings occur in "normal" leukocytosis. Thus, while such B_{12} abnormalities may be helpful in diagnosing CML, they are not pathognomonic and may not always help to distinguish leukemic from nonleukemic leukocytosis. In vivo studies of the B_{12} metabolism in such patients should shed additional light on their abnormalities.

We thank Dr. Karl Eurenius of the Institute for Surgical Research, Fort Sam Houston, Texas, for the opportunity to study patient DB. We also thank Airman Leonard J. DeLallo for technical assistance and Mrs. JoAnn Honn for secretarial help.

Addendum

Since submission of this manuscript, a study has appeared demonstrating that granulocytes actively secrete B_{12}-binding protein.[25] This would help further in explaining the relationship between total B_{12}-binding capacity and leukocyte levels. In addition it is probable that the raised "beta globulin" binding capacity as seen in some of our patients is actually due to a third serum B_{12} binder found frequently in other patients with leukocytosis.[26]

REFERENCES

1. Beard MF, Pitney WR, and Sanneman EH: Serum concentrations of vitamin B_{12} in patients suffering from leukemia. Blood 9: 789-794, 1954.
2. Mollin DL, and Ross GIM: Serum vitamin B_{12} concentrations in leukaemia and in some other haematological conditions. Brit J Haemat 1: 155-172, 1955.
3. Miller A, and Sullivan JF: The in vitro binding of cobalt-60-labeled vitamin B_{12} by normal and leukemic sera. J Clin Invest 37: 556-566, 1958.
4. Miller A, and Sullivan JF: Electrophoretic studies of the vitamin B_{12}-binding protein of normal and chronic myelogenous leukemia serum. J Clin Invest 38: 2135-2143, 1959.

5. Mendelsohn RS, Watkin DM, Horbett AP, et al: Identification of the vitamin B_{12}-binding protein in the serum of normals and of patients with chronic myelocytic leukemia. Blood 13: 740-747, 1958.
6. Hall CA, and Finkler AE: Measurement of the amounts of the individual vitamin B_{12}-binding proteins in plasma. II. Abnormalities in leukemia and pernicious anemia. Blood 27: 618-622, 1966.
7. Gottlieb CW, Retief FP, Pratt PW, et al: Correlation of B_{12}-binding proteins with disorders of B_{12} metabolism. J Clin Invest 45: 1016, 1966.
8. Meyer LM, Cronkite EP, Miller IF, et al: Co^{60} vitamin B_{12}-binding capacity of human leukocytes. Blood 19: 229-235, 1962.
9. Simons K, and Weber T: The vitamin B_{12}-binding protein in human leukocytes. Biochim Biophys Acta 117: 201-208, 1966.
10. Stenman V-H, Simons K, and Gräsbeck R: Vitamin B_{12}-binding protein in normal and leukemic human leukocytes and sera. Scand J Lab Clin Invest 21: 202-210, 1968.
11. Carmel R, and Coltman, CA: Radioassay for serum vitamin B_{12} with the use of saliva as the vitamin B_{12} binder. J Lab Clin Med 74: 967-975, 1969.
12. Gottlieb C, Lau KS, Wasserman LR, et al: Rapid charcoal assay for intrinsic factor (IF), gastric juice–unsaturated B_{12} binding capacity, antibody to IF, and serum-unsaturated B_{12}-binding capacity. Blood 25: 875-884, 1965.
13. Silverstein E, and Herbert V: Rapid determination of vitamin B_{12}-binding alpha and beta globulin in serum. Blood 31: 518-521, 1968.
14. Retief P, Gottlieb CW, and Herbert V: Delivery of $Co^{57}B_{12}$ to erythrocytes from alpha and beta globulin of normal, B_{12}-deficient, and chronic myeloid leukemia serum. Blood 29: 837-851, 1967.
15. Simons K: Vitamin B_{12} binders in human body fluids and blood cells. Comment Biol Soc Sci Fenn 27: 5, 1964.
16. Beard MF, Pitney WR, Sanneman EH, et al: Serum concentrations of vitamin B_{12} in acute leukemia. Ann Intern Med 41: 323-327, 1954.
17. Erdmann-Oehlecker S, and Heinrich HC: Der Vitamin B_{12}-Stoffwechsel bei Hämoblastosen. Clin Chim Acta 1: 269-286, 1956.
18. Rachmilewitz M, Izak G, Hochman A, et al: Serum vitamin B_{12} in leukemias and malignant lymphomas. Blood 12: 804-813, 1957.
19. Meyer LM, Bertcher RW, Cronkite EP, et al: Co^{60} vitamin B_{12}-binding capacity of serum in persons with hematologic disorders, various medical diseases, and neoplasms. Acta Med Scand 169: 557-575, 1961.
20. Mikkonen L, Finkler AE, and Hall CA: A diagnostic test for chronic myelogenous leukemia based on abnormalities of B_{12}-binding. Acta Med Scand 181: 591-596, 1967.
21. Hall CA, and Finkler AE: Vitamin B_{12}-binding protein in polycythemia vera plasma. J Lab Clin Med 73: 60-69, 1969.
22. Carmel R, and Coltman CA: Serum vitamin B_{12} binding in nonleukemic leukocytosis. Clin Res 18: 40, 1970.
23. Carmel R and Coltman CA: Serum vitamin B_{12}-binding capacity and muramidase changes with cyclic neutropenia induced by cytosine arabinoside. Blood 37: 31-39, 1971.
24. Olesen H, Andersen MP, and Amris A: Serum vitamin B_{12}-binding capacity in patients with anemia. Scand J Haemat 5: 235-240, 1968.
25. Corcino J, Krauss S, Waxman S, et al: Release of vitamin B_{12}—binding protein by human leukocytes in vitro. J Clin Invest 49: 2250-2255, 1970.
26. Carmel R: Vitamin B_{12}—binding protein abnormality in subjects without myeloproliferative disease. II. The presence of a third B_{12}—binding protein in serum. Submitted for publication.

Dis. Nerv. Syst. 31: 566-569

Serum Vitamin B.12 Values in Psychiatric In-Patients

M.W.P. CARNEY, M.D., M.R.C.P.I., D.P.M.

Introduction

It is widely held that vitamin B.12 deficiency is an aetiological factor in mental illness. Some authors have found strikingly high proportions of psychiatric patients to have low serum B.12 levels[7,3,8] but Murphy et al. (1969), finding a much lower incidence, doubted whether this was any higher than in the general population.

It has been stressed that mental changes in vitamin B.12 deficiency may long precede evidence of haematological or neurological damage[2]. Not all low serum levels, however, indicate vitamin B.12 *deficiency*[10]. The aetiological links (if any) between low serum B.12 deficiency and mental illness are obscure: McCallum (1965) found depression frequently associated with low serum levels and responsive to cyanocobalamen therapy; on the other hand, Shulman (1968), in a controlled trial of cyanocobalamen, found only memory impairment to be significantly improved.

Consultant Psychiatrist, Blackpool & Fylde Hospital Group and Lancaster Moor Hospital.

The present study was undertaken as part of a survey of vitamin B.12 and folate levels in 432 in-patients admitted consecutively from a defined geographical area (Blackpool and Fylde) of population 300,000. Serum B.12 values were obtained for 374 patients of mean age 53.1 years (260 females and 114 males). Only one patient had received Cytamen (cyanocobalamen) prior to admission and none had received folic acid or was diagnosed as suffering from any form of megaloblastic anaemia.

Method

Blood was obtained from the fasting patient and, because of the suspected influence of certain drugs in lowering serum vitamin B.12, as soon as possible after admission and nearly always before treatment commenced. The serum B.12 was estimated by the method of Meynell (1957), using L.leichmannii, and the serum folate by that of Waters and Mollin (1961), using L.casei. Though these assays were performed in two different laboratories the procedures in both were identical.

The low B.12 (<150 pg.ml.) patients were investigated for aetiological factors by clinical examination, further laboratory studies, social history taking from relatives, and consultation with the appropriate general practitioners (for details of any pre-admission medication).

Results

Incidence: 53 (14.2%) were found to have serum B.12 levels of 150 pg.ml. or less and 16 (4.3%) had values below 100 pg.ml.

Psychiatric diagnosis

Table I shows diagnosis with mean ages and mean serum B.12 values ranked in ascending order of magnitude. "Organic psychoses" has the lowest mean value. The difference between the means for "organic psychoses" and the other 294 patients is significant ($t = 2.04$; $P<0.05$). This may be because the mean age for organic psychoses is so much higher than for the other conditions but in the present study the mean ages for the normal and low

TABLE I

Diagnoses, Mean Ages and Mean Serum Vitamin B.12 Levels with Significance Tests for Differences from Organic Psychoses

Diagnosis	*No.*	*Mean Age*	*Mean Serum Vit. B.12 (pg)* (± Standard deviations)	*Significance Tests*
Drug Addiction	10	47.5	418.3 ± 177.8	N.S.*
Chronic Alcoholism	22	46.4	400.0 ± 193.1	t = 2.1:P.<0.05
Mania	14	54.0	400.0 ± 167.5	t = 1.97:P.<0.05
Neurosis (neurotic depression excepted)	26	48.9	385.0 ± 202.2	t = 2.0:P.<0.05
Epilepsy	8	47.5	382.5 ± 162.9	N.S.*
Abnormal Personality	13	34.5	348.8 ± 132.3	N.S.*
Endogenous Depression	68	55.0	338.3 ± 173.6	N.S.*
Schizophrenia	70	41.8	328.0 ± 144.8	N.S.*
Neurotic/Reactive Depression	63	47.7	318.2 ± 182.3	N.S.*
Organic Psychoses	80	73.0	303.4 ± 183.5	
Totals	374	53.7 ± 17.15.	340.4 ± 177.1	

*Not Significant

TABLE II

Psychiatric Diagnoses and Low Serum B.12

Serum B.12 (pg.)	*Organic*	*Endogenous Depression*	*Neurotic Depression*	*Schizophrenia*	*Others*	*Totals*
>150	60	56	55	61	89	321
100 — 150	13	10	5	6	2	36
<100	7	2	3	3	2	17
Total >150	20	12	8	9	4	53

(<150 pg.ml.) B.12 patients did not differ significantly (P>0.05). The means for "neuroses", "chronic alcoholism" and "mania" are also significantly higher than for "organic psychoses".

Table II analyses low and very low (<100 pg.ml) B.12 patients by diagnosis. "Organic psychoses" has the highest number of each category and "others" (mania, neurosis (depression excepted), epilepsy, drug-addiction, chronic alcoholism and abnormal personality) the lowest. The diagnostic distribution of all the low B.12 patients differs significantly from that of the normal B.12 patients ($X^2 = 16.78$; d.f. = 4; $P<0.01$).

Haematological data

Twelve (22.6%) low serum B.12 patients had a haemoglobin of less than 80% against 56 (17.4%) of normal serum B.12 patients, a non-significant difference (P = 0.2). Of the low B.12 patients with a haemoglobin of less than 80%, 5 showed another abnormality (macrocytosis, 4; anisocytosis, 1) and of 11 specimens of sternal marrow from them only 2 were megaloblastic: neither of these was associated with histamine-fast achlorhydria, so that in no instance was the diagnosis of pernicious anaemia confirmed. Only 6 patients had white cell counts of less than 5,000 cu.mm. Thus haematological abnormality and low serum B.12 were infrequently associated.

Low serum folate: 19 of the low serum B.12 patients had serum folate levels less than 2.0 ng/ml.

TABLE III

Drugs and Low Serum Vitamin B.12.

Drug	*No. of Patients*
Phenothiazines (including Chlorpromazine = 5)	12
Barbiturates	7
Chlordiazepoxide	5
Tricyclic antidepressants	5
Mono-amine oxidase inhibitors	2
Butazolidine	1
Phenytoin	1
Others	8

Low B.12 Patients.

Drugs: 26 (49%) had received drugs (... in Table III) of whom nearly half had ... on phenothiazines.

Chronicity: In assessing the duration of ... sent illness prior to admission, frequent ... sodes, with only a few days or weeks re... sion between, were regarded as one ill... Thirty-two (60.4%) had been ill for ... than one year — a very high proportion ... pared with the generality of psychiatric ... tients.

Nutritional status: 16 (30.2%) were ... scribed on admission as poorly nourished ... whom four showed dependent oedema ... two others, the clinical signs of Wernicke's encephalopathy, almost certainly due ... chronic avitaminosis.

Associated physical illness: 15 (28.3%) ... evidence on admission of physical illness ... than malnutrition: cardiovascular di... patients; chronic bronchitis, 2; gynaecol... conditions, 2; hepatic impairment, 2; P... sonism, 1; myxoedema 1; chronic retent... urine, 1. Also, one patient had had a l... tomy.

Pregnancy: Two patients were pregnant.

Stomach operation: One patient had ... gastrotectomy.

Fractional test meal: Five patients had ... investigation, only one of whom showed h... mine-fast achlorydria (and a normob... marrow).

Malabsorption: There were no cases of ... absorption syndrome.

Discussion and Conclusions

The most striking result of this inve... tion was the high incidence of low serum ... levels in a representative sample of m... mental illness in a defined community — ... finding in agreement with studies previ... mentioned. However, other authors give m... lower figures, possibly because they emp... different methods of estimating serum vit... B.12 e.g. Murphy et al. (1969) used a ... with E.gracilis. This study, however, con... with those who failed to report an incre... liability to pernicious anaemia among p... chiatric patients (in fact no case was s... nor were the low levels associated ...

0453

haematological or neurological abnormality. There was considerable evidence of possible serum B.12 — lowering factors, especially pre-admission drugs (mainly phenothiazines and barbiturates), impaired nutrition, associated physical illness and chronicity of illness.

The present investigation was not designed to explore the precise aetiology of these low levels, nor their exact relationship with various forms of psychiatric illness. Nevertheless there was a striking excess of organic psychoses, psychiatric syndromes said to be typical of B.12 deficiency[8] — presumably the psychiatric counterpart of sub-acute combined degeneration of the cord. Even if organic psychoses are excluded on the grounds that advanced age could have depressed the serum B.12 values, the remaining low B.12 patients have a diagnostic distribution significantly different from the normal B.12 patients ($X^2 = 7.65$; d.f. = 2; $P<0.05$), endogenous depression predominating.

Although evidence of links between low serum B.12 values in psychiatric patients and mental symptoms must, in the present state of knowledge, remain speculative, there are two good reasons why assay of the serum B.12 is valuable: firstly, in order to treat low B.12 patients with cyanocobalamen in the hope of improving their physical and mental state; and secondly, since low folate is aetiologically important in mental illness[6] and low folate levels frequently occur in psychiatric patients[1], serum B.12 values should be known before treatment with folic acid is given in case sub-acute combined degeneration of the cord is precipitated.

Summary

Serum B.12 estimations were carried out on 374 psychiatric inpatients, consecutive admissions to a general hospital and a mental hospital serving the same catchment area. Fifty-three (14.2%) gave values below 150 pg.ml., 49 of whom belonged to the following diagnosis (in numerical order); organic psychoses, endogenous depression, schizophrenia and neurotic depression: this distribution varied significantly from that of the whole group. Sixteen patients (4.3%) gave values below 100 pg.ml. Haematological abnormalities were not significantly more common among the low serum B.12 patients than among the rest of low serum B.12 patients, 49% had received drugs (mainly phenothiazines and barbiturates) during the pre-admission three weeks, 60.4% had been ill for more than one year, 30.2% were classified as severely undernourished and 28.3% had concomitant physical disease: two patients were pregnant and one had had a gastrectomy.

Neither low serum B.12 patient with a megaloblastic marrow was suffering from pernicious anaemia.

It was concluded that low serum vitamin B.12 values were common in psychiatric patients and that the serum vitamin B.12 estimation was a useful screening procedure.

Present Address: 382 Clifton Drive North, St. Annes on Sea, Lancashire, England.

Acknowledgements: I wish to thank the following: Drs. F. Yates, P. Harvey, and F. Hayes, Messrs. Orrell, Curd, Singleton and Kilgarriff, Sisters Hetherington and Morton, for advice and co-operation; Mrs. J. Taylor and Mrs. E. Jackson, for clerical assistance.

REFERENCES

1. Carney, M.W.P.: Serum folate values in 423 psychiatric patients. *Brit. med. J.* 4, 512-516, 1967.
2. Hunter, R. and Matthews, D.M.: Mental symptoms in vitamin B.12 deficiency. *Lancet*, 2, 738, 1965.
3. McCallum, W.A.G.: Recoverable psychiatric illness occurring with low serum vitamin B.12 levels. *J. Irish med. Assoc.*, 57, 187-192, 1965.
4. Meynell, M.J., Cooke, W.T., Cox, E.W. and Gaddie, R.: Serum—cyanocobalamin levels in chronic intestinal disorders. *Lancet*, 1, 901-904, 1957.
5. Murphy, F., Scrivastaun, P.C., Varadi, S. and Elwis, A.: Screening of psychiatric patients for hypovitaminosis B.12. *Brit. med. J.* 3, 559-560, 1969.
6. Reynolds, E.H.: Vitamin B.12 and folate deficiencies. *Brit. P. Psychiat.* 119, 681-683, 1967.
7. Schrumpf, A.: Mental symptoms in vitamin B.12 deficiency. *Lancet*, 2. 856, 1965.
8. Shulman, R.: A survey of vitamin B.12 deficiency in an elderly psychiatric population. *Brit. J. Psychiat.* 113, 241-251, 1967.
9. Shulman, R.: Psychiatric aspects of pernicious anaemia: a prospective controlled investigation. *Brit. med. J.* 3, 266-270, 1968.
10. Watson-Williams, E.J.: Personal communication.

Amer. J. Physiol. 224(4): 758-762 0557

AMERICAN JOURNAL OF PHYSIOLOGY
Vol. 224, No. 4, April 1973 Printed in U.S.A.

Evidence for complexing of transcobalamin-2 in canine and human plasma and serum

BERNARD A. COOPER AND JOHN DIRKS (With the Technical Assistance of Barbara Mudd)
Hematology and Renal-Electrolyte Divisions, Department of Medicine, Royal Victoria Hospital, and McGill University, Montreal, Quebec, Canada

COOPER, BERNARD A., AND JOHN DIRKS. *Evidence for complexing of transcobalamin-2 in canine and human plasma and serum.* Am. J. Physiol. 224(4): 758–762. 1973.—Human and dog transcobalamin-2, a vitamin B_{12}-binding plasma protein with molecular weight of about 40,000, has been found to complex when filtered through Sephadex with dog plasma or human serum, suggesting that it may circulate in vivo as a complex in these species. Fluid from the proximal convoluted tubule of the dog obtained after intravenous injection of radioactive B_{12} did not contain measurable radioactivity except when the radioactive B_{12} was injected after a large dose of nonradioactive B_{12}. This indicates that transcobalamin-2 does not cross the glomerulus in the dog, supporting the suggestion that it circulates as a complex with radius in excess of that of the transcobalamin-2 monomer.

vitamin B_{12}; micropuncture; proximal tubule

TRANSCOBALAMIN-2, one of the proteins that bind vitamin B_{12} in human serum (10), filters through Sephadex gels as if its molecular weight were 35,000–40,000 (13). The Stokes radius of transcobalamin-2 associated with cyanocabalamin has been calculated to be 25.5 ± 0.6 A, whereas the human glomerulus appears to sieve molecules 24 A and above (15). Proteins and peptides of small molecular size (insulin, ribonuclease, lysozyme, α_2-microglobulin, β_2-microglobulin) pass the glomerulus to be absorbed and degraded by renal tubular cells (2–4, 12, 17). The protein concentration in fluid from the lumen of the proximal convoluted tubule of the dog (21) has been reported to be very low and that from the rat is somewhat higher (7). Newmark et al. (19, 20) have reported that the rat kidney, which concentrates injected vitamin B_{12} more than do those of the dog or man, accumulates vitamin B_{12} in a lysosomal fraction. Vitamin B_{12} on human transcobalamin-2 has been reported to have a rapid turnover rate (14) and to be capable of forming complexes when filtered through Sephadex in 100% human plasma (22). We have undertaken to determine the effect of serum and plasma concentrations on the apparent size of transcobalamin-2 during filtration through Sephadex, and to determine if this binder can be recovered from the lumen of the proximal convoluted renal tubule of the dog (6). These studies were designed to determine if transcobalamin-2 exists in vivo as a monomer or as a complex, and if renal filtration represents a significant catabolic pathway for this material.

METHODS

Blood was obtained from patients receiving oral anticoagulants. Serum was collected, pooled, and stored as aliquots at −20 C. Dog plasma was obtained in commercial ACD solution (citric acid, sodium citrate, and dextrose; Abbott Laboratories) from healthy mongrel dogs. It was partially defibrinated by removing the cryoprecipitate which formed during incubation at 4 C after prior freezing.

Gel filtration utilized a column 2.5 cm in diameter and 34–40 cm high containing approximately 200 ml of Sephadex G-200 gel. The filtration buffer was 0.16 M sodium chloride containing 0.01 M sodium phosphate, pH 7.0, or various mixtures of this with pooled human serum or defibrinated dog plasma. The column was maintained at 1–4 C by means of a cooling jacket, and collected fractions were kept at 2 C in a water bath. Microbial growth was inhibited with 0.02% (w/v) sodium azide in the filtration buffer.

Fractions labeled with radioactive B_{12} were applied to the column as follows: plasma or serum or a fraction previously separated was allowed to thaw at 1 C in a water bath. The liquid plasma or serum was mixed with the required concentration of radioactive vitamin B_{12} and incubated at 37 C for 30 min. In experiments in which only a single isotope was filtered, the B_{12}-labeled material was brought to a volume of 5 ml with filtration buffer containing 2 mg of Blue Dextran 2000 (Pharmacia Canada Ltd., Montreal), 50 mg glucose, and an appropriate number of counts of ^{131}-labeled human serum albumin (Charles E. Frosst and Co., Montreal). The sample was layered on the column with a perfusion pump. In experiments in which two isotopes of radioactive cyanocobalamin were to be filtered simultaneously, plasma or serum was incubated with cyanocobalamin labeled with one or the other isotope at 37 C for 30 min and cooled to 1 C in a water bath, and the two preparations were mixed at this temperature with filtration buffer, Blue Dextran 2000, and glucose immediately prior to application to the gel.

Transcobalamin-2 was prepared by filtering a mixture of 6 ml of pooled human serum and 12,000 pg of B_{12}-^{57}Co through a Sephadex column as described above. Radioactivity in an aliquot of each fraction was determined and then the appropriate samples were collected and used.

The rate of dissociation of B_{12}-^{57}Co from transcobalamin-2 was measured by incubating transcobalamin-2 labeled

372

MATERIAL AND METHODS

Transplantable rat leukaemias

Young A × C rats inbred since 1949 and weighing from 120 to 275 g were used throughout this work. Acute leukaemias LR III and LR XII developed spontaneously in these animals and were made transplantable by serial passage at the Institute of Experimental Medicine, Santiago, Chile, by Dr. D. Iglesias who kindly supplied the animals to our laboratory.

These leukaemias cause death between 13 to 18 days after inoculation. LR III produces large tumours at the site of inoculation with moderate liver and spleen enlargement. LR XII, on the other hand, produces slight local tumefaction and pronounced hepato- and splenomegaly. Both types give 100% takes and are always fatal. A detailed haematological study of LR III leukaemia with daily follow-up of the blood picture and of normal controls has been made in our laboratory by Ojeda and Bernardi (unpublished observations). The main findings are summarized in Table I.

The more striking features of the normal blood picture in these animals are the high proportion of agranulocytes—mainly small lymphocytes—and the presence of circulating mature and immature plasma cells.

In the leukaemic animals, neutrophils, red cells and haemoglobin decrease steadily from the third or fourth day after inoculation and there is a rapid decrease of platelets with a sharp increase in white cells after the first week. Towards the end of the disease (14th day) the red cells average 2.045×10^6 and white cells 260,600 per mm³. Neutrophils increase to 40% in the first five days, then decrease steadily, while lymphocytes and monocytes decrease continuously to less than 0.1% and blast cells increase to 95% at the 14th day. Abnormal lymphocytes and monocytoid cells were found and erythroblasts appeared in the last stages. The most abundant type of blast cell was large (20 to 40 μ), usually round with strongly basophilic cytoplasm and a large, often eccentric nucleus with coarse purple chromatin, one or two nucleoli and pale perinuclear zone.

TABLE I

HAEMATOLOGICAL DATA ON LR III LEUKAEMIA AND NORMAL CONTROLS

	Controls	*LR III*[1]
Erythrocytes/mm³	$7.787 \pm 0.069 \times 10^6$	$4.614 \pm 0.343 \times 10^6$
Leucocytes/mm³	$10{,}716 \pm 314$	$169{,}223 \pm 20{,}961$
Platelets/mm³	$1.053 \pm 0.007 \times 10^6$	$0.354 \pm 0.047 \times 10^6$
Haemoglobin (g/100 ml)	13.98 ± 0.10	8.83 ± 0.58
Differential cell count (%)		
Blasts	0	61
Neutrophils	17	28
Lymphocytes	69	7
Monocytes	7.2	1.6
Eosinophils	4	1.4
Basophils	0.1	0.1
Plasmocytes	1.6	0.2

[1] At 11 days after inoculation, when samples for B_{12} estimations were taken.

Sampling

Serial transplants were made by injecting subcutaneously into the interscapular region 0.1 ml of a 1% suspension of finely minced leukaemic spleen in isotonic saline.

Blood samples from animals with leukaemia LR III were taken at the 11th day, and from animals with LR XII at the 14th day after inoculation. Haemolysed blood was obtained by heart puncture with a syringe containing an

with B_{12}-^{57}Co with an excess of unlabeled cyanocobalamin-^{59}Co at 37 C (30,000 pg B_{12} = ^{59}Co/pg of B_{12}-^{57}Co). At intervals, aliquots were taken and the B_{12}-^{57}Co bound was determined by adding an ice suspension of a bovine-serum-albumin-charcoal mixture (Norit A) (9) in saline to the sample, mixing it in the cold, and centrifuging out the charcoal to which unbound B_{12}-^{57}Co had absorbed.

Equilibrium dialysis utilized bags of Visking casing partly filled with 4 ml of buffered saline placed in screw-capped test tubes containing the test solution made up to the same volume in the same buffer. The tubes were capped with Parafilm secured by the screw caps, and rotated slowly at 4 C for 20 hr. A small bubble in each arm of the Visking casing served to mix the contents of the bags. At the end of the rotation the bags were removed from the test tubes, wiped dry, and emptied. One milliliter of the dialysate and of the dialysand were counted in a well-type scintillation counter. The concentration of free B_{12} in the bags after 20 hr of rotation was consistently within 5% of that in the solution outside of the bags.

Details of the micropuncture technique in the dog were as described previously (5, 7). For the experiments described here, the following procedure was adopted. Mongrel dogs weighing 10–15 kg were prepared for micropuncture in the usual fashion. Arterial and venous catheters were kept patent with Krebs-Ringer-bicarbonate solution containing 2.5 μ/ml of heparin. Before the experiment the catheters were flushed with heparin-free Ringer. The test solution was injected into the venous catheter and flushed into the circulation with 7 ml of Ringer solution. Blood samples were taken from the arterial catheter after removal of 7 ml of fluid, which then was replaced with fresh Ringer solution after each sampling. All blood samples clotted within 10–20 min, confirming the absence of significant concentrations of heparin.

Proximal convoluted tubules were punctured close to the glomerulus immediately after injection of the radioactive bolus. Early proximal tubules had previously been identified by renal arterial injections of FD & C green dye. Long oil blocks were maintained to exclude retrograde collection. All samples were examined under the microscope to exclude contamination with red blood cells. During the experiment described below, timed samples were taken sequentially from 10 tubules during each 15-min test period. Micropuncture samples were expressed into capillary tubes of uniform bore, and the volume of the sample was measured. In one experiment, similar volumes of serum were placed in such capillary tubes and counted. The results obtained were similar to those with 0.5 ml of serum, confirming the validity of counting radioactivity of samples in capillary tubes.

A well-type scintillation counter with a 3-inch crystal (Arrowell 11) was utilized for the counting of radioactivity. For counting of samples in capillary tubes, the following procedure was used. Twelve clean, acid-washed counting tubes were selected, and the capillary tubes containing samples from the desired collection periods were placed in six of these. The 12 tubes, together with appropriate standards, were counted repeatedly for 20 min each until each had been counted 8 times. The capillary tubes then were transferred to the other six tubes, and the complete counting procedure was repeated for all 12 tubes. In this way, each counting tube served as both a sample tube and a background tube. Mean counts for 20 min in both ^{57}Co and ^{58}Co channels were calculated, and the probability that they differed from background was calculated using the Student t test. The windows used for radioactive counting excluded ^{57}Co counts from the ^{58}Co channel, but disintegrations emanating from ^{58}Co appeared in the ^{57}Co channel. The ratio of counts from ^{58}Co counted in the ^{58}Co channel to those appearing in the ^{57}Co channel was 3.26:1.

Cyanocobalamin labeled with ^{57}Co and ^{58}Co was purchased from The Radiochemical Centre, Amersham, Buckinghamshire, England. It was confirmed to be cyanocobalamin by its partition coefficient at various pHs between benzyl alcohol and buffer (26).

Plasma clearances of vitamin B_{12} were calculated from radioactivity detected in samples of urine and plasma. Mean plasma concentration was calculated by adding one-third of the difference between plasma radioactivity in samples obtained at the beginning and at the end of the interval studied to the radioactivity in samples at the end of each collection period. Because the collection periods were short, and the time required for urine to traverse the kidney was short, clearance rates are only approximate. Inulin clearance was not determined.

RESULTS

When 210 ng (30 μc) of B_{12}-^{57}Co were injected intravenously into a dog, only a very small proportion of the radioactivity injected appeared in the urine (Table 1). This radioactivity was dialyzable through Visking 8/22 casing (92–98% of the radioactivity equilibrated in 20 hr at 4 C). When 433 ng (26 μc) or B_{12}-^{58}Co were injected after a massive injection of nonradioactive cyanocobalamin (1,000 μg), considerable radioactivity appeared in the urine. In Table 1, the plasma renal clearance of each isotope of vitamin B_{12} is indicated, based on its mean concentration in serum and urine during the collection period and the volume

TABLE 1. *Plasma clearance of ^{57}Co- and ^{58}Co-labeled B_{12} calculated from plasma and urine counts*

Time, min	Urine Vol, ml/min	Counts B_{12}-^{57}Co/0.1 ml		B_{12}-^{57}Co Clearance, ml/min	Counts B_{12}-^{58}Co/0.1 ml		B_{12}-^{58}Co Clearance, ml/min
		Plasma	Urine		Plasma	Urine	
Right (unpunctured) kidney							
0–5*	0.640	18,000	60,852	2.16			
5–20	0.633	12,000	11,802	0.61			
20–35†	0.680	8,000	5,390	0.45			
35–45	0.66	6,600	13,629	1.36	3,400	94,996	18.4
45–55	0.65	6,000	10,916	1.18	1,200	66,884	36.2
Left (punctured) kidney							
0–5*	0.16	18,000					
5–20	0.12	12,000	76,895	0.76			
20–35†	0.12	8,000	21,407	0.32			
35–45	0.14	6,600	35,309	0.88	3,400	58,438	6.20
45–55	0.12	6,000	68,259	1.48	1,200	479,812	48.5

* B_{12}-^{57}Co injected intravenously at 0 min. † B_{12}-^{58}Co injected intravenously at 20 min immediately after 1,000 μg of nonradioactive cyanocobalamin.

TABLE 2. *B_{12} in fluid from proximal convoluted renal tubule*

Sample No.	Time, min	Vol, μl	^{57}Co Channel		^{58}Co Channel	
			counts/ 20 min	P	counts/ 20 min	P
1	0-0:45	10.5	326	0.36	232	0.31
2	1:35-3:05	48	324	0.50	231	0.38
3	6:25-20:00	383	321	0.74	228	
4	35:05-38:00	195	350	0.005	259	0.001
5	38:40-45:15	394	338	0.07	251	0.001
6	47:30-55:00	269	321	0.74	235	0.31
Background			318 ± 8.9		229 ± 6.0	
B_{12}, 1 pg			4,800		569	

of urine collected. The plasma clearance calculated for B_{12}-^{58}Co injected after the large dose of nonradioactive cyanocobalamin was similar to the calculated glomerular filtration rate of the animal (29 ml/min per kidney), whereas that of the B_{12}-^{57}Co injected, most of which could have been bound by the B_{12}-binding proteins of the animal, was considerably smaller.

Data from studies of micropuncture fluid obtained from the lumen of proximal convoluted tubules are summarized in Table 2. The volume of micropuncture fluid obtained during each collection period is listed together with the average counts for 20 min in each counting channel and the probability (Student *t* test) that these exceed the background. Significant radioactivity was not observed in micropuncture fluid until after injection of the large quantity of nonradioactive cyanocobalamin. ^{58}Co was detected in puncture fluid without difficulty after this. A small but significant number of counts appeared in the ^{57}Co channel at this time. These were in excess of the counts expected from the ^{58}Co appearing in this channel, and probably represented appearance of some B_{12}-^{57}Co in the puncture fluid at this time. This may have resulted from release of of B_{12}-^{57}Co from binding proteins or from sites of adsorption.

The experiment of determining ^{57}Co counts in proximal convoluted tubule fluid and urine after intravenous injection of B_{12}-^{57}Co was conducted in four different dogs. Those in the other three animals differed from the one described in detail here in not being followed by injection of a massive dose of nonradioactive B_{12} and then B_{12}-^{58}Co. The minimum quantity of radioactivity detectable by the counting procedures used in those studies was 10% or less of that which would have been filtered if B_{12}-^{57}Co clearance were equal to calculated GFR. In only 1 of 10 samples was any radioactivity detected, and that specimen, the 0- to 3-min collection, was contaminated with some surface blood.

Mean renal plasma clearance of B_{12}-^{57}Co in these animals was 0.38 ± 0.09 ml/min (mean ± SD), and in none was the clearance greater than 1 ml/min. The first sample in the experiment described in this paper (Table 1) was the greatest plasma clearance of B_{12}-^{57}Co observed in any of the studies.

Size of transcobalamin in vitro. To determine the size of transcobalamin-2 in vitro, citrated dog plasma previously incubated with B_{12}-^{57}Co was filtered through Sephadex G-200 in buffered saline. The pattern of radioactivity in the effluent was compared with that of B_{12}-^{58}Co previously incubated with normal human serum and was applied to the column simultaneously (Fig. 1, broken lines). Transcobalamin-2 from both human and dog filtered with mobility similar to that reported previously (23) for human transcobalamin-2. No unsaturated transcobalamin-1 was observed in this dog plasma, but some was found in plasma from other dogs. When the same mixture was filtered through the same column using as filtering solution citrated dog plasma diluted in buffered saline to 4.0 g of protein per 100 ml, pH 7.2, both B_{12}-^{57}Co and B_{12}-^{58}Co, representing dog and human transcobalamins, filtered before albumen with apparent molecular weights of about 65,000 and 85,000 daltons, respectively (Fig. 1, solid lines). The presence of dilute dog plasma in the filtering buffer increased the apparent molecular size of these B_{12}-binding proteins in both human serum and dog plasma. The mobility of ^{131}I-labeled albumin was not affected by the dog plasma.

To exclude exchange of radioactivity between binders as an explanation, further studies were conducted with pooled normal serum. Serum mixed with B_{12}-^{57}Co was filtered through Sephadex G-200, and a fraction corresponding to transcobalamin-2 was collected (Fig. 2). This material, when refiltered through the gel in buffered saline, contained only a small amount of large-molecular-weight binder (Fig. 3). When filtered through the same column using 50% human serum in buffered saline as filtering solution, all the radioactivity filtered with an apparent molecular weight about 85,000–90,000 daltons (Fig. 4). When two fractions of this material were collected and refiltered with the same filtering solution, the pattern was

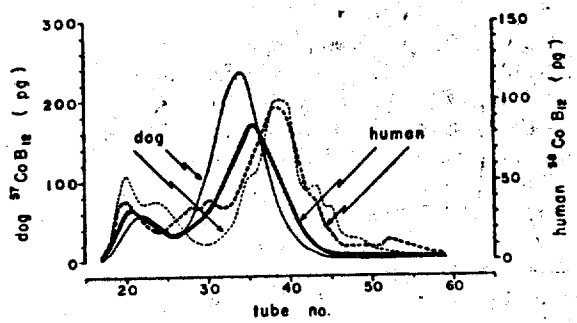


FIG. 1. B_{12}-^{57}Co-dog plasma and B_{12}-^{58}Co-human serum filtered through Sephadex G-200 in buffered saline and in dog plasma.

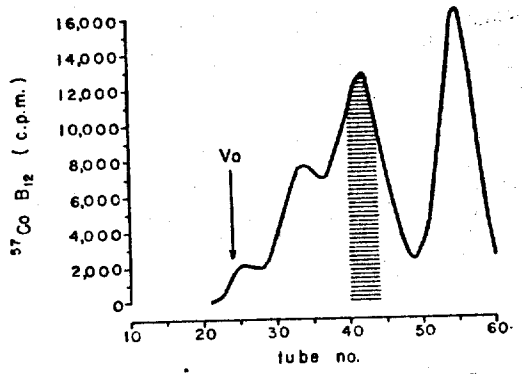


FIG. 2. Preparation of human transcobalamin-2.

identical (Fig. 5 *A* and *B*, solid lines) with that of the original transcobalamin-2 solution filtered in 50% serum. When these fractions were refiltered in buffered saline, however, much of the radioactivity filtered as if smaller than albumin, and with mobility appropriate for transcobalamin in saline (Fig. 5 *A* and *B*, broken lines). In order to use the same column for all studies with these fractions, the material filtered in buffered saline in experiments illustrated in Fig. 5 *A* and *B* was stored at 4 C for 5 days. Some irreversible polymerization may have occurred during this storage period. These studies illustrate, however, that most of the transcobalamin-2 fraction can undergo reversible complexing in 50% serum solution.

Dissociation of B_{12}-^{57}Co from transcobalamin-2. Turnover studies of transcobalamin-2 labeled with B_{12}-^{57}Co may indicate a more rapid turnover than is true of the protein itself if B_{12}-^{57}Co dissociates from it faster than from other serum binders. The B_{12}-^{57}Co remaining bound to the preparation of transcobalamin-2 at 37 C in the presence of a 30,000-fold excess of nonradioactive cyanocobalamin decreased with time and approximated a first-order reaction with $T_{1/2}$ of 22 hr. The $T_{1/2}$ of a transcobalamin-1 preparation prepared from the same serum was in excess of 60 hr. At 0 C, dissociation from whole serum was so slow that a meaningful $T_{1/2}$ could not be calculated. These studies indicate that studies of the rate of disappearance of the ^{57}Co label from transcobalamin-2 may result in calculation of erroneously fast turnover and that observations of shift of B_{12}-^{57}Co from transcobalamin-2 to transcobalamin-1 may be due to exchange.

DISCUSSION

These studies indicate that human and dog transcobalamin-2 may exist as a larger molecule in 50% serum or plasma than in saline and extend the previous observation that human transcobalamin-2 filters as a large molecule in 100% human plasma at 37 C (22). The apparent size of transcobalamin-2 reported in whole plasma was greater than that observed in our studies, suggesting that the size of the complex is determined by the concentration of plasma or serum components. Human transcobalamin in dog plasma filtered as a smaller molecule than did dog transcobalamin-2, although both appeared similar in saline. In addition, human transcobalamin-2 appeared to be smaller in dog plasma than in 50% human serum, suggesting some species specificity in complex formation. The mobility of ^{131}I-labeled human serum in Sephadex albumen gel was unaffected by either 100% (22) or 50% serum.

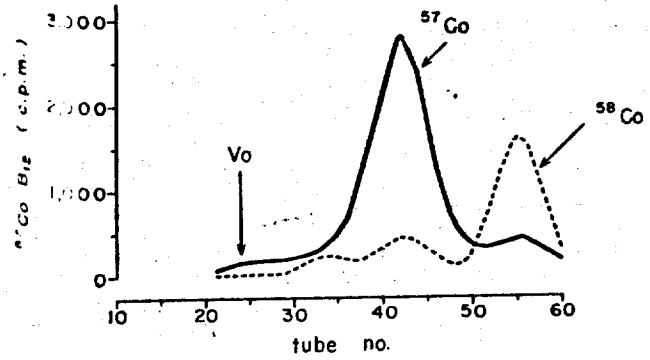


FIG. 3. Refiltration of transcobalamin-2 through Sephadex. A small amount of unfractionated normal human serum previously incubated with B_{12}-^{58}Co was filtered simultaneously as a reference.

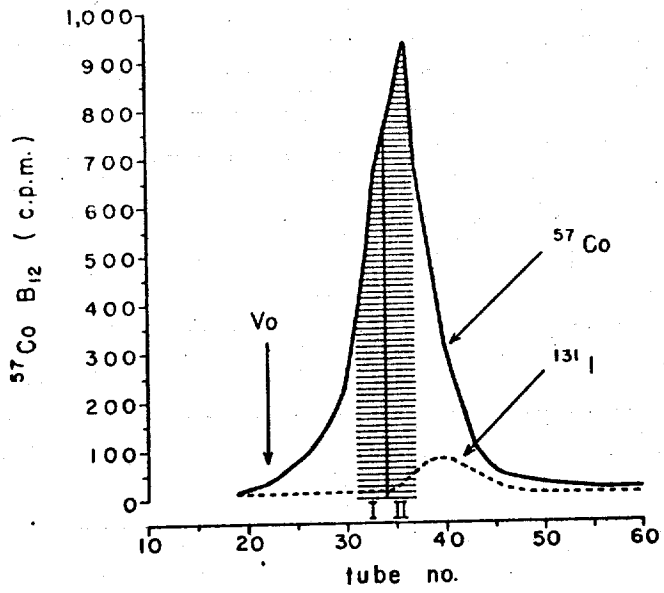


FIG. 4. Refiltration through Sephadex G-200 of transcobalamin-2 fraction from Fig. 2 using 50% human serum as filtering buffer.

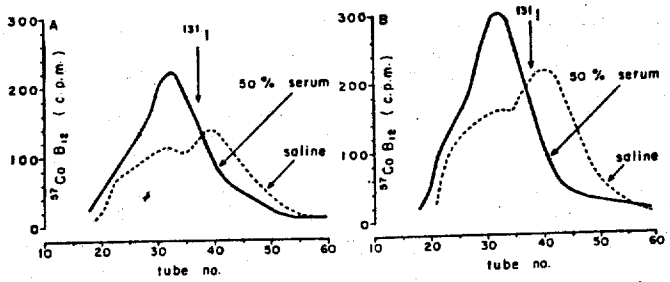


FIG. 5. Refiltration of fractions from Fig. 4 through Sephadex G-200 in buffered saline and in 50% serum. *A*: front portion of peak from Fig. 4, possibly containing larger molecules. *B*: center portion of peak from Fig. 4.

Our micropuncture data indicate that B_{12}-^{57}Co in significant quantities did not enter the proximal convoluted tubule, suggesting that it does not cross the glomerulus in the dog. The glomerulus has been reported to act as a molecular sieve (17), although it has been suggested (18) that the distribution of pore sizes in the human and dog glomerulus may be wide and may follow a log-normal distribution. The sieving coefficient, the renal clearance of a test molecule compared with that of inulin or creatinine, has been used as a measure of glomerular filtration for molecules unaffected by reabsorption in the renal tubules and has been found to vary inversely with molecular radius (17). The Stokes radius of transcobalamin-2-binding cyanocobalamin has been reported to be between 25 and 26 A (14). For a protein of this size, the sieving coefficient expected, calculated from data reported by Hardwicke et al. (11) for humans and by Lambert et al. (17) for dogs, would be about 10–15% of the creatinine clearance. For dextrans, the expected sieving coefficient would be about 2–3% of

the creatinine clearance for humans and about 18% for dogs, whereas for labeled fractions of polyvinylpyrrolidone, it would be considerably higher. The values in Table 1 are considerably lower that these, and most of the radioactivity appearing in the dog urine was dialyzable, indicating that only a very small proportion of the radioactivity filtered into the proximal tubular lumen could have been protein bound. Dialysis was carried out with 23/32 Visking tubing, the pore size of which is smaller than that of 8/23 casing and retains particles to 10,000–15,000 daltons (2). It thus would appear that transcobalamin-2 in dog plasma may be presented to the glomerulus as a particle with a radius larger than that observed for transcobalamin-2 filtered in saline. The sieving coefficient of a complex with a radius corresponding to the apparent molecular size observed in the experiment illustrated in Fig. 1 would be 0.1–1% of creatinine clearance and more consistent with the data presented in Table 2.

It has been reported that injected cyanocobalamin accumulates in a lysozomal fraction prepared from rat kidney (19, 20), and that after injection of radioactive cyanocobalamin in the rat and mouse, radioactivity is seen in cells of the proximal convoluted tubule (8, 25). These observations suggest that either a small quantity of free cyanocobalamin can be absorbed from the lumen of the proximal tubule in these animals, that mouse and rat transcobalamins may not form complexes as readily as do those of man and the dog, or that the pore size of the glomerulus in these animals is larger than in the dog. One also might speculate that these materials were accumulated from the peritubular surface of the cells.

A small quantity of vitamin B_{12} appears in normal urine, and one report indicates that in acute liver disease some of this is bound to material with a low affinity for DEAE-cellulose (24). The wide variety of proteins in urine (2, 16) and the renal source of some of these make identification of transcobalamin-2 in human urine difficult (23). The observations described above do not exclude the possibility that some transcobalamin-2 enters the urine, either by synthesis in renal tissue or by some other mechanism. They do, however, indicate that little or no transcobalamin-2-bound cyanocobalamin filters through the glomerulus in the dog and demonstrate complexing of transcobalamin-2 in vitro in dog plasma and human serum, which could provide a mechanism whereby transcobalamin-2-bound cyanocobalamin might be excluded from the glomerular sieve.

This work was supported by Grant MT-802 of the Medical Research Council of Canada.

Some of these observations have been reported previously in an abstract (6).

Requests for reprints should be sent to B. A. Cooper.

Received for publication 22 August 1972

REFERENCES

1. Berggård, I. Studies on the plasma proteins in normal human urine. *Clin. Chim. Acta* 6: 413–429, 1961.
2. Berggård, I. Plasma proteins in normal human urine. In: *Proteins in Normal and Pathological Urine*, edited by Y. Manuel, J. P. Revillard, and H. Betuel. Basel: Karger, p. 7–19.
3. Bernier, G. M., and M. E. Conrad. Catabolism of human β_2-microglobulin by the rat kidney. *Am. J. Physiol.* 217: 1359–1362, 1969.
4. Chamberlain, M. J., and L. Stimmler. The renal handling of insulin. *J. Clin. Invest.* 46: 911–919, 1967.
5. Clapp, J. R., J. F. Watson, and R. W. Berliner. Osmolality, bicarbonate concentration, and water reabsorption in proximal tubule of the dog nephron. *Am. J. Physiol.* 205: 273–280, 1963.
6. Cooper, B. A. Apparent polymerization of transcobalamin-2-B_{12} binding protein in human plasma. *Blood* 34: 856, 1969.
7. Dirks, J. H., J. R. Clapp, and R. W. Berliner. The protein concentration in the proximal tubule of the dog. *J. Clin. Invest.* 43: 916–921, 1964.
8. Flodh, H. Distribution and kinetics of labelled vitamin B_{12}. *Acta Radiol.* Suppl. 284, 1968.
9. Gottlieb, C., K.-S. Lau, L. R. Wasserman, and V. Herbert. Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated B_{12} binding capacity, antibody to IF, and serum unsaturated binding capacity. *Blood* 25: 875–884, 1965.
10. Hall, C. A., and A. E. Finkler. A second vitamin-B_{12} binding substance in human plasma. *Biochim. Biophys. Acta* 78: 234–236, 1963.
11. Hardwicke, J., J. S. Cameron, J. F. Harrison, B. Hulme, and J. F. Soothill. Proteinuria, studied by clearances of individual macromolecules. In: *Proteins in Normal and Pathological Urine*, edited by Y. Manuel, J. P. Revillard, and H. Betuel. Basel: Karger, 1970, p. 111–152.
12. Harrison, J. F., G. S. Lunt, P. Scott, and J. D. Blainey. Urinary lysozyme, ribonuclease, and low-molecular weight protein in renal disease. *Lancet* 1: 371–374, 1968.
13. Hom, B. L. Stokes radius of ^{57}Co-labelled vitamin B_{12}-transcobalamin I and II determined by Sephadex gel filtration. *Biochim. Biophys. Acta* 175: 20–23, 1969.
14. Hom, B. L., and H. A. Olesen. Plasma clearance of ^{57}cobalt-labelled vitamin B_{12} bound in vitro and in vivo to transcobalamin I and II. *Scand. J. Clin. Lab. Invest.* 23: 201–211, 1969.
15. Hulme, B., and J. Hardwicke. Human glomerular permeability to macromolecules in health and disease. *Clin. Sci.* 34: 515–529, 1968.
16. Kallee, E., S. Debiasi, C. Karypidis, K. Heide, and H.-G. Schwick. ^{60}Co-Vitamin B_{12}-Bindende Glykoproteine. *Acta Isotopica* 4: 103–137, 1964.
17. Lambert, P. P., J. P. Gassee, and R. Askenasi. Physiological basis of protein excretion. In: *Proteins in Normal and Pathological Urine*, edited by Y. Manuel, J. P. Revillard, and H. Betuel. Basel: Karger, 1970, p. 67–82.
18. Lambert, P. P., J. P. Gassee, A. Verniory, and P. Ficheroulle. Measurement of the glomerular filtration pressure from sieving data for macromolecules. *Arch. Ges. Physiol.* 329: 34–58, 1971.
19. Newmark, P. The mechanism of uptake of vitamin B_{12} by the kidney of the rat in vivo. *Biochim. Biophys. Acta* 261: 85–93, 1972.
20. Newmark, P., G. E. Newman, and J. R. P. O'Brien. Vitamin B_{12} in the rat kidney. Evidence for an association with lysozomes. *Arch. Biochem. Biophys.* 141: 121–130, 1970.
21. Oken, D. E., and W. Flamenbaum. Micropuncture studies of proximal tubule albumin concentrations in normal and nephrotic rats. *J. Clin. Invest.* 50: 1498–1505, 1971.
22. Olesen, H., J. Rehfeld, B. L. Hom, and E. Hippe. Stokes radius of ^{57}Co-labelled vitamin B_{12}-transcobalamin I and II and ^{125}-I-labelled insulin estimated by Sephadex G-200 gel filtration in human plasma at 37°. *Biochim. Biophys. Acta* 194: 67–70, 1969.
23. Poortmans, J. R., and R. W. Jeanloz. Quantitative immunological determination of twelve plasma proteins excreted in human urine collected before and after exercise. *J. Clin. Invest.* 47: 386–393, 1968.
24. Retief, F. P., L. Vandenplas, and H. Visser. Vitamin B_{12} binding proteins in liver disease. *Brit. J. Haematol.* 16: 231–240, 1969.
25. Salmi, H. A. Comparative studies on vitamin B_{12} in developing organism and placenta. Human and animal investigations with reference to the effects of low vitamin B_{12} diet on tissue vitamin B_{12} concentrations in rat. *Ann. Acad. Sci. Fennicae, Ser. A5* 103: 1–91, 1963.
26. Smith, E. L., S. Ball, and D. M. Ireland. B_{12} vitamins (cobalamins). II. Neutral, basic, and acidic cobalamins. *Biochem. J.* 52: 395–400, 1952.

[CANCER RESEARCH 32, 167–172, January 1972]

Distribution of Radioactive and Nonradioactive Vitamin B_{12} in Normal and Malignant Tissues of an Infant with Neuroblastoma[1]

Jack M. Cooperman

Hematology and Nutrition Laboratory, Department of Pediatrics, New York Medical College, New York, New York 10029

SUMMARY

An infant with disseminated neuroblastoma was given radioactive vitamin B_{12} 30, 21, and 9 days before he succumbed to the disease. The retention of radioactive vitamin B_{12} was determined by scanning the external hepatic area for radioactivity. At autopsy, the organs and tumors were obtained, cleaned, and weighed, and either the whole organ or a section was taken for analysis. The normal and malignant tissues were assayed for radioactivity and microbiologically for vitamin B_{12}. Tumors associated with the liver, pancreas, and adrenals had considerably lower vitamin B_{12} concentrations and radioactivity retention than did adjacent normal tissue. Two large mediastinal tumors were also uniformly low in vitamin B_{12} concentration and radioactivity. Neuroblastoma cells can proliferate rapidly and presumably synthesize nucleic acids in the presence of low concentrations of vitamin B_{12}. Results of the assays also revealed that normal liver contained by far the highest vitamin B_{12} concentration, the highest total vitamin B_{12} content, and the greatest retention of radioactivity of the organs studied. The liver contained 82.6 and 80.8%, respectively, of the total vitamin B_{12} and radioactivity recovered in the organs of the infant with neuroblastoma. The liver also contained 42% of the radioactive vitamin B_{12} retained by the infant. The distribution of this vitamin among the organs of this child differed considerably from that in most laboratory animals.

INTRODUCTION

Bodian (3, 4) reported a marked regression of tumors in a significant number of children with neuroblastoma to whom vitamin B_{12} was administered. His rationale for this mode of therapy was that this vitamin might be necessary for the maturation of neuroblastoma towards ganglioneuroma. Others, however, have subsequently questioned the effectiveness of vitamin B_{12} administration to children with neuroblastoma (18, 34).

In an attempt to assess the role of this vitamin in the metabolism of this tumor, we compared the retention of vitamin B_{12} by malignant and normal tissues of a child with neuroblastoma who was given radioactive vitamin B_{12} before he succumbed. In addition, it was possible to study the distribution of vitamin B_{12} and the accumulation of radioactivity among the various organs of this child.

[1] Supported by Grants AM-01778, NIAMD and HD-01162, NICHHD from the NIH, USPHS.

Received February 25, 1971; accepted June 30, 1971.

MATERIALS AND METHODS

A male infant had been treated at another institution when he was 3 months old for neuroblastoma by surgery and X-ray irradiation. At 7 months of age, when he entered this hospital, he had disseminated neuroblastoma, which subsequently did not respond to X-ray treatment. His serum vitamin B_{12} levels during the course of these studies were within normal limits, ranging from 300 to 400 pg/ml.

At 8 months of age, when he weighed 16.5 pounds, he was given 0.135 μg of vitamin B_{12} containing 0.0967 μCi of ^{60}Co in 0.13 ml of water by stomach tube. Distilled water, 60 ml, was then passed slowly through the tube to wash down the vitamin B_{12}. The hepatic area, stomach, and left lower quadrant of the infant were scanned for radioactivity daily with a probe-type scintillation counter containing a 1-inch, thallium-activated sodium iodide crystal.

Fourteen days after the 1st dose, when the counts over the liver had stabilized, 0.13 ml of the above solution (now containing 0.135 μg of vitamin B_{12} and 0.0963 μCi of ^{60}Co) was injected s.c. into the lateral aspect of the right leg below the gastrocnemius muscle. The daily scanning of the infant was continued.

After 7 days, the counts over the liver stabilized, and another s.c. injection of 0.13 ml of solution containing 0.135 μg of vitamin B_{12} and 0.0960 μCi of ^{60}Co was given in the same area.

The scanning was continued on a daily basis. The child succumbed to the disease 9 days after the last injection.

At autopsy, most of the organs and tissues (with the exception of brain, bone, skin, muscle, and blood) were carefully dissected out, cleaned, weighed, and then frozen and stored at −20° until prepared for assay. Tumors were carefully separated from the tissues in which they occurred.

Preparation of Tissue Homogenates and Radioactivity Counting. Aliquots of the various organs were individually homogenized with specific amounts of triple-distilled water in a Waring Blendor. At least 2 aliquots of each homogenate were then added to 57- x 27-mm screw top vials, which were previously tared and counted for radioactivity. The radioactivity of the homogenates was determined in a well-type scintillation counter containing a 3-inch, thallium-activated sodium iodide crystal with a 1-7/8-x 1-1/8-inch well. The preparation of standards, methods of calculation, corrections for physical decay of ^{60}Co, and other details have been previously described (6).

Microbiological Assay for Vitamin B_{12} Content of the Organs. The microbiological assay of Cooperman *et al.* (6) for vitamin B_{12} in tissues with the use of *Lactobacillus lactis*

Dorner (ATCC 8000) was used in these studies. Methods of tissue extraction, correction of non-vitamin B_{12} stimulators, and other steps in the assay were performed as previously described (6).

RESULTS

Uptake and Retention of ^{60}Co-labeled Vitamin B_{12} in an Infant with Neuroblastoma. The uptake and retention of radioactive vitamin B_{12} was calculated from the increments in counts over the hepatic area according to the method of Glass *et al.* (14). The results are shown in Table 1, from which it can be seen that 0.0608 μg and 0.0435 μCi, representing 45% of the p.o. dose, were absorbed and retained by the infant. When the 1st parenteral dose was given, calculations revealed that 0.112 μg and 0.0796 μCi of ^{60}Co-labeled vitamin B_{12} were retained. If the assumption is made that a similar percentage of the 2nd parenteral dose was retained, then a total of 0.285 μg and 0.203 μCi of ^{60}Co-labeled vitamin B_{12} was retained by the infant at the time of death. These values were used to determine the recovery of radioactivity in the various organs analyzed.

Tissue Distribution of Vitamin B_{12}. The total vitamin B_{12} content determined by microbiological assay and the distribution of radioactivity in the tissues of the infant with neuroblastoma are shown in Table 2. The values for the liver, pancreas, and adrenals include the radioactivity and vitamin B_{12} content of the tumors found in these tissues. Although the tumors accounted for 37, 80, and 47% of the total weight of the liver, pancreas, and adrenals, they contained only 16, 48, and 11% of the total B_{12} content of the respective tissues.

Table 1

Uptake and retention of ^{60}Co-labeled vitamin B_{12} by an infant with neuroblastoma

Calculations were made from peak external-hepatic-area radioactivity measurements after p.o. and s.c. doses of ^{60}Co-labeled vitamin B_{12}.

Dose of B_{12}-^{60}Co		Route of administration	EHAR[a] (cpm)	Calculated B_{12}-^{60}Co retained		% dose retained
μg	μCi			μg	μCi	
0.135	0.0967	p.o.	300	0.0608	0.0435	45
0.135	0.0963	s.c.	960	0.112	0.0796	83
0.135	0.0960	s.c.	1740			

[a] EHAR, external-hepatic-area radioactivity.

Table 2

Total vitamin B_{12} and total radioactivity in the major visceral organs of an infant with neuroblastoma given ^{60}Co-labeled vitamin B_{12}

Vitamin B_{12} was determined in aliquots of tissues homogenized in extracting liquid and autoclaved, and the supernatants were assayed by *L. lactis* microbiological assay. Radioactivity was measured in aliquots homogenized with distilled water and counted in a well-type scintillation counter.

Organ	Total vitamin B_{12} (μg/organ)	Total radioactivity (μμCi/organ)	% of dose retained
Liver	45.53	85,107	42.0
Kidneys	3.12	6,281	3.1
Gastrointestinal tract			
Stomach	0.408	515	
Duodenum	0.458	1,118	
Jejunum and upper ileum	0.196	511	
Ileum, terminal	0.382	497	
Colon	0.331	919	
Total	1.78	3,560	1.8
Lungs	1.46	5,064	2.5
Heart	1.19	2,566	1.3
Pancreas	0.518	864	0.4
Spleen	0.458	1,274	0.6
Adrenals	0.399	763	0.4
Bladder	0.073	87	0.04
Diaphragm	0.057	91	0.04
Testes	0.039	24	0.01
Superior mediastinal tumor	0.441	1,187	0.6
Inferior mediastinal tumor	0.06	114	0.06
Total	55.12	105,309	52.9

378

The liver had the highest vitamin B_{12} content, containing a total of 45.53 μg. A total of 0.085 μCi of ^{60}Co-labeled B_{12} was recovered in the liver, which represented 42% of the ^{60}Co-labeled B_{12} retained by the infant. Another 9.7% of the retained ^{60}Co-labeled vitamin B_{12} was found in the other tissues and tumors that were assayed. The other 48% of the retained radioactivity was probably distributed among the muscles, skin, bone and brain, bone marrow, and blood, samples of which were not obtainable at autopsy.

The kidneys, with 3.12 μg of vitamin B_{12}, had the next highest total content. This amount was about one-fifteenth of that in the liver. The other tissues in Table 2 had lesser amounts of vitamin B_{12}.

Relative Distribution of Radioactive and Nonradioactive Vitamin B_{12}. Eight organs (liver, kidneys, gastrointestinal tract, lungs, heart, pancreas, spleen, and adrenals) accounted for 98.8% of the total vitamin B_{12} found in the tissues assayed. These are listed in Table 3, from which it can be seen that these 8 organs also contained 98.5% of the total radioactivity recovered in the infant. The liver had by far the highest percentages of vitamin B_{12} and of radioactivity, 82.6 and 80.8%, respectively. The distribution of radioactivity paralleled that of vitamin B_{12} content in each organ, with the possible exception of the lungs, which contained 4.8% of the recovered radioactivity and only 2.6% of the total vitamin B_{12} content. However, the concentrations of vitamin B_{12} and of radioactivity were so low in the samples of this organ (Table 4) that the values were near the lower limits of sensitivity of the assay methods. The differences between the percentages of recovered radioactivity and vitamin B_{12} content of the lungs are probably not significant.

Table 3

Percentage of the total radioactivity and the total vitamin B_{12} recovered in each of 8 visceral organs of an infant with neuroblastoma

Organ	% total radioactivity	% total vitamin B_{12}
Liver	80.8	82.6
Kidneys	6.0	5.7
Lungs	4.8	2.6
Gastrointestinal tract	3.4	3.2
Heart	2.4	2.2
Spleen	1.2	0.83
Pancreas	0.75	0.94
Adrenals	0.64	0.72
Total	98.5	98.8

Concentration of Vitamin B_{12} and Radioactivity in Normal and Malignant Tissues. The concentrations of vitamin B_{12} (μg/g, wet weight) and of radioactivity (μμCi/g of wet weight) for the tissues and tumors of the infant with neuroblastoma are shown in Table 4. Normal liver tissue contained the highest concentrations of both vitamin B_{12} and radioactivity. Next in order of decreasing concentration were the normal adrenals, with one-half of the concentration of vitamin B_{12} of the liver, followed by the normal pancreas tissue, kidney, and heart.

The tumors separated from the liver, pancreas, and adrenals had a considerably lower concentration of vitamin B_{12} and accumulation of radioactive vitamin B_{12} than the normal tissues with which they were associated. For example, normal liver tissue contained 0.162 μg of vitamin B_{12} and 371.5 μμCi/g, compared to 0.031 μg of vitamin B_{12} and 53 μμCi/g for the liver tumor. Similarly, normal adrenal tissue contained between 7 and 8 times the radioactivity and vitamin B_{12} concentration of adrenal tumor, and the normal pancreas tissue contained 4 to 5 times the radioactivity and vitamin B_{12} concentration of the pancreas tumor.

Table 4

Concentration of vitamin B_{12} and radioactivity in normal and malignant tissues of an infant with neuroblastoma

Tissues	Concentration (μg/g, wet weight)	Radioactivity (μμCi/g, wet weight)
Normal tissues		
Liver	0.162	372
Adrenals	0.081	152
Pancreas	0.055	80.7
Kidneys	0.048	99.9
Heart	0.030	64.7
Ileum, terminal	0.024	31.4
Spleen	0.017	47.3
Stomach	0.015	28.9
Colon	0.012	33.4
Testes	0.012	23.8
Duodenum	0.011	26.8
Diaphragm	0.010	12.1
Bladder	0.009	10.8
Jejunum and upper ileum	0.009	23.5
Lungs	0.008	27.8
Bile	0.005	10.4
Malignant tissues		
Liver tumor	0.031	53.3
Pancreatic tumor	0.012	22.7
Adrenal tumor	0.010	22.3
Superior mediastinal tumor	0.006	11.7
Inferior mediastinal tumor	0.006	11.4

Two large, solid masses which were dissected out and assayed, superior mediastinal and inferior mediastinal tumors, were uniformly low in vitamin B_{12} and radioactivity, containing 0.006 μg of vitamin B_{12} per g and 11.7 and 11.4 μμCi/g.

It was not possible to obtain the pituitary of this child. This gland was of interest, since we have previously shown that in animals it is the tissue with the highest concentration of vitamin B_{12} (6). However, both the pituitary and the liver of a 3-year-old girl who died of infection secondary to leukemia were obtained at autopsy. The liver appeared normal under gross examination. The vitamin B_{12} concentration of the pituitary was 0.195 μg/g, compared to 0.143 μg/g for the liver. Because of its considerably larger size, the liver had a larger total content of this vitamin.

DISCUSSION

Vitamin B_{12} in Tumors. Bodian (3, 4) reported a marked regression of tumors in many children with neuroblastoma treated with massive doses of vitamin B_{12}. His reason for this treatment was that, since vitamin B_{12} was necessary for the maturation of hematopoietic cells, it might enhance the maturation of neuroblastic tissue towards ganglioneuroma. However, in those cases where vitamin B_{12} was effective, a regression of the tumor rather than a maturation of immature cells was found.

In further studies with mice and rats, Rigby and Bodian (29) demonstrated that the C1300 neuroblastoma avidly took up and retained radioactivity in mice given injections of radioactive vitamin B_{12}. In rats, the accumulation of radioactivity by the PWAZ fibrosarcoma was considerable. They interpreted these results as supporting their concept that vitamin B_{12} had an important role in the metabolism of the tumors.

Further evidence that tumors take up and accumulate relatively large amounts of vitamin B_{12} was presented by Miller *et al.* (23), who showed that hamster methylcholanthrene-induced sarcoma and rat Walker carcinoma concentrated considerable radioactivity when the animals were given ^{60}Co-labeled vitamin B_{12}. In addition, Flodh and Ullberg (11) injected radioactive vitamin B_{12} into mice in which 4 different tumors were induced and reported that all the tumors accumulated radioactivity at a rate equal to that of kidney and liver. They correlated the accumulation of radioactive vitamin B_{12} with nucleic acid synthesis.

In the study reported here, the neuroblastoma tissue of the infant had both a low concentration of vitamin B_{12} and a low accumulation of radioactivity (0.006 μg/g and 11 μμCi/g, compared to 0.162 μg/g and 372 μμCi/g in normal liver tissue). The tumor associated with the liver had about 20% of the vitamin B_{12} concentration of normal liver. Similarly, the tumors of the pancreas and adrenals had considerably lower vitamin B_{12} concentrations than the adjacent normal tissues.

These results indicate that the neuroblastoma tissue can proliferate and presumably synthesize nucleic acids in the presence of low concentrations of vitamin B_{12}. These results, which differed from those in the animal studies, were somewhat unexpected in view of the role of vitamin B_{12} in nucleic acid metabolism (21).

However, whether vitamin B_{12} is necessary for tumor metabolism and whether uptake and retention of this vitamin parallels this function has not been elucidated.

Vitamin B_{12} is essential for the growth of implants of Rous sarcoma in chicks (27) and enhances the carcinogenicity of some azo dyes in laboratory animals (9, 12, 25).

In the human, Hsu *et al.* (16) found that liver tissue containing tumor cells had a higher concentration of vitamin B_{12} than normal liver tissue. Meyer *et al.* (22) noted that organs with tumors obtained at autopsy from patients with cancers who had been given radioactive vitamin B_{12} before death occasionally contained extremely high radioactivity. It was also shown in *in vitro* studies that HeLa cells are capable of taking up large amounts of vitamin B_{12} (5).

On the other hand, Stein *et al.* (36) demonstrated that when hepatoma-bearing rats were given ^{60}Co-labeled vitamin B_{12} parenterally the retention of radioactivity by the tumor cells was considerably lower than that by normal liver tissue. Stein (37) then reported that when patients with liver metastases were given radioactive vitamin B_{12} the neoplastic tissue had considerably less radioactivity than normal liver tissue. He proposed a test for the detection of liver tumors based upon the scanning of livers of patients after administering radioactive vitamin B_{12} to them. If areas of the liver were found containing little radioactivity, this would be indicative of tumor tissue. Others have also found that carcinomas contained less vitamin B_{12} than normal tissue (2, 26, 33).

It is apparent that, in some cases at least, the metabolic role of vitamin B_{12} in malignant cells differs from that in proliferating normal cells. Of considerable interest would be the elaboration of the mechanism whereby some of these neoplastic cells proliferate and presumably make nucleic acid in the absence of appreciable levels of vitamin B_{12}. Such information may have important therapeutic implications.

Organ Distribution of Vitamin B_{12}. We have determined the concentration (μg of vitamin B_{12} per g, wet tissue) and whole organ content of both radioactive and nonradioactive vitamin B_{12}. In 2 recent reports on the distribution of vitamin B_{12} among human tissues, the author has discussed previous investigations by others on this subject (28, 35). With few exceptions, only the concentration of vitamin B_{12} was given in these studies, since specimens were obtained either by needle biopsy or from sections of tissues at autopsy. However, whole organ content is important for studies involving storage, uptake, and transport of vitamin B_{12}.

It is readily apparent that the liver is the main repository of vitamin B_{12} in the human. This is shown by the fact that 80.8% of the total radioactivity and 82.6% of the total vitamin B_{12} recovered in the child with neuroblastoma was found in the liver. In addition, 42% of the radioactive vitamin B_{12} retained by the infant was recovered in the liver.

The total of 45.53 μg of vitamin B_{12} found in the liver of this infant is slightly lower than that reported by Ross and Mollin (33) for infants under 1 year of age. However, these investigators found that the relative distribution of vitamin B_{12} among the organs was similar regardless of the absolute vitamin B_{12} content of the liver. It is therefore probable that the relative distribution found in the infant with neuroblastoma reflects that in normal infants.

In the infant with neuroblastoma, normal liver tissue had

the highest concentration of vitamin B_{12} of the organs assayed. However, it was not possible to obtain the pituitary, which we have previously shown to contain high concentrations of vitamin B_{12} in several species of animals (6). However, the pituitary was obtained from a 3-year-old child who died of leukemia, and it contained a higher vitamin B_{12} concentration than the liver (0.195 μg/g compared to 0.143 μg/g). This high concentration of vitamin B_{12} in the pituitary is of considerable interest. However, no role has yet been found for this vitamin in this gland.

The human liver contains both the highest concentration and the highest total vitamin B_{12} content of the visceral organs. In contrast, in the chicken (7, 8), bovine (20), dog (6), hamster (24), mouse (24), and rat (1, 7, 10, 15, 19), the kidney has a higher concentration of vitamin B_{12} than the liver. In the pig (17), the kidney and liver have about the same concentration of this vitamin. The kidney of the rabbit (30, 31) has a somewhat higher vitamin B_{12} concentration than the liver. In all the above species, with the exception of the rat, the liver had the greater total vitamin B_{12} content primarily because of its larger size. In the guinea pig (17), the liver contained by far the highest concentration and total vitamin B_{12} content and, in this respect, was similar to man.

Thus, in addition to different modes of absorption (13) and different biological turnover rates of vitamin B_{12} (32), several species of animals differ from man in the distribution of vitamin B_{12} among the organs. It is therefore apparent that results of studies with vitamin B_{12} in animals may not be applicable to man.

From the parallel distribution of radioactivity and total vitamin B_{12} content among the organs of the infant with neuroblastoma in this study, it was shown that the radioactivity does not dissociate from the vitamin B_{12} molecule. Radioactive vitamin B_{12} is therefore a valid tracer for this vitamin in studies with humans.

REFERENCES

1. Barbee, K. W., and Johnson B. C. Metabolism of Radioactive Vitamin B_{12} by the Rat. Proc. Soc. Exptl. Biol. Med., *76:* 720–721, 1951.
2. Blum, K.-U., and Heinrich, H. C. Neoplasma und Organ-Vitamin B_{12}-Gehalt des Menschen. Vitamine Hormone, *7:* 486–496, 1957.
3. Bodian, M. Neuroblastoma. Pediat. Clin. N. Am., *6:* 449–472, 1959.
4. Bodian, M. Neuroblastoma. An Evaluation of Its Natural History and the Effects of Therapy with Particular Reference to Treatment by Massive Doses of Vitamin B_{12}. Arch. Disease Childhood, *38:* 6[illegible]–619, 1963.
5. Cooper, B. A., and Paranchych, W. Selective Uptake of Specifically Bound Cobalt-58 Vitamin B_{12} by Human and Mouse Tumour Cells. Nature, *191:* 393–395, 1961.
6. Cooperman, J. M., Luhby, A. L., Teller, D. N., and Marley, J. F. Distribution of Radioactive and Nonradioactive Vitamin B_{12} in the Dog. J. Biol. Chem., *235:* 191–194, 1960.
7. Cooperman, J. M., Tabenkin, B., and Drucker, R. Growth Response and Vitamin B_{12} Tissue Levels in Vitamin B_{12} Deficient Rats and Chicks Fed Riboflavin, 5,6-Dimethylbenzimidazole and Related Compounds J. Nutr., *46:* 467–478, 1952.
8. Couch, J. R., and Orlando, O. The Vitamin B_{12} Content of Chick Tissues as Influenced by Diet. J. Nutr., *42:* 337–345, 1950.
9. Day, P. L., Payne, L. D., and Dinning, J. S. Procarcinogenic Effect of Vitamin B_{12} on *p*-Dimethylaminoazo-benzine-fed Rats. Proc. Soc. Exptl. Biol. Med., *74:* 854–857, 1950.
10. Dryden, L. P., and Hartman, A. M. Relative Concentration of Vitamin B_{12} in the Organs of the Male Rat as Affected by Its Intake of the Vitamin. J. Nutr., *90:* 382–386, 1966.
11. Flodh, H., and Ullberg, S. Accumulation of Labelled Vitamin B_{12} in Some Transplanted Tumours. Intern. J. Cancer, *3:* 694–699, 1968.
12. Georgadze, G. E. The Influence of Vitamins B_1 and B_{12} on the Induction of Malignant Tumours in Hamsters. Probl. Oncol. USSR English Transl., *6:* 1459–1464, 1960.
13. Glass, G. B. J. Gastric Intrinsic Factor and Its Function in the Metabolism of Vitamin B_{12}. Physiol. Rev., *43:* 529–849, 1963.
14. Glass, G. B. J., Boyd, L. J., Gellin, G. A., and Stephanson, L. Uptake of Radioactive Vitamin B_{12} by the Liver in Humans. Test for Measurement of Intestinal Absorption of Vitamin B_{12} and Intrinsic Factor. Arch. Biochem., *51:* 251–257, 1954.
15. Harte, R. A., Chow, B. F., and Barrows, L. Storage and Elimination of Vitamin B_{12} in the Rat. J. Nutr., *49:* 669–678, 1953.
16. Hsu, J. M., Miner, P., Mitchell, J. A., and Chow, B. F. Vitamin B_{12} Content in Normal and Pathological Human Tissues. *In:* Abstracts of the 136th Meeting, American Chemical Society, Atlantic City, N. J., September 13–18, 1959, pp. 75C–76C, 1959.
17. Kline, E. A., Kastelic, J., and Ashton, G. C. The Effect of Vitamin B_{12}, Cobalt and Antibiotic Feeding on the Composition of Pork Tissue of 100-pound Pigs. J. Nutr., *56:* 321–334, 1955.
18. Langman, M. J. S. Treatment of Neuroblastoma with Vitamin B_{12}. A Report to the Working Party of the Medical Research Council. Arch. Disease Childhood, *45:* 385–387, 1970.
19. Lewis, U. J., Register, U. D., and Elvehjem, C. A. The Vitamin B_{12} Content of Various Organs and Tissues of the Rat. Proc. Soc. Exptl. Biol. Med., *71:* 509–511, 1949.
20. Lewis, U. J., Register, U. D., Thompson, H. T., and Elvehjem, C. A. Distribution of Vitamin B_{12} in Natural Materials. Proc. Soc. Exptl. Biol. Med., *72:* 479–482, 1949.
21. Luhby, A. L., and Cooperman, J. M. Aminoimidazole Excretion in Vitamin B_{12} and Folic Acid Deficiencies. Lancet, *2:* 1381–1382, 1962.
22. Meyer, L. M., Driscoll, D. H., and Bertcher, R. W. Organ and Tissue Distribution of Co^{60} Vitamin B_{12} (Abstract). Ninth Congress of the International Society of Hematology, Mexico City, August 1962.
23. Miller, A., Gaull, G., Lemon, H. M., and Ross, J. F. Studies on the Uptake of Vitamin B_{12}-Co^{60} by the Hamster Methylcholanthrene-induced Sarcoma and the Rat Walker Carcinosarcoma. Cancer Res., *16:* 842–847, 1956.
24. Miller, A., Gaull, G., Ross, J. F., and Lemon, H. M. The Distribution of Parenteral Co^{60} Vitamin B_{12} in Mouse, Hamster, Rat and Guinea Pig. Proc. Soc. Exptl. Biol. Med., *93:* 33–35, 1956.
25. Miller, E. C., Plescia, A. M., Miller, J. A., and Heidelberger, C. The Metabolism of Methylated Aminoazo Dyes. I. The Demethylation of 3'-Methy-4-dimethyl-C^{14}-aminoazobenzene *in Vivo.* J. Biol. Chem., *196:* 863–873, 1952.
26. Nelson, R. S., and Doctor, V. M. Vitamin B_{12} Content of Liver and Serum in Malignant Neoplasia. Gastroenterology, *42:* 414–418, 1962.
27. Oleson, J. J., and Little, P. A. Effect of Pteroylglutamic Acid and Vitamin B_{12} on Growth of Rous Tumor Implants. Proc. Soc. Exptl. Biol. Med., *71:* 226–227, 1949.
28. Rapazzo, M. B., Salmi, H. A., and Hall, C. A. The Content of Vitamin B_{12} in Adult and Foetal Tissue. A Comparative Study. Brit. J. Haematol., *18:* 425–433, 1970.
29. Rigby, C. C., and Bodian, M. Experimental Study of the Relationship between Vitamin B_{12} and Two Animal Tumour Systems. Brit. J. Cancer, *17:* 90–99, 1963.
30. Rosenthal, H. L. The Uptake and Turnover of Radioactive Vitamin B_{12} in Rabbit Tissues. J. Nutr., *68:* 297–311, 1959.
31. Rosenthal, H. L., and Cravitz, L. Organ, Urine and Feces Vitamin

B_{12} Content of Normal and Starved Rabbits. J. Nutr., *64:* 281–290, 1958.

32. Rosenthal, W. S., and Glass, G. B. J. Vitamin B_{12} and the Liver. *In:* H. Popper and F. Schaffner (eds.), Progress in Liver Disease, Vol. 3, pp. 118–146. New York: Grune and Stratton, Inc., 1970.
33. Ross, C. I. M., and Mollin, D. L. Vitamin B_{12} in Tissues in Pernicious Anaemia and Other Conditions. Vitamin B_{12} and Intrinsic Factor. *In:* Europaisches Symposion Hamburg 1956, Vol. 1, pp. 437–443. Stuttgart: Ferdinand Enke, 1957.
34. Sawitsky, A., and Deposito, F. A Survey of American Experience with Vitamin B_{12} Therapy of Neuroblastoma. J. Pediat., *67:* 99–103, 1965.
35. Stahlberg, K. G., Radner, S., and Norden, A. Liver B_{12} in Subjects with and without Vitamin B_{12} Deficiency. A Quantitative and Qualitative Study. Scand J. Haematol., *4:* 312–320, 1967.
36. Stein, O., Stein, Y., Aronovitch, J., Grossowicz, N., and Rachmilewitz, M. Isotopic and Microbiological Studies of Vitamin B_{12} Distribution in Normal and Hepatoma-bearing Rats. Cancer Res., *18:* 849–852, 1958.
37. Stein, Y. Detection of Space Occupying Lesions of the Liver by Directional Counting of Co^{60}-Labelled B_{12}. J. Lab. Clin. Med., *56* 290–293, 1960.

J. clin. Path., 1971, 24, 449-455

Cobalamins in human pregnancy and lactation

I. L. CRAFT, D. M. MATTHEWS, AND J. C. LINNELL

From the Department of Obstetrics and Gynaecology, Westminster Hospital, and the Department of Experimental Chemical Pathology, Westminster Medical School, London

SYNOPSIS Samples of maternal and foetal blood and of human milk, cow's milk, and dried cow's milk have been analysed for cobalamins by chromatography and bioautography. Plasma cobalamins in late pregnancy and after delivery differed quantitatively from those in healthy non-pregnant women. Differences in the proportions of plasma cobalamins were also observed between mothers and their newborn infants. The findings suggest that methylcobalamin may be a more labile plasma form of the vitamin than 5-deoxyadenosyl cobalamin. Breast milk from healthy mothers contained more deoxyadenosylcobalamin and proportionately less methylcobalamin than their plasma. Cyanocobalamin given parenterally to lactating women was detected in the plasma, and high concentrations appeared unchanged in the milk. The B_{12} in fresh cow's milk was found to be largely 5-deoxyadenosyl cobalamin whereas dried milk contained only hydroxocobalamin and a little cyanocobalamin. The nutritional implications of the findings, and their bearing on problems of cobalamin transport, are discussed.

Relatively little is known about the role of vitamin B_{12} in the developing foetus and the mechanisms by which it acquires this vitamin from the mother. Early animal studies (O'Dell, Whitley, and Hogan, 1951; Grainger, O'Dell, and Hogan, 1954) showed that B_{12} deficiency in the mother is frequently associated with hydrocephalus and other congenital abnormalities in the offspring. In man, it has been observed that patients with pernicious anaemia may be infertile (Adams, 1958; Sharp and Witts, 1962; Jackson, Doig, and McDonald, 1967), and even when conception occurs, a normal pregnancy rarely ensues without some form of B_{12} supplementation.

Vitamin B_{12} is required increasingly by the foetus as development proceeds. Cobalamins are supplied via the placenta from the maternal circulation (Boger, Wright, Beck, and Bayne, 1956; Ball and Giles, 1964), whose total of B_{12} may temporarily fall to a level well below that found in healthy non-pregnant women. The newborn infant, whether breast or bottle-fed, obtains its B_{12} from the milk. Many workers have estimated total B_{12} in milk and in maternal and foetal blood (Karlin and Dumont, 1955; Baker, Ziffer, Pasha, and Zobotka, 1958; Baker, Jacob, Rajan, and Swaminathan, 1962). Nothing is known, however, about the individual cobalamins making up the total B_{12} of foetal blood or milk, and knowledge concerning the mechanisms of transport of vitamin B_{12} into milk is scanty.

The development of a sensitive bioautographic method for the separate estimation of cobalamins (Linnell, Mackenzie, and Matthews, 1969a; Linnell, Mackenzie, Wilson, and Matthews, 1969b) has enabled us to carry out the present study of the plasma cobalamins in healthy non-pregnant women, and in women near term, following delivery and in the puerperium. In the last group, cobalamins were estimated in breast milk before and after systemic B_{12} administration. In addition, cobalamins were estimated in samples of liquor amnii and in the plasma of newborn infants.

Material and Methods

Heparinized blood (10 ml) was taken from 12 healthy non-pregnant women of childbearing age for the estimation of total B_{12} and plasma cobalamins. In four patients approaching term samples of liquor amnii were collected and venous blood was taken at the same time. In a further four subjects peripheral and cord blood was sampled at delivery. Breast milk (10 ml) was collected from 10 mothers four to eight days after delivery when lactation was well established and venous blood was drawn simultaneously. In order to study the transfer of cobalamins into breast milk, two volunteers were given intramuscular

Received for publication 10 December 1970.

cyanocobalamin, 100 μg and 1 mg respectively, and samples of blood and milk taken eight hours later. For comparison, cobalamins were estimated in several commercial milk preparations and in two specimens of fresh cow's milk collected by dim light.

All blood samples were collected using syringes and heparin tubes covered with aluminium foil in order to protect the B_{12} coenzymes from photolytic conversion to hydroxocobalamin. Plasma was separated within one hour of collection in a dark-room illuminated only by red light. Samples of amniotic fluid were handled similarly. Breast milk was expressed manually in a dark room and all samples were stored in foil-covered containers at −20°.

Total B_{12} in plasma and liquor amnii was determined by radioisotopic assay (Matthews, Gunasegaram, and Linnell, 1967). The Euglena method (Hutner, Bach, and Ross, 1956) was used for the assay of total B_{12} in milk since it was found that the high fat content interfered with binding in the radioisotopic method. Individual cobalamins were estimated by the chromato-bioautographic method previously described (Linnell *et al*, 1969a,b). Deoxyadenosylcobalamin was not routinely separable from hydroxocobalamin in this study and is referred to as Co-OH B_{12}. However, a method for separating deoxyadenosylcobalamin from hydroxocobalamin has subsequently been developed (Linnell, Hussein, and Matthews, 1970) and has permitted the separate estimation of these two cobalamins in one or two cases.

Results

Total plasma B_{12} and plasma cobalamins in 12 healthy non-pregnant women are shown in Table I; the values fall within the normal range published previously (Linnell *et al*, 1969a). The separate estimation of hydroxocobalamin was undertaken in two subjects and the results indicated that approximately 10% of the plasma total B_{12} was present in this form.

Plasma cobalamins in eight subjects at or approaching delivery are shown in Table II. The

Subject	*Age*	*Plasma Cobalamins (pg/ml)*[1]				*Ratio*
		Total B_{12}	*MeB_{12}*	*CNB_{12}*	*Co-OHB_{12}*	*MeB_{12}/Co-OHB_{12}*
HM	21	380	250	0	130	1·9
GB	25	450	340	0	110	3·1
AO	26	375	280	0	170	1·6
DM	27	375	250	40	85	2·9
JB	28	265	175	0	90	1·9
EP	26	490	280	0	210	1·3
AW	21	250	160	15	75	2·1
MM	40	320	215	0	105	2·0
DS	31	440	330	0	110	3·0
JG	19	460	305	50	105	2·9
MY	16	550	435	0	115	3·8
JJ	29	600	300	75	225	1·3
Mean (± SE) n = 12	25·8	413 (± 76)	277 (± 22)	15 (± 7)	128 (± 14)	2·3 (± 0·2)

Table I *Plasma cobalamins in normal female subjects of childbearing age*

[1]MeB_{12} = methylcobalamin, CNB_{12} = cyanocobalamin, Co-OHB_{12} = deoxyadenosylcobalamin + hydroxocobalamin

Subject	*Age*	*Plasma Cobalamins (pg/ml)*				*Ratio*
		Total B_{12}	*MeB_{12}*	*CNB_{12}*	*Co-OHB_{12}*	*MeB_{12}/Co-OHB_{12}*
BK	23	220	147	0	73	2·0
CB	25	190	127	0	63	2·0
TG	25	300	225	0	75	3·0
SM	24	210	120	0	90	1·3
DH	30	600	346	39	215	1·6
EA	36	330	195	13	122	1·6
RS	30	355	230	0	125	1·8
GC	18	250	123	17	110	1·1
Mean (± SE) (n = 8)	26·4	307 (± 47)	189 (± 28)	8·6 (± 5·0)	109 (± 17)	1·8 (± 0·1)

Table II *Plasma cobalamins at or approaching delivery*

plasma total B_{12} (mean 307 pg/ml) was less than that in controls (mean 413 pg/ml) but the difference was not significant. However, the methylcobalamin levels (mean 189 pg/ml) were considerably lower than the control values (mean 277 pg/ml), the difference being statistically significant.

Results in the group of lactating mothers are seen in Table III, and a chromato-bioautogram from the milk and plasma of one subject is shown in Figure 1. The plasma total B_{12} (mean 490 ± 56 pg/ml) was not significantly different from that of the controls although the Me/Co-OH ratio was slightly raised (this difference was not quite significant at the 5% level). A comparison of both total B_{12} and Me/Co-OH ratio in the full-term and lactating women revealed highly significant differences between the groups. Total B_{12} in the milk from seven of the 10 mothers was higher than the plasma level but as a group (mean 605 ± 185 pg/ml) this increase was not significant since individual values varied widely. The proportion of Co-OH B_{12} was markedly higher in milk than plasma and a statistical comparison of the Me/Co-OH ratios showed the difference to be highly significant ($P < 0.001$). Separation of cobalamins in three milk samples using the second solvent system indicated that the proportion of hydroxocobalamin was less than 5% of the total milk B_{12}.

Information concerning the transfer of cobalamins into breast milk was obtained following the parenteral administration of cyanocobalamin. Figure 2 shows that the rise in cyanocobalamin in the milk was greater than that in the plasma at either dosage and accounted for much of the increase in total B_{12}. Following 1 mg cyanocobalamin intramuscularly the plasma total B_{12} rose to four times the pre-dosage level but the milk B_{12} level went up more than 10 times. Similar results were obtained after the dose of 100 μg. While it is uncertain to what extent concentration changes in the milk parallel those in blood, the experiment nonetheless shows that parenterally administered cyanocobalamin is transferred into the milk on a substantial scale.

Plasma cobalamins in the newly delivered infant

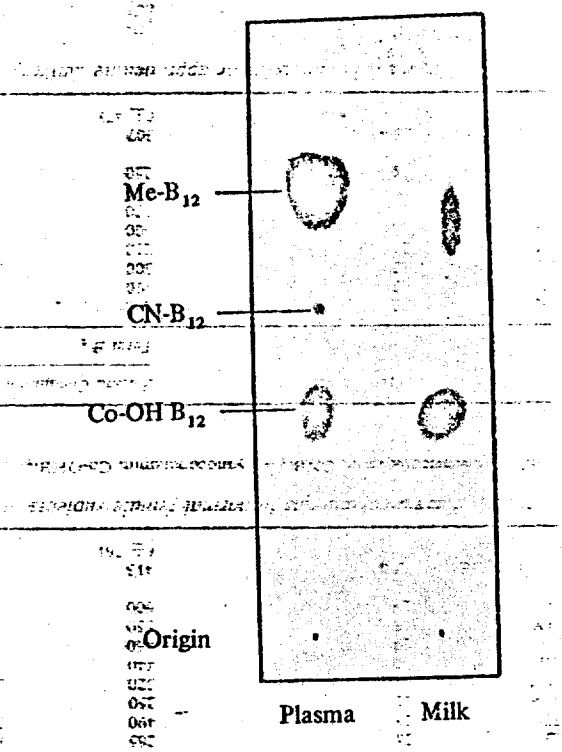


Fig. 1 *Chromato-bioautogram of plasma and milk cobalamins in a normal mother. A trace of cyanocobalamin may be seen in the plasma, although none was detected in the milk.*

Subject	Age	Days after Delivery	Plasma Cobalamins (pg/ml) Total B_{12}	MeB_{12}	CNB_{12}	Co-OHB_{12}	Ratio MeB_{12}/Co-OHB_{12}	Milk Cobalamins (pg/ml) Total B_{12}	MeB_{12}	CNB_{12}	Co-OHB_{12}	Ratio MeB_{12}/Co-OHB_{12}
YJ	23	6	905	638	54	213	3·2	1,480	845	0	635	1·4
AA	27	5	405	286	24	95	3·1	530	350	0	180	1·9
ES	18	4	530	398	0	132	3·4	20	8	0	12	0·7
EB	36	4	555	417	0	138	3·2	780	445	0	335	1·3
JC	27	6	365	313	0	52	6·0	320	183	0	137	1·3
PE	24	7	335	250	0	85	3·0	420	240	0	180	1·3
PA	25	4	—	—	—	—	—	1,860	990	125	745	1·3
NC	28		330	283	0	47	6·0	500	375	0	125	3·0
JA	25	4	475	407	0	68	6·0	80	46	0	34	1·4
MF	18	8	510	380	0	130	2·9	60	35	0	25	1·4
Mean (±SE) n = 10	25·1	5·2	490 (±59)	375 (±39)	8·7 (±6·3)	107 (±18)	4·1 (±0·4)	605 (±185)	352 (±106)	12·5 (±12·5)	241 (±81)	1·5 (±0·2)

Table III *Plasma and milk cobalamins during lactation*

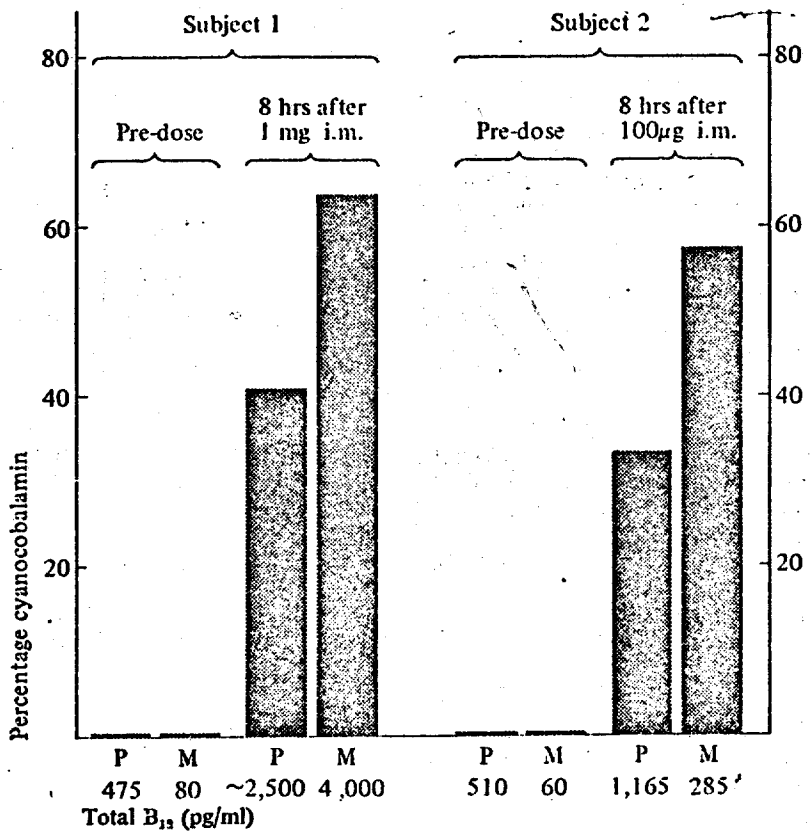


Fig. 2

Fig. 2 *Appearance of cyanocobalamin in breast milk following parenteral administration. P = plasma; M = milk.*

Fig. 3 *Plasma cobalamins in maternal and foetal blood* ▨ = *Me-B₁₂* ■ = *CN-B₁₂* □ = *Co-OH B₁₂*

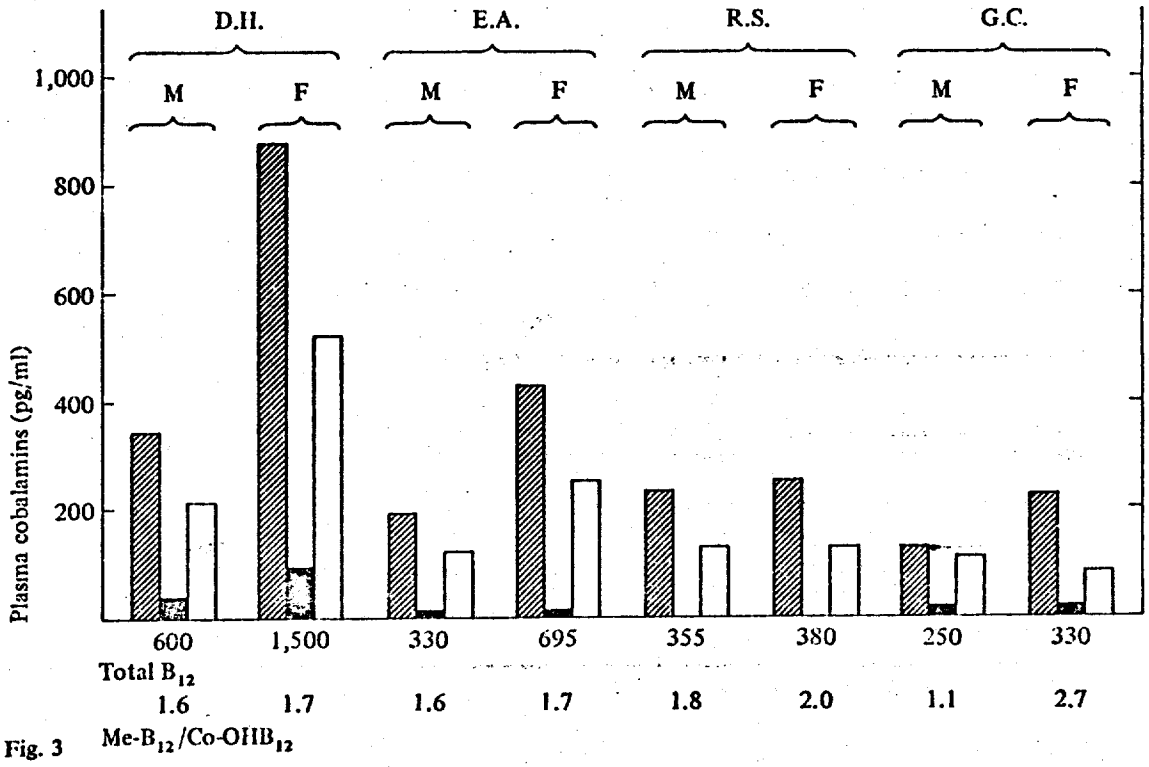


Fig. 3

are shown in Figure 3. Total B_{12} in cord blood was 50%-100% above the maternal level, and the proportion of methylcobalamin also was slightly but consistently higher. Small amounts of cyanocobalamin were detected in blood from three of the four mothers and their respective infants but levels were within the normal range. In three of the subjects samples from the umbilical artery were assayed alongside those from the umbilical vein, but no significant difference was observed in either total B_{12} or plasma cobalamins.

Results in the four samples of amniotic fluid revealed no consistent pattern. In two the total B_{12} (205, 145 pg/ml) was similar to the plasma level (210, 190 pg/ml). The third specimen had a high B_{12} concentration (1,160 pg/ml), five times that of the plasma, while in the fourth the total B_{12} was low (10 pg/ml) although the plasma level was quite normal (300 pg/ml). Chromatographically, a single spot corresponding to Co-OH B_{12} was detected in the two samples with the higher total B_{12} but in the two remaining specimens no cobalamins were detected even when loading of the chromatogram was increased. These results appear similar to the chromatographic findings in normal urine (unpublished) where the B_{12} is almost certainly present as hydroxocobalamin.

Parameter	*Groups Compared*[1]	*t*	*P*
Total B_{12} concentration	A vs B	0·43	> 0·5
	A vs C	1·31	0·2 > P < 0·3
	B vs C	4·17	< 0·001
	C (plasma) vs C (milk)	0·60	> 0·5
Me/Co-OH	A vs B	1·11	0·2 > P < 0·3
	A vs C	2·07	0·05 > P < 0·1
	B vs C	4·63	< 0·001
	C (plasma) vs C (milk)	5·50	< 0·001
Me-B_{12} concentration	A vs B	2·65	0·01 > P < 0·02

Table IV *Statistical comparison of groups using Student's t test*

[1]A = non-pregnant controls (n = 12), B = subjects at parturition (n = 8), C = subjects in puerperium (n = 10)

Total B_{12} concentrations in eight samples of branded milk food diluted as for a week-old infant gave the results in Table V. Two of the products were described as 'half-cream', the remainder as 'full cream'. All were derived from cow's milk. The fat content appeared to make little difference to the total B_{12} which in seven out of eight ranged from 2,000 to 4,000 pg/ml prepared feed. The remaining product was a tinned liquid whose total B_{12} was approximately one-sixth the mean value of the powdered milks. Chromatography was carried out on product no. 1; Co-OH B_{12} and a little cyanocobalamin were detected. Since the products were exposed to light during processing and again in preparing feeds it may be assumed that the major portion of the total B_{12} was present as hydroxocobalamin.

Product	*Cream*	*Total B_{12} Concentration*	
		Whole Product	*pg/ml Feed*[1]
Cow and Gate—half cream	Half	24·4 ng/g	2,900
National dried—half cream	Half	36·4 ng/g	4,000
S.M.A.	Full	16·8 ng/g	2,200
Ostermilk—One	Full	24·6 ng/g	3,300
Ostermilk—Two	Full	35·6 ng/g	3,500
Cow and Gate—full cream	Full	22·2 ng/g	2,400
National dried—full cream	Full	29·3 ng/g	3,000
Carnation (liquid)	Full	1·6 ng/ml	450

Table V *Total vitamin B_{12} content of infant milk foods*

[1]Feed prepared as for 1 week infant

Chromatography of two samples of fresh cow's milk revealed the presence of a single spot with the R_f of Co-OH B_{12}. Neither methylcobalamin nor cyanocobalamin was detected.

Discussion

Changes in total serum B_{12} during pregnancy and in the puerperium have been thoroughly studied by many workers (Karlin and Dumont, 1955; Boger *et al*, 1956b; Boger, Wright, and Bayne, 1957; Izak, Rachmilewitz, Stein, Berkovici, Sadovsky, Arnovitch, and Grossovicz, 1957; Baker *et al*, 1958; Baker *et al*, 1962; Low-Beer, McCarthy, Austad, Brzechwa-Ajdukiewicz, and Read, 1968; Hibberd and Spencer, 1970; Jathar, Kamath, Parikh, Rege, and Satoskar, 1970). The normal picture is of a slowly declining blood level, steepening somewhat towards term but rising again rapidly and spontaneously after delivery. Our results accord with this pattern and show the difference in total plasma B_{12} between full-term and lactating groups to be highly significant. Concurrent with this change is an alteration in the proportion of the plasma cobalamins making up the total B_{12}. Before term there is placental transfer of the vitamin to the foetus and, compared with the mother, there is a raised level of methylcobalamin in the foetal plasma. Following delivery lactation commences, and a mixture of cobalamins appears in the breast milk in which the proportion of methylcobalamin is lower than that in the mother's plasma. Together these observations may explain the postpartum rise in maternal plasma Me/Co-OH ratio. Previous evidence (Linnell *et al*,

1969b, Linnell, Hoffbrand, Peters, and Matthews, 1971) suggests that methylcobalamin may be a more labile plasma component than deoxyadenosylcobalamin, a contention which is supported by the present findings.

Rappazzo, Salmi, and Hall (1970) found the total B_{12} of various foetal tissues, including bone marrow, spleen, and thyroid, to be significantly higher than that of identical adult tissues. The foetus receives its B_{12} via the placenta but the reasons for the higher total B_{12} and the raised proportion of methylcobalamin in foetal plasma are not clear. Active transport processes or binding phenomena could be responsible. Flodh (1968) has suggested that B_{12} may be transferred to the foetus as methylcobalamin. Kumento, Lopez, Luhby, and Hall (1967) have described a plasma B_{12} binder peculiar to blood of the newborn, although it may be identical to a protein isolated from the serum in polycythaemia vera. Should the B_{12} binding proteins have, as seems possible, different affinities for the various cobalamins and their coenzymes, Kumento's binder may have especial significance in the maintenance of the raised methylcobalamin in the foetal plasma.

The newborn infant is dependent on the milk to meet its continued B_{12} requirement. The present results in breast milk sampled four to eight days after delivery show that the increase in Co-OH B_{12} largely accounts for the rise in total milk B_{12} above the plasma level. Whether this alteration is due to interconversion of cobalamins or to preferential binding of deoxyadenosylcobalamin by the milk is not known. Metz, Zalusky, and Herbert (1968) found that breast milk contained a potent binder for folate and that orally administered folic acid attained considerably higher levels in breast milk than in the serum, albeit after a delay of some hours. Studies on the B_{12}-binding capacity of milk from different species (Glass, 1963) indicate, however, that total B_{12} correlates poorly with binding capacity (eg, cow's milk, total B_{12} approximately 3,000 pg/ml, B_{12} BC 5,000 pg/ml; breast milk, total B_{12} approximately 600 pg/ml, B_{12} BC 80,000 pg/ml). The possibility that interconversion of cobalamins takes place during membrane transport is shown by the finding that cyanocobalamin is partly converted to deoxyadenosylcobalamin during intestinal absorption (Peters, Linnell, Matthews, and Hoffbrand, 1971). Some cyanocobalamin, however, escapes conversion, appearing as such in the peripheral blood (Linnell *et al*, 1971). The present experiments show that unchanged cyanocobalamin can also pass from blood to milk.

The mother's B_{12} intake is normally adequate both for herself and her child and the maternal plasma total B_{12} declines only after prolonged lactation (Shapiro, Alberts, Welch, and Metz, 1965). The total B_{12} concentration of the milk falls progressively, but this is compensated by the mother's increasing milk output. Karlin (1954) recorded mean values from two mothers which showed a decline of 400 pg B_{12} per millilitre milk in a period of eight months but the average daily milk output rose by over 400 g in the same time. The breast-fed baby receives a mixture of B_{12} coenzymes in which deoxyadenosylcobalamin may predominate. The child reared by bottle obtains its B_{12} largely as hydroxocobalamin, although generally at higher concentration. Hence an infant of 1 to 2 weeks taking some 800 ml milk a day would obtain perhaps 0·5 μg B_{12} if breast fed against a daily 2·5 μg from modified cow's milk. Were the total B_{12} intake considered alone it might appear that the breast-fed child was at a disadvantage by comparison with its bottle-fed contemporary. However, the significance of the high coenzyme levels in breast milk may be to furnish the infant with a form of the vitamin immediately usable by the actively growing tissues. It is known that the newborn are deficient in many enzyme systems, which develop only slowly over the first few weeks or months of life. Little is known of the B_{12}-dependent enzyme systems at this time but it may be assumed from the progress made by the majority of bottle-fed children that an ability to utilize hydroxocobalamin normally exists from the earliest age. Children suffering from a rare inborn error of B_{12} metabolism, however, may not be so capable of handling hydroxocobalamin or cyanocobalamin. Such a case was described recently (Mudd, Levy, and Abeles, 1969, Levy, Mudd, Schulman, Dreyfus, and Abeles, 1970) where a 4-week-old child presented with a combined homocystinuria/methylmalonic aciduria which was exacerbated by parenteral cyanocobalamin. The infant died at 7½ weeks. Postmortem studies revealed abnormally low levels of deoxyadenosylcobalamin in both liver and kidney coexisting with a deficiency of methyltetrahydrofolate methyltransferase. While it is unlikely that a child with such manifold biochemical lesions could survive, had breast-feeding been employed it may well be that the deficiency in cobalamin coenzymes might, at least in part, have been remedied.

This work was supported by grants from the Wellcome Trust and Glaxo Laboratories Limited. We are most grateful to Dr R. J. Wrighton for providing certain samples and much helpful information. Our sincere thanks are due to the volunteers who participated in this study, to Miss Heather Mackenzie and Mr H. A-A. Hussein for skilled technical assistance, and to Mr P. W. Pramod for so ably undertaking the *Euglena* B_{12} assay.

References

Adams, J. F. (1958). Pregnancy and Addisonian pernicious anaemia. *Scot. med. J.*, 3, 21-25.

Baker, H., Zifler, H., Pasher, I., and Sobotka, H. (1958). A comparison of maternal and foetal folic acid and vitamin B_{12} at parturition. *Brit. med. J.*, 1, 978-979.

Baker, S. J., Jacob, E., Rajan, K. T., and Swaminathan, S. R. (1962). Vitamin B_{12} deficiency in pregnancy and the puerperium. *Brit. med. J.*, 1, 1658-1661.

Ball, E. W., and Giles, C. (1964). Folic acid and vitamin B_{12} levels in pregnancy and their relation to megaloblastic anaemia. *J. clin. Path.*, 17, 165-174.

Boger, W. P., Wright, L. D., Beck, G. D., and Bayne, G. M. (1956). Vitamin B_{12}: correlation of serum concentrations and pregnancy. *Proc. Soc. exp. Biol. (N.Y.)*, 92, 140-143.

Boger, W. P., Wright, L. D., Bayne, G. M. (1957). Serum vitamin B_{12} concentrations of pregnant women and newborn infants. In *Vitamin B_{12} and Intrinsic Factor.* (1st European Symposium), edited by H. C. Heinrich, p. 443, Enke, Stuttgart.

Flodh, H. (1968). Distribution and kinetics of labelled vitamin B_{12}. *Acta radiol. (Stockh.)*, Suppl. 284.

Glass, G. B. J. (1963). Gastric intrinsic factor and its function in the metabolism of vitamin B_{12}. *Physiol. Rev.*, 43, 529-849 (see p. 566).

Grainger, R. B., O'Dell, B. L., and Hogan, A. G. (1954). Congenital malformations as related to deficiencies of riboflavin and vitamin B_{12}, source of protein, calcium to phosphorus ratio and skeletal phosphorus metabolism. *J. Nutr.*, 54, 33-48.

Hibbard, E. D., and Spencer, W. J. (1970). Low serum B_{12} levels and latent Addisonian anaemia in pregnancy. *J. Obstet. Gynaec. Brit. Cwlth*, 77, 52-57.

Hutner, S. H., Bach, M. K., and Ross, G. I. M. (1956). Sugar-containing basal medium for vitamin B_{12} assay with *Euglena:* application to body fluids. *J. Protozool.*, 3, 101-112.

Izak, G., Rachmilewitz, M., Stein, Y., Berkovici, B., Sadovsky, A., Arnovitch, Y., and Grossovicz, N. (1957). Vitamin B_{12} and iron deficiencies in anaemia of pregnancy and puerperium. *Arch. intern. Med.*, 99, 346-355.

Jackson, I. M. D., Doig, W. B., and McDonald, G. (1967). Pernicious anaemia as a cause of infertility. *Lancet*, 2, 1159-1160.

Jathar, V. S., Kamath, S. A. Parikh, M. N., Rege, D. V., and Satoskar, R. S. (1970). Maternal milk and serum vitamin B_{12}, folic acid and protein levels in Indian subjects. *Arch. Dis. Childh.*, 45, 236-241.

Karlin, R. (1954). La vitamine B_{12} dans le lait humain et bovin. *C. R. Soc. Biol. (Paris)*, 148, 371-373.

Karlin, R., and Dumont, M. (1955). Sur le taux de vitamine B_{12} dans le serum sanguin chez la femme au cours de l'accouchement, dans celui du cordon et dans le placenta. *C. R. Soc. Biol. (Paris)*, 149, 1986 1988.

Kumento, A., Lopez, R., Luhby, A. L., and Hall, C. A. (1967). B_{12} binders in human cord serum. (Abstr.) *Clin. Res.*, 15, 283.

Levy, H. L., Mudd, S. H., Schulman, J. 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.

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.* 40, 1-16.

Linnell, J. C., Hussein, H. A-A. and Matthews, D. M. (1970). A two-dimensional chromato-bioautographic method for complete separation of individual plasma cobalamins. *J. clin. Path.*, 23,820-821.

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

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

Low-Beer, T. S., McCarthy, C. F., Austad, W. I., Brzechwa-Ajdukiewicz, A., and Read, A. E. (1968). Serum vitamin B_{12} levels and vitamin B_{12} binding capacity in pregnant and non-pregnant Europeans and West Indians. *Brit. med. J.*, 4, 160-161.

Matthews, D. M., Gunasegaram, R., and Linnell, J. C. (1967). Results with radioisotopic assay of serum B_{12} using serum binding agent. *J. clin. Path.*, 20, 683-686.

Metz, J., Zalusky, R., and Herbert, V. (1968). Folic acid binding by serum and milk. *Amer. J. clin. Nutr.*, 21, 289-297.

Mudd, S. H., Levy, H. L., and Abeles, R. H. (1969). A derangement in B_{12} metabolism leading to homocystinemia, cystathioninemia and methylmalonic aciduria. *Biochem. biophys. Res. Commun.*, 35, 121-126.

O'Dell, B. L., Whitley, J. R., and Hogan, A. G. (1951). Vitamin B_{12}, A factor in prevention of hydrocephalus in infant rats. *Proc. Soc. exp. Biol. (N.Y.)*, 76, 349-353.

Peters, T. J., Linnell, J. C., Matthews, D. M., and Hoffbrand, A. V. (1971). The absorption of vitamin B_{12} in the guinea-pig. III The forms of vitamin B_{12} in ileal mucosa and portal plasma in the fasting state and during absorption of cyanocobalamin. *Brit. J. Haemat.*, 20, 299-305.

Rappazzo, M. E., Salmi, H. A., and Hall, C. A. (1970). The content of vitamin B_{12} in adult and foetal tissue: a comparative study. *Brit. J. Haemat.*, 18, 425-433.

Shapiro, J., Alberts, H. W., Welch, P., and Metz, J. (1965). Folate and vitamin B_{12} deficiency associated with lactation. *Brit. J. Haemat.*, 11, 498-504.

Sharp, A. A. and Witts, L. J. (1962). Seminal vitamin B_{12} and sterility. (Letter to Editor). *Lancet*, 2, 779.

References

Adams, J. F. (1958). Pregnancy and Addisonian pernicious anaemia. *Scot. med. J.*, 3, 21-25.

Baker, H., Zifler, H., Pasher, I., and Sobotka, H. (1958). A comparison of maternal and foetal folic acid and vitamin B_{12} at parturition. *Brit. med. J.*, 1, 978-979.

Baker, S. J., Jacob, E., Rajan, K. T., and Swaminathan, S. R. (1962). Vitamin B_{12} deficiency in pregnancy and the puerperium. *Brit. med. J.*, 1, 1658-1661.

Ball, E. W., and Giles, C. (1964). Folic acid and vitamin B_{12} levels in pregnancy and their relation to megaloblastic anaemia. *J. clin. Path.*, 17, 165-174.

Boger, W. P., Wright, L. D., Beck, G. D., and Bayne, G. M. (1956). Vitamin B_{12}: correlation of serum concentrations and pregnancy. *Proc. Soc. exp. Biol. (N.Y.)*, 92, 140-143.

Boger, W. P., Wright, L. D., Bayne, G. M. (1957). Serum vitamin B_{12} concentrations of pregnant women and newborn infants. In *Vitamin B_{12} and Intrinsic Factor*. (1st European Symposium), edited by H. C. Heinrich, p. 443, Enke, Stuttgart.

Flodh, H. (1968). Distribution and kinetics of labelled vitamin B_{12}. *Acta radiol. (Stockh.)*, Suppl. 284.

Glass, G. B. J. (1963). Gastric intrinsic factor and its function in the metabolism of vitamin B_{12}. *Physiol. Rev.*, 43, 529-849 (see p. 566).

Grainger, R. B., O'Dell, B. L., and Hogan, A. G. (1954). Congenital malformations as related to deficiencies of riboflavin and vitamin B_{12}, source of protein, calcium to phosphorus ratio and skeletal phosphorus metabolism. *J. Nutr.*, 54, 33-48.

Hibbard, E. D., and Spencer, W. J. (1970). Low serum B_{12} levels and latent Addisonian anaemia in pregnancy. *J. Obstet. Gynaec. Brit. Cwlth*, 77, 52-57.

Hutner, S. H., Bach, M. K., and Ross, G. I. M. (1956). Sugar-containing basal medium for vitamin B_{12} assay with *Euglena*: application to body fluids. *J. Protozool.*, 3, 101-112.

Izak, G., Rachmilewitz, M., Stein, Y., Berkovici, B., Sadovsky, A., Arnovitch, Y., and Grossovicz, N. (1957). Vitamin B_{12} and iron deficiencies in anaemia of pregnancy and puerperium. *Arch. intern. Med.*, 99, 346-355.

Jackson, I. M. D., Doig, W. B., and McDonald, G. (1967). Pernicious anaemia as a cause of infertility. *Lancet*, 2, 1159-1160.

Jathar, V. S., Kamath, S. A. Parikh, M. N., Rege, D. V., and Satoskar, R. S. (1970). Maternal milk and serum vitamin B_{12}, folic acid and protein levels in Indian subjects. *Arch. Dis. Childh.*, 45, 236-241.

Karlin, R. (1954). La vitamine B_{12} dans le lait humain et bovin. *C. R. Soc. Biol. (Paris)*, 148, 371-373.

Karlin, R., and Dumont, M. (1955). Sur le taux de vitamine B_{12} dans le serum sanguin chez la femme au cours de l'accouchement, dans celui du cordon et dans le placenta. *C. R. Soc. Biol. (Paris)*, 149, 1986 1988.

Kumento, A., Lopez, R., Luhby, A. L., and Hall, C. A. (1967). B_{12} binders in human cord serum. (Abstr.) *Clin. Res.*, 15, 283.

Levy, H. L., Mudd, S. H., Schulman, J. 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.

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.* 40, 1-16.

Linnell, J. C., Hussein, H. A-A. and Matthews, D. M. (1970). A two-dimensional chromato-bioautographic method for complete separation of individual plasma cobalamins. *J. clin. Path.*, 23,820-821.

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

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

Low-Beer, T. S., McCarthy, C. F., Austad, W. I., Brzechwa-Ajdukiewicz, A., and Read, A. E. (1968). Serum vitamin B_{12} levels and vitamin B_{12} binding capacity in pregnant and non-pregnant Europeans and West Indians. *Brit. med. J.*, 4, 160-161.

Matthews, D. M., Gunasegaram, R., and Linnell, J. C. (1967). Results with radioisotopic assay of serum B_{12} using serum binding agent. *J. clin. Path.*, 20, 683-686.

Metz, J., Zalusky, R., and Herbert, V. (1968). Folic acid binding by serum and milk. *Amer. J. clin. Nutr.*, 21, 289-297.

Mudd, S. H., Levy, H. L., and Abeles, R. H. (1969). A derangement in B_{12} metabolism leading to homocystinemia, cystathioninemia and methylmalonic aciduria. *Biochem. biophys. Res. Commun.*, 35, 121-126.

O'Dell, B. L., Whitley, J. R., and Hogan, A. G. (1951). Vitamin B_{12}, A factor in prevention of hydrocephalus in infant rats. *Proc. Soc. exp. Biol. (N.Y.)*, 76, 349-353.

Peters, T. J., Linnell, J. C., Matthews, D. M., and Hoffbrand, A. V. (1971). The absorption of vitamin B_{12} in the guinea-pig. III The forms of vitamin B_{12} in ileal mucosa and portal plasma in the fasting state and during absorption of cyanocobalamin. *Brit. J. Haemat.*, 20, 299-305.

Rappazzo, M. E., Salmi, H. A., and Hall, C. A. (1970). The content of vitamin B_{12} in adult and foetal tissue: a comparative study. *Brit. J. Haemat.*, 18, 425-433.

Shapiro, J., Alberts, H. W., Welch, P., and Metz, J. (1965). Folate and vitamin B_{12} deficiency associated with lactation. *Brit. J. Haemat.*, 11, 498-504.

Sharp, A. A. and Witts, L. J. (1962). Seminal vitamin B_{12} and sterility. (Letter to Editor). *Lancet*, 2, 779.

References

Adams, J. F. (1958). Pregnancy and Addisonian pernicious anaemia. *Scot. med. J.*, 3, 21-25.

Baker, H., Zifter, H., Pasher, I., and Sobotka, H. (1958). A comparison of maternal and foetal folic acid and vitamin B_{12} at parturition. *Brit. med. J.*, 1, 978-979.

Baker, S. J., Jacob, E., Rajan, K. T., and Swaminathan, S. R. (1962). Vitamin B_{12} deficiency in pregnancy and the puerperium. *Brit. med. J.*, 1, 1658-1661.

Ball, E. W., and Giles, C. (1964). Folic acid and vitamin B_{12} levels in pregnancy and their relation to megaloblastic anaemia. *J. clin. Path.*, 17, 165-174.

Boger, W. P., Wright, L. D., Beck, G. D., and Bayne, G. M. (1956). Vitamin B_{12}: correlation of serum concentrations and pregnancy. *Proc. Soc. exp. Biol. (N.Y.)*, 92, 140-143.

Boger, W. P., Wright, L. D., Bayne, G. M. (1957). Serum vitamin B_{12} concentrations of pregnant women and newborn infants. In *Vitamin B_{12} and Intrinsic Factor.* (1st European Symposium), edited by H. C. Heinrich, p. 443, Enke, Stuttgart.

Flodh, H. (1968). Distribution and kinetics of labelled vitamin B_{12}. *Acta radiol. (Stockh.)*, Suppl. 284.

Glass, G. B. J. (1963). Gastric intrinsic factor and its function in the metabolism of vitamin B_{12}. *Physiol. Rev.*, 43, 529-849 (see p. 566).

Grainger, R. B., O'Dell, B. L., and Hogan, A. G. (1954). Congenital malformations as related to deficiencies of riboflavin and vitamin B_{12}, source of protein, calcium to phosphorus ratio and skeletal phosphorus metabolism. *J. Nutr.*, 54, 33-48.

Hibbard, E. D., and Spencer, W. J. (1970). Low serum B_{12} levels and latent Addisonian anaemia in pregnancy. *J. Obstet. Gynaec. Brit. Cwlth*, 77, 52-57.

Hutner, S. H., Bach, M. K., and Ross, G. I. M. (1956). Sugar-containing basal medium for vitamin B_{12} assay with *Euglena:* application to body fluids. *J. Protozool.*, 3, 101-112.

Izak, G., Rachmilewitz, M., Stein, Y., Berkovici, B., Sadovsky, A., Arnovitch, Y., and Grossovicz, N. (1957). Vitamin B_{12} and iron deficiencies in anaemia of pregnancy and puerperium. *Arch. intern. Med.*, 99, 346-355.

Jackson, I. M. D., Doig, W. B., and McDonald, G. (1967). Pernicious anaemia as a cause of infertility. *Lancet*, 2, 1159-1160.

Jathar, V. S., Kamath, S. A. Parikh, M. N., Rege, D. V., and Satoskar, R. S. (1970). Maternal milk and serum vitamin B_{12}, folic acid and protein levels in Indian subjects. *Arch. Dis. Childh.*, 45, 236-241.

Karlin, R. (1954). La vitamine B_{12} dans le lait humain et bovin. *C. R. Soc. Biol. (Paris)*, 148, 371-373.

Karlin, R., and Dumont, M. (1955). Sur le taux de vitamine B_{12} dans le serum sanguin chez la femme au cours de l'accouchement, dans celvi du cordon et dans le placenta. *C. R. Soc. Biol. (Paris)*, 149, 1986 1988.

Kumento, A., Lopez, R., Luhby, A. L., and Hall, C. A. (1967). B_{12} binders in human cord serum. (Abstr.) *Clin. Res.*, 15, 283.

Levy, H. L., Mudd, S. H., Schulman, J. 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.

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.* 40, 1-16.

Linnell, J. C., Hussein, H. A-A. and Matthews, D. M. (1970). A two-dimensional chromato-bioautographic method for complete separation of individual plasma cobalamins. *J. clin. Path.*, 23,820-821.

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

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

Low-Beer, T. S., McCarthy, C. F., Austad, W. I., Brzechwa-Ajdukiewicz, A., and Read, A. E. (1968). Serum vitamin B_{12} levels and vitamin B_{12} binding capacity in pregnant and non-pregnant Europeans and West Indians. *Brit. med. J.*, 4, 160-161.

Matthews, D. M., Gunasegaram, R., and Linnell, J. C. (1967). Resu'ts with radioisotopic assay of serum B_{12} using serum binding agent. *J. clin. Path.*, 20, 683-686.

Metz, J., Zalusky, R., and Herbert, V. (1968). Folic acid binding by serum and milk. *Amer. J. clin. Nutr.*, 21, 289-297.

Mudd, S. H., Levy, H. L., and Abeles, R. H. (1969). A derangement in B_{12} metabolism leading to homocystinemia, cystathioninemia and methylmalonic aciduria. *Biochem. biophys. Res. Commun.*, 35, 121-126.

O'Dell, B. L., Whitley, J. R., and Hogan, A. G. (1951). Vitamin B_{12}, A factor in prevention of hydrocephalus in infant rats. *Proc. Soc. exp. Biol. (N.Y.)*, 76, 349-353.

Peters, T. J., Linnell, J. C., Matthews, D. M., and Hoffbrand, A. V. (1971). The absorption of vitamin B_{12} in the guinea-pig. III The forms of vitamin B_{12} in ileal mucosa and portal plasma in the fasting state and during absorption of cyanocobalamin. *Brit. J. Haemat.*, 20, 299-305.

Rappazzo, M. E., Salmi, H. A., and Hall, C. A. (1970). The content of vitamin B_{12} in adult and foetal tissue: a comparative study. *Brit. J. Haemat.*, 18, 425-433.

Shapiro, J., Alberts, H. W., Welch, P., and Metz, J. (1965). Folate and vitamin B_{12} deficiency associated with lactation. *Brit. J. Haemat.*, 11, 498-504.

Sharp, A. A. and Witts, L. J. (1962). Seminal vitamin B_{12} and sterility. (Letter to Editor). *Lancet*, 2, 779.

References

Adams, J. F. (1958). Pregnancy and Addisonian pernicious anaemia. *Scot. med. J.*, 3, 21-25.

Baker, H., Ziffer, H., Pasher, I., and Sobotka, H. (1958). A comparison of maternal and foetal folic acid and vitamin B_{12} at parturition. *Brit. med. J.*, 1, 978-979.

Baker, S. J., Jacob, E., Rajan, K. T., and Swaminathan, S. R. (1962). Vitamin B_{12} deficiency in pregnancy and the puerperium. *Brit. med. J.*, 1, 1658-1661.

Ball, E. W., and Giles, C. (1964). Folic acid and vitamin B_{12} levels in pregnancy and their relation to megaloblastic anaemia. *J. clin. Path.*, 17, 165-174.

Boger, W. P., Wright, L. D., Beck, G. D., and Bayne, G. M. (1956). Vitamin B_{12}: correlation of serum concentrations and pregnancy. *Proc. Soc. exp. Biol. (N.Y.)*, 92, 140-143.

Boger, W. P., Wright, L. D., Bayne, G. M. (1957). Serum vitamin B_{12} concentrations of pregnant women and newborn infants. In *Vitamin B_{12} and Intrinsic Factor.* (1st European Symposium), edited by H. C. Heinrich, p. 443, Enke, Stuttgart.

Flodh, H. (1968). Distribution and kinetics of labelled vitamin B_{12}. *Acta radiol. (Stockh.)*, Suppl. 284.

Glass, G. B. J. (1963). Gastric intrinsic factor and its function in the metabolism of vitamin B_{12}. *Physiol. Rev.*, 43, 529-849 (see p. 566).

Grainger, R. B., O'Dell, B. L., and Hogan, A. G. (1954). Congenital malformations as related to deficiencies of riboflavin and vitamin B_{12}, source of protein, calcium to phosphorus ratio and skeletal phosphorus metabolism. *J. Nutr.*, 54, 33-48.

Hibbard, E. D., and Spencer, W. J. (1970). Low serum B_{12} levels and latent Addisonian anaemia in pregnancy. *J. Obstet. Gynaec. Brit. Cwlth*, 77, 52-57.

Hutner, S. H., Bach, M. K., and Ross, G. I. M. (1956). Sugar-containing basal medium for vitamin B_{12} assay with *Euglena:* application to body fluids. *J. Protozool.*, 3, 101-112.

Izak, G., Rachmilewitz, M., Stein, Y., Berkovici, B., Sadovsky, A., Arnovitch, Y., and Grossovicz, N. (1957). Vitamin B_{12} and iron deficiencies in anaemia of pregnancy and puerperium. *Arch. intern. Med.*, 99, 346-355.

Jackson, I. M. D., Doig, W. B., and McDonald, G. (1967). Pernicious anaemia as a cause of infertility. *Lancet*, 2, 1159-1160.

Jathar, V. S., Kamath, S. A. Parikh, M. N., Rege, D. V., and Satoskar, R. S. (1970). Maternal milk and serum vitamin B_{12}, folic acid and protein levels in Indian subjects. *Arch. Dis. Childh.*, 45, 236-241.

Karlin, R. (1954). La vitamine B_{12} dans le lait humain et bovin. *C. R. Soc. Biol. (Paris)*, 148, 371-373.

Karlin, R., and Dumont, M. (1955). Sur le taux de vitamine B_{12} dans le serum sanguin chez la femme au cours de l'accouchement, dans celui du cordon et dans le placenta. *C. R. Soc. Biol. (Paris)*, 149, 1986 1988.

Kumento, A., Lopez, R., Luhby, A. L., and Hall, C. A. (1967). B_{12} binders in human cord serum. (Abstr.) *Clin. Res.*, 15, 283.

Levy, H. L., Mudd, S. H., Schulman, J. 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.

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.* 40, 1-16.

Linnell, J. C., Hussein, H. A-A. and Matthews, D. M. (1970). A two-dimensional chromato-bioautographic method for complete separation of individual plasma cobalamins. *J. clin. Path.*, 23,820-821.

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

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

Low-Beer, T. S., McCarthy, C. F., Austad, W. I., Brzechwa-Ajdukiewicz, A., and Read, A. E. (1968). Serum vitamin B_{12} levels and vitamin B_{12} binding capacity in pregnant and non-pregnant Europeans and West Indians. *Brit. med. J.*, 4, 160-161.

Matthews, D. M., Gunasegaram, R., and Linnell, J. C. (1967). Results with radioisotopic assay of serum B_{12} using serum binding agent. *J. clin. Path.*, 20, 683-686.

Metz, J., Zalusky, R., and Herbert, V. (1968). Folic acid binding by serum and milk. *Amer. J. clin. Nutr.*, 21, 289-297.

Mudd, S. H., Levy, H. L., and Abeles, R. H. (1969). A derangement in B_{12} metabolism leading to homocystinemia, cystathioninemia and methylmalonic aciduria. *Biochem. biophys. Res. Commun.*, 35, 121-126.

O'Dell, B. L., Whitley, J. R., and Hogan, A. G. (1951). Vitamin B_{12}, A factor in prevention of hydrocephalus in infant rats. *Proc. Soc. exp. Biol. (N.Y.)*, 76, 349-353.

Peters, T. J., Linnell, J. C., Matthews, D. M., and Hoffbrand, A. V. (1971). The absorption of vitamin B_{12} in the guinea-pig. III The forms of vitamin B_{12} in ileal mucosa and portal plasma in the fasting state and during absorption of cyanocobalamin. *Brit. J. Haemat.*, 20, 299-305.

Rappazzo, M. E., Salmi, H. A., and Hall, C. A. (1970). The content of vitamin B_{12} in adult and foetal tissue: a comparative study. *Brit. J. Haemat.*, 18, 425-433.

Shapiro, J., Alberts, H. W., Welch, P., and Metz, J. (1965). Folate and vitamin B_{12} deficiency associated with lactation. *Brit. J. Haemat.*, 11, 498-504.

Sharp, A. A. and Witts, L. J. (1962). Seminal vitamin B_{12} and sterility. (Letter to Editor). *Lancet*, 2, 779.

J. clin. Path., 1970, 23, 563-568

Vitamin B_{12} absorption in some neurological and neuroendocrine disorders

D. DONALDSON[1] AND P. T. LASCELLES

From the Department of Chemical Pathology, The Institute of Neurology, The National Hospitals for Nervous Diseases, Queen Square, London

SYNOPSIS An assessment of vitamin B_{12} absorption in neurological patients has been made by both serum and urine counting of ^{57}Co cyanocobalamin during the conventional Schilling test. Although patients with pernicious anaemia, some with subacute combined degeneration of the cord, have been studied, emphasis in the discussion is placed on the significantly increased excretion of the radioactive vitamin in a group of patients with pituitary insufficiency. Results are also given for epileptic patients who have developed folate deficiency (as assessed by serum folate levels) coincidental with anticonvulsant therapy, as well as for some patients who have had neurological symptoms or signs following partial gastrectomy operations.

The Schilling test has been used for establishing the diagnosis of pernicious anaemia since 1953. It has also been a valuable laboratory investigation in patients with malabsorption syndromes. The parameters of normality have been accurately established both for urine and serum (Donaldson, Blight, and Lascelles, 1970), and the present communication describes applications of this technique to the investigation of certain neurological and neuroendocrine diseases.

Methods

COUNTING TECHNIQUE

This is described on page 559.

SELECTION OF PATIENTS

The following groups of patients were selected from an in-patient hospital population over a two-year period:

Received for publication 3 March 1970.

[1] Present address; Chemical Pathology Department, Redhill General Hospital, Earlswood Common, Redhill, Surrey. Requests for reprints should be addressed to D.D. at this address.

1 Patients with pernicious anaemia

Parameters used for studying this group included the following: full blood count; serum vitamin B_{12} level (*Lactobacillus leichmanii*); serum folate level (*Lactobacillus casei*); augmented histamine test meal (in most patients); sternal marrow examination; and antibodies to gastric parietal cells and intrinsic factor (not examined in all patients).

These were all assessed before the Schilling test was performed. Table I shows the haemoglobin values at the time of diagnosis and indicates that many of the patients were only slightly anaemic. One patient (J.S., Table I) had coincidental hypopituitarism.

2 Patients with pituitary insufficiency

This state was as assessed by clinical and biochemical criteria. Part I Schilling tests were done on five patients in this group. Four of the patients had pituitary tumours removed (two before and two after the Schilling test), and were examined histologically. Three of these were reported to be chromophobe adenomas, and one was stated to be an adenoma. In the remaining patient the cause of the hypopituitarism had not been established. In addition there was one patient, not included in Table II, who had coincidental

Patient	Sex	Haemoglobin Level (g/100 ml)	Serum Vitamin B_{12} Level (μμg/ml)	Schilling Test			
				Part 1		Part 2	
				24-Hour Urine Excretion (% of dose administered)	Eight-hour Serum Level (% of dose/l)	24-Hour Urine Excretion (% of dose)	Eight-hour Serum Level (% of dose/l)
M.C.	F	12·2	65	2·4	—	25·0	1·65
D.T.	F	5·8	95	0·8	0·00	—	0·20
B.F.	M	14·1	130	1·3	0·11	12·2	1·08
A.F.	M	10·5	67	2·1	0·00	17·5	0·74
B.M.	F	10·0	80	1·1	0·00	12·6	1·22
G.G.	F	11·9	81	1·7	0·20	11·6	1·58
T.L.	M	12·2	—	2·4	0·18	21·3	0·84
J.S.	F	13·0	150	2·7	0·26	11·3	0·99
E.T.	F	12·6	30	0·6	0·01	20·8	0·95
E.N.	F	7·9	56	0·5	0·04	11·7	0·88
M.E.	F	11·4	130	2·2	0·18	21·6	1·41
W.C.	F	12·8	—	0·4	0·00	14·0	1·22
Mean				1·5	0·09	16·4	1·07
SD				0·8	0·09	5·2	0·39
Normal mean				18·1	1·05	—	—
SD				3·8	0·25		

Table I *Results of Schilling tests on patients with pernicious anaemia*

Patient	Sex	24-Hour Urine Excretion (% of dose administered)	Eight-hour Serum Level (% of dose administered/l)
W.M.	M	31·5	1·03
J.C.	M	21·2	2·67
V.T.	F	23·9	1·70
S.S.	M	29·6	1·07
P.C.	M	20·5	0·81
Mean		25·3	1·46
SD		5·0	0·76

Table II *Results of part 1 Schilling tests on patients with hypopituitarism*

Patient	Sex	Hb Level (g/100 ml)	Serum Folate Level (mμg/ml)	24-Hour Urine Excretion (% of dose)	Eight-hour Serum Level (% of dose/l)
D.T.	M	14·9	1·8	32·2	0·84
A.R.	M	13·2	2·9	19·6	0·93
S.P.	F	13·2	1·4	27·2	1·80
P.K.	M	14·4	3·0	5·3	0·47
F.R.	F	13·2	1·8	12·0	0·53
J.S.	F	5·8	3·0	10·0	0·60
J.W.	M	13·0	1·9	33·0	1·58
J.M.	M	14·1	2·0	12·9	1·43
J.U.	F	12·6	3·6	28·9	—
M.B.	F	7·4	1·8	14·6	2·91
Mean				19·0	1·18
SD				9·7	0·72

Table III *Results of part 1 Schilling tests on folate-deficient epileptic patients on anticonvulsant drugs*

pernicious anaemia, and her result is therefore included under that heading. Three of the five patients investigated were receiving no replacement therapy at the time of the Schilling test. One patient (V.T., Table II) was receiving both thyroxine and cortisone acetate, and one (P.C., Table II) was only receiving cortisone acetate replacement.

3 Epileptic patients

All had been treated with anticonvulsant drugs, and except for one had folate levels of 3·0 mμg/ml or less. Red cell folate levels were not arranged as a parameter for investigation in this group.

4 Patients after partial gastrectomy

The patients had had partial gastrectomy operations between one and 14 years previously. All the patients in this group had neurological symptoms and signs following the partial gastrectomy operation. In most the neurological involvement was of a type suggestive of vitamin B_{12} deficiency, but the vitamin B_{12} levels were in the normal range in a number of patients, possibly due to short-term vitamin B_{12} administration before admission.

Five out of the seven patients had proved duodenal ulcers before operation. In two patients (R.S. and A.B., Table IV) the only information available is that they had peptic ulcers.

Serum vitamin B_{12} was estimated by the microbiological method using *Lactobacillus leichmanii*, and serum folate was measured by the *Lactobacillus casei* method.

Results

PATIENTS WITH PERNICIOUS ANAEMIA

Table I shows the distribution of our data for urine and serum in patients with pernicious anaemia (for part 1 and for part 2 with intrinsic factor). In part 1 of the test the mean values for the urine excretion of ^{57}Co vitamin B_{12} were significantly lower than normal, and similarly for the serum levels, usually rising to normal levels when the test was repeated with the addi-

Patient	Age (yr)	Sex	Hb Level (g/100 ml)	Serum Vitamin B_{12} (μμg/ml)	Time since Gastrectomy (yr)	Diagnosis	Schilling Test: Part 1 — 24-Hour Urine Excretion (% of dose)	Part 1 — Eight-hour Serum Level (% of dose/l)	Part 2 — 24-Hour Urine Excretion (% of dose)	Part 2 — Eight-hour Serum Level (% of dose/l)
V.R.	60	M	12·6	160	6	DU	6·0	0·68	9·6	1·06
R.S.	54	M	12·6	190	14	PU	7·5	0·66	16·2	1·00
W.C.	67	M	14·5	517	2	DU	16·1	0·83	10·5	0·66
									10·0	0·58
A.W.	62	M	13·0	—	14	DU	17·0	1·00	10·4	0·58
J.McS.	38	M	12·3	650	10	DU	14·2	1·39	10·5	1·22
A.B.	69	F	12·1	544	1	PU	3·6	0·50	9·2	0·85
R.D.	46	M	11·8	330	1	DU	11·2	0·70	—	—
Mean							10·8	0·83	11·1	0·89
SD							5·2	0·30	2·6	0·24

Table IV *Results of Schilling tests on patients following partial gastrectomy*
DU = duodenal ulcer
PU = peptic ulcer

Group	Number of Patients	Urine Values	Number of Patients	Serum Values
Ten normal controls and part 2 Schilling tests on patients with pernicious anaemia	11	0·50 > P > 0·40	12	0·95 > P > 0·90
Ten normal controls and part 1 Schilling tests on patients with hypopituitarism	5	0·01 > P > 0·001	5	0·20 > P > 0·10
Ten normal controls and part 1 Schilling tests on epileptic patients with folate deficiency on anticonvulsant drugs	12	0·70 > P > 0·60	11	0·60 > P > 0·50
Ten normal controls and part 1 Schilling tests on gastrectomy patients	7	0·01 > P > 0·001	7	0·20 > P > 0·10

Table V *Significance between results in different groups*

tion of intrinsic factor. It should be noted that neither for urine nor serum values was there an overlap with the normal range. As seen from Table I there was one patient who showed no significant change for urine or serum values when the test was repeated with intrinsic factor.

STUDIES ON PATIENTS WITH HYPOPITUITARISM

Table II shows the data for this group of patients. The mean for urine and serum is seen to be higher than for normal controls. Table V shows that there is a significant difference between the two groups for the urine values. For serum values the difference is seen to be just not significant; but the number of patients was small.

EPILEPTIC PATIENTS TREATED WITH ANTICONVULSANT DRUGS INDUCING FOLATE DEFICIENCY

Table III shows the urine and serum levels of ^{57}Co vitamin B_{12} for these patients. Table V illustrates that there are no significant differences between these values and the part 1 Schilling test results on normal controls. All the patients in this group had near normal haemoglobin levels except for two (J.S. and M.B., Table III) who had levels of 5·8 g/100 ml and 7·4 g/100 ml respectively.

STUDIES ON PATIENTS AFTER PARTIAL GASTRECTOMY

The data for this group of patients are given in Table IV. Parts 1 and 2 of the Schilling test were performed in each case except for the last patient in the list (R.D., Table IV).

Table V shows that there are no significant differences between the first and second parts of the test, either for urine or serum if the results are taken together, but individually it is seen that both R.S. and A.B. (Table IV) showed a significant rise. However, patients W.C. and A.W. both showed a fall in the second part of the test.

Discussion

Table I shows that there was clear demarcation between the part 1 Schilling test results (urine and serum levels) for patients with pernicious anaemia (including those with no evidence of anaemia) and those of normal controls, and that there was no overlap between the two groups, confirming the observations of many previous workers.

One patient (D.T., Table I) failed to show a rise in the urinary excretion of ^{57}Co vitamin B_{12} when intrinsic factor was given. This may be in keeping with the observations of several investigators that there is sometimes a malabsorption for vitamin B_{12} in patients who have suffered deficiency of the vitamin for a considerable time (Haurani, Sherwood, and Goldstein, 1964; Brody, Estren, and Herbert, 1966; Carmel and Herbert, 1967). These authors have demonstrated that this specific malabsorption is reversible when patients are treated with vitamin B_{12}, and they

showed that the Schilling test part 2 became normal after periods ranging from one to 20 months. Some of the patients had only been re-investigated after an elapse of several months or more, and it is therefore possible that the time of response may have been considerably shorter. Goldberg, Bickel, and Fudenberg (1969) similarly described a patient who showed increased absorption of vitamin B_{12} after six months of treatment; in addition these authors draw attention to the possibility that the presence of gastric antibody to intrinsic factor may be the cause of a low absorption of vitamin B_{12} in the second part of the Schilling test.

It is of interest that the patient (D.T.) had the lowest haemoglobin in the group (5·8 g/100 ml) and therefore may have been deficient in vitamin B_{12} for a long period. Unfortunately there was no opportunity to investigate this patient further. There was no clinical evidence of generalized malabsorption. Sauli, Astaldi, and Malossini (1963) studied the histopathology of the intestinal mucosa in patients with pernicious anaemia, before and after vitamin B_{12} therapy. They showed that there were appreciable changes toward normality after one month of treatment with 500 gamma per day. Foroozan and Trier (1967) demonstrated that after two or more months of vitamin B_{12} administration the small intestines of all their eight patients were histologically indistinguishable from those of the control subjects.

Apart from this one patient there was no difference between the mean value for part 2 of the Schilling test on patients with pernicious anaemia and the part 1 tests on normal controls (see Table V).

The five patients in the hypopituitary group all showed a urinary excretion of ^{57}Co vitamin B_{12} greater than 20·0%. Two of the patients had excretions greater than the upper limit of the normal range (using 95% confidence limits). In addition, two of the three remaining patients had serum levels of ^{57}Co vitamin B_{12} in excess of the normal range. Hence, four of the five patients would have been separable from the normal controls taking either the urine or serum values alone as significant.

From Table II it is noted that the highest two urinary excretions belonged to patients who were not receiving replacement therapy at the time of the test (W.M. and S.S., Table II). However, the excretion in the third patient not receiving therapy was lower than these, although the eight-hour serum level was the highest of the group.

It is possible that the physiological mechanism for this increased excretion of vitamin B_{12} in hypopituitarism is in some way related to deficiency of certain hormonal factors, either the trophic hormones produced by the pituitary, or deficient production of hormones by the target organs for these trophic hormones, namely, the thyroid, adrenals, and the gonads.

Also to be considered is the possibility that the adenomatous tissue of the pituitary tumours themselves may be producing a substance which has an effect on vitamin B_{12} metabolism. It is hoped that further work will establish whether or not pituitary adenomas are the necessary factor, or whether hypopituitarism *per se*, whatever its cause, will produce the same effect.

It is established in humans that the thyroid gland influences xylose metabolism. Broitman, Bondy, Yachnin, Hoskins, Ingbar, and Zamcheck (1964) showed that although the absorption of xylose was normal in states of thyrotoxicosis and myxoedema, the urinary excretion was low in myxoedema and increased in hyperthyroidism. Furthermore, they showed that the excretory pattern was similar when the xylose was given intravenously. They calculated that there was a decreased rate of renal clearance and an apparently normal rate of extrarenal disposal in patients with myxoedema, and that there was a decreased rate of extrarenal disposal with a concomitant increase in renal excretion in patients with hyperthyroidism.

Also of importance in this connexion are experiments involving the use of rats in which there was shown to be increased absorption of vitamin B_{12} when the animals were fed on large doses of desiccated thyroid together with a casein-containing diet, and a decreased absorption of the vitamin when the animals were thyroidectomized (Okuda, Steelman, and Chow, 1956).

It would appear, however, that these animal experiments were in conflict with our findings in hypopituitary patients if the thyroid were the factor responsible for the difference from the normal controls. One might thus expect a lowered excretion of vitamin B_{12} if there was a degree of hypothyroidism secondary to hypopituitarism, instead of the increase observed. On the other hand, the aetiology of the hypothyroidism is different in the two examples, and this fact may be of importance in the interpretation of the results. The hypothyroidism of pituitary insufficiency is mediated through the pituitary, whereas that of the hypothyroid rats was induced by excision of the thyroid gland, in which case the pituitary gland in each animal of the series was intact. Experiments on vitamin B_{12} absorption in patients with thyrotoxicosis and myxoedema are in progress.

The question arises whether the thyroid could have an effect on overall vitamin B_{12} metabolism, or simply on transport across the intestinal mucosa. Thus it is established that the intracellular accumulation *in vitro* of the amino acids cycloleucine, histidine, and valine, and the sugar α-methyl-D-glucopyranoside, were increased in the intestinal segments of rats made hypothyroid by thyroidectomy and radioactive ^{131}I (London and Segal, 1967). When the hypothyroid animals were treated with triiodo-

thyronine the accumulation of substrate fell toward the control level. The authors suggested that as this phenomenon was seen with an unutilizable amino acid and sugar, it strongly suggested an effect on transport rather than on metabolism.

So far we have considered only the hypothyroid element of the hypopituitary state. Another possible explanation to be considered is that the replacement therapy itself may be responsible for the increased vitamin B_{12} absorption in the hypopituitary group. That this is not tenable is shown by the fact that three of the five patients were, at the time of the Schilling test, not on any form of therapy.

There is known to be an increased incidence of pernicious anaemia in myxoedema (Leithold, David, and Best, 1958; Forssell and Halunen, 1958; Ardeman, Chanarin, Krafchik, and Singer, 1966; and Strickland, 1969), and also of low serum vitamin B_{12} levels in hypothyroidism without pernicious anaemia (Tudhope and Wilson, 1962). Again this observation does not account for our hypopituitary observations. Ziffer, Gutman, Pasher, Sobotka, and Baker (1957), however, demonstrated that the whole-blood vitamin B_{12} values were lower in a group of hyperthyroid patients than in a normal control group and a myxoedematous group. The mean urinary excretion after the vitamin B_{12} dose was considerably lower in hyperthyroid patients than in the normal group, and the myxoedematous group showed an increased excretion. These authors suggested that vitamin B_{12} turnover and demand is greater in hyperthyroid patients and lower in myxoedema.

There have been reports of pernicious anaemia in Addison's disease (Berlin, 1952; Strickland, 1969), but no literature is available regarding vitamin B_{12} levels. Because of the increased absorption of vitamin B_{12} in Schilling tests in which the dose is given to fasting patients, it does not necessarily follow that there is an increased absorption of vitamin B_{12} from the food under physiological conditions in these patients.

In order to define the mechanism for the increased excretion of vitamin B_{12} in the Schilling test we are currently investigating a further group of such patients, some of whom are on replacement therapy. We are also establishing the effect of ACTH, prednisone, and dexamethasone on the absorption of vitamin B_{12} in a control group of patients, as well as studying the relationship of the thyroid function of the hypopituitary group to the problem. In addition, patients with non-pituitary cerebral tumours and raised intracranial pressure are being similarly investigated.

It is well established that patients who are treated with anticonvulsant drugs, such as phenobarbitone, phenytoin, and primidone, for epilepsy are liable to develop folate deficiency (Reynolds, Chanarin, Milner, and Matthews, 1966). It is also known that the metabolism of folate and vitamin B_{12} is closely interdependent (Vilter, 1964). This can be seen by the changes in serum levels of the one in response to changes in serum levels of the other: for example, the tendency to high serum folate levels in pernicious anaemia (Waters and Mollin, 1961; Waters and Mollin, 1962; Waters and Mollin, 1963), and the reduction in the serum vitamin B_{12} in epileptic patients who are treated with folic acid for folate deficiency induced by anticonvulsant therapy (Hunter, Barnes, and Matthews, 1969).

It was with these facts in mind that Schilling tests were done on a series of folate-deficient epileptic patients who had been treated with various combinations of the drugs mentioned above. Lees (1961) reported that the Schilling test indicated a lower absorption of vitamin B_{12} in patients who had developed megaloblastic anaemia while on anticonvulsant drugs, and that in six of eight patients a considerable increase occurred when folic acid therapy was given.

In our series, however, there was no significant difference (see Table V) between the series of normal controls and the epileptic patients who were folate deficient. Only two of our series of 10 patients had a significant anaemia, and the explanation of the disparity between our results and those of Lees may lie here.

As shown in Tables III and V, there is no significant difference for the urine and serum levels of ^{57}Co vitamin B_{12} in the Schilling test between the folate-deficient epileptic patients and the series of normal controls.

Table IV illustrates the importance of studying individual patients separately, as certain changes between part 1 and 2 tests do not show up when the group is studied as a whole.

It should be noted that none of these patients was given a vitamin B_{12}-free meal at the time the radioactive vitamin B_{12} was given in the Schilling test. Deller, Germar, and Witts (1961) noted that when a vitamin B_{12}-free meal was given with the labelled vitamin B_{12} in the Schilling test absorption was significantly enhanced in patients who had undergone partial gastrectomy. Turnbull (1967) observed similar results in a series of patients with anaemia after Polya partial gastrectomy, and suggested that the improved absorption of vitamin B_{12} with food was likely to be due to stimulation of a small residual secretion of intrinsic factor.

These initial findings by the more precisely standardized Schilling test counting both urine and serum radioactivity confirm and extend the observations of others. The explanations for the mechanism of increased excretion of radioactive vitamin B_{12} in patients with pituitary tumours and the fall in excretion in part 2 in some post-gastrectomy patients with neurological complications are not at present apparent and clearly need further investigation.

We wish to thank the physicians and surgeons

of the National Hospitals, Queen Square and Maida Vale, for permission to investigate patients under their care.

We are also grateful to Mrs Carol Pye at the Hospital for Sick Children, Great Ormond Street, for the serum folate assays.

References

Ardeman, S., Chanarin, I., Krafchik, B., and Singer, W. (1966). Addisonian pernicious anaemia and intrinsic factor antibodies in thyroid disorders. *Quart. J. Med.*, 35, 421-431.

Berlin, R. (1952). Addison's disease: familial incidence and occurrence in association with pernicious anaemia. *Acta med. scand.*, 144, 1-6.

Brody, E. A., Estren, S., and Herbert, V. (1966). Coexistent pernicious anaemia and malabsorption in four patients (including one whose malabsorption disappeared with vitamin B_{12} therapy). *Ann. intern. Med.*, 64, 1246-1251.

Broitman, S. A., Bondy, D. C., Yachnin, I., Hoskins, L. C., Ingbar, S., and Zamcheck, N. (1964). Absorption and disposition of D-xylose in thyrotoxicosis and myxoedema. *New Engl. J. Med.*, 270, 333-337.

Carmel, R., and Herbert, V. (1967). Correctable intestinal defect of vitamin B_{12} absorption in pernicious anaemia. *Ann. intern. Med.*, 67, 1201-1207.

Deller, D. J., Germar, H., and Witts, L. J. (1961). Effect of food on absorption of radioactive vitamin B_{12}. *Lancet*, 1, 574-577.

Donaldson, D., Blight, B. J. N., and Lascelles, P. T. (1970). An assessment of serum ^{57}Co cyanocobalamin as an index of vitamin B_{12} absorption. *J. clin. Path.*, 23, 588-562

Foroozan, P., and Trier, J. S. (1967). Mucosa of the small intestine in pernicious anaemia. *New Engl. J. Med.*, 277, 553-559.

Forssell, J., and Halunen, P. I. (1958). Thyroid function and pernicious anaemia. Comments on some typical cases. *Acta med. scand.*, 162, 61-68.

Goldberg, L. S., Bickel, Y. B., and Fudenberg, H. H. (1969.) Immunologic approaches to malabsorption of vitamin B_{12}. *Arch. intern. Med.*, 123, 397-400.

Haurani, F. I., Sherwood, W., and Goldstein, F. (1964). Intestinal malabsorption of vitamin B_{12} in pernicious anaemia. *Metabolism*, 13, 1342-1348.

Hunter, R., Barnes, J., and Matthews, D. M. (1969). Effect of folic acid supplement on serum-vitamin-B_{12} levels in patients on anticonvulsants. *Lancet*, 2, 666-667.

Lees, F. (1961). Radioactive vitamin B_{12} absorption in the megaloblastic anaemia caused by anticonvulsant drugs. *Quart. J. Med.*, 30, 231-248.

Leithold, S. L., David, D., and Best, W. R. (1958). Hypothyroidism with anaemia demonstrating abnormal vitamin B_{12} absorption. *Amer. J. Med.*, 24, 535-548.

London, D. R., and Segal, S. (1967). *In vitro* studies on the intestinal transport of amino acids and a sugar in hypothyroid rats. *Endocrinology*, 80, 623-628.

Okuda, K., Steelman, S., and Chow, B. F. (1956). Absorption of vitamin B_{12} in hyper- and hypothyroid rat. *Fed. Proc.*, 15, 567.

Reynolds, E. H., Chanarin, I., Milner, G., and Matthews, D. M. (1966). Anticonvulsant therapy, folic acid and vitamin B_{12} metabolism and mental symptoms. *Epilepsia*, 7, 261-270.

Sauli, S., Astaldi, G., and Malossini, L. (1963). Histopathology of intestinal mucosa obtained by Crosby's biopsy in pernicious anemia before and after vitamin B_{12} treatment. *Acta vitamin. (Milano)*, 17, 143-152.

Strickland, R. G. (1969). Pernicious anemia and polyendocrine deficiency. *Ann. intern. Med.*, 70, 1001-1005.

Tudhope, G. R., and Wilson, G. M. (1962). Deficiency of vitamin B_{12} in hypothyroidism. *Lancet*, 1, 703-706.

Turnbull, A. L. (1967). Absorption of vitamin B_{12} in patients with anaemia after Polya partial gastrectomy. *Brit. J. Haemat.*, 13, 752-763.

Vilter, R. W. (1964). Inter-relationships between folic acid, vitamin B_{12} and ascorbic acid in the megaloblastic anaemias. *Medicine (Baltimore)*, 43, 727-730.

Waters, A. H., and Mollin, D. L. (1961). The folic acid activity of serum in normal subjects and patients with megaloblastic anaemia. In *Proceedings of the 8th Congress of the European Society of Haematology*, Vienna (Paper 332), 1961, vol. 2, Karger, Basle and New York.

Waters, A. H., and Mollin, D. L. (1962). Studies on the folic acid activity of human serum. *J. clin. Path.*, 14, 335-344.

Waters, A. H., and Mollin, D. L. (1963). Observations on the metabolism of folic acid in pernicious anaemia. *Brit. J. Haemat.*, 9, 319-327.

Ziffer, H., Gutman, A., Pasher, I., Sobotka, H., and Baker, H. (1957). Vitamin B_{12} in thyrotoxicosis and myxoedema. *Proc. Soc. exp. Biol. (N.Y.)*, 96, 229-230.

PROC. SOC. EXPTL BIOL AND MED 72(2):388-390

Lipotropic Effects of Vitamin B_{12} Concentrate. (17442)

VICTOR A. DRILL AND HARRY M. MCCORMICK

From the Department of Physiology and Pharmacology, Wayne University College of Medicine, Detroit, Mich.

It has recently been reported that crude liver extract exerts a lipotropic effect in rats with dietary induced liver injury.[1] The effect of the liver extract was not due to stimulation of the appetite with a resultant increase in protein intake, nor did the lipotropic effect seem to be related to the choline content of the liver extract. It was also demonstrated that other supplements. with a higher choline content than that of liver extract, exert a lesser degree of lipotropic activity when compared with liver extract.[2] In the present studies the lipotropic effect of a vitamin B_{12} concentrate was determined in rats fed a high-fat diet.

Methods. Male rats of the Sprague-Dawley strain, weighing between 115 and 149 grams were used. The high-fat diet (51% lard) and the control diet (6% lard) as recently described,[3] were fed *ad libitum* for the period listed in Tables I and II. When the animals were placed on the synthetic diets injections of vitamin B_{12} concentrate were begun and were administered subcutaneously 3 times a week.* At the end of the experiment sections were taken from the left lobe of the liver and stained with hematoxylin and eosin. Frozen sections were also made and stained with Sudan III. The remainder of the liver was analyzed for total fat.[4]

Results. In the first study treatment with a vitamin B_{12} concentrate, in doses of 0.2 μg of vitamin B_{12} 3 times a week, reduced the average fat content of the liver from 31.5%

[1] Hall, C. A., and Drill, V. A., PROC. SOC. EXP. BIOL. AND MED., 1948, **69**, 3.

[2] Drill, V. A., and Hall, C. A., *Am. J. Med. Sci.*, in press.

[3] Hall, C. A., and Drill, V. A., PROC. SOC. EXP. BIOL. AND MED., 1949, **70**, 202.

* The Oleum Percomorphum used in the diet was kindly supplied by Mead Johnson and Company.

The vitamin B_{12} concentrate was supplied by Dr. T. H. Jukes of Lederle Laboratories Division of the American Cyanamid Company.

[4] Outhouse, E. L., and Forbes, J. C., *J. Lab. and Clin. Med.*, 1939, **25**, 1157.

TABLE I.
Effect of Vitamin B_{12} Concentrate on Hepatic Changes of Rats Fed High-Fat Diet for 29 Days.

No. of rats	Avg wt change, g	Avg liver wt, g	Avg liver wt g/100 g rat	No. animals with hepatic fatty changes* 0	1+-2+	3+-4+	Avg liver fat, %
High fat diet, untreated							
6	55 ± 6.12	17.1 ± 1.25	8.1 ± 0.47	0	0	6	31.5 ± 1.18
High fat diet, + B_{12} conc. ≈ 0.2 μg B_{12} 3 times a wk							
5	48 ± 8.13	10.4 ± 0.66	5.1 ± 0.17	1	4	0	16.3 ± 1.47

* Grading of fatty change.
0 = None or only a rare large droplet.
1+ = Few large droplets in each lobule.
2+ = More than 1+, but less than half of lobule involved.
3+ = Over half of lobule involved, but some cells fat-free.
4+ = Almost no fat-free cells.

TABLE II.
Effect of Vitamin B_{12} Concentrate on Hepatic Changes of Rats Fed High-Fat Diet for 66 Days

No. of rats	Avg wt change, g	Avg liver wt, g	Avg liver wt g/100 g rat	No. animals with hepatic fatty changes* 0	1+-2+	3+-4+	Avg liver fat, %
Normal diet, control							
4	160 ± 3.14	12.1 ± 1.13	4.0 ± 0.37	3	1	0	5.6 ± 1.08
High fat diet, untreated							
8	43 ± 9.67	15.7 ± 1.07	8.5 ± 0.62	0	1	7	24.6 ± 0.59
High fat diet, + B_{12} conc. ≈ 1.0 μg B_{12} 3 times a wk							
10	84.6 ± 8.44	10.7 ± 0.71	4.7 ± 0.22	8	1	1	7.8 ± 0.89

* Fatty changes graded as in Table I.

in untreated animals to 16.3% in treated rats (Table I). Histological examination also showed a partial effect of the treatment. The vitamin B_{12} concentrate also prevented the increase in liver weight that usually occurs in untreated rats fed a high-fat diet. There was no significant difference in the gain in weight between the two groups of animals.

Treatment with a higher amount of vitamin B_{12} concentrate (1 μg of vitamin B_{12} 3 times a week) over a period of 66 days demonstrated a marked lipotropic activity of the concentrate. The fat content of the liver of the treated animals was not significantly different from the amount present in rats fed a normal diet (Table II). Histological examinations of the liver also demonstrated the lipotropic activity of the vitamin B_{12} concentrates. The weight of the liver was similarly affected by the treatment and was in the range of the normal controls. Furthermore, there was a significantly greater weight gain in the treated group as compared with the animals fed a high-fat diet alone.

Discussion. A vitamin B_{12} concentrate administered over a period of 29 and 66 days to rats receiving a high-fat diet showed a significant lipotropic effect. This effect is comparable to the previous results obtained with liver extract.[1,2] Both vitamin B_{12} concentrate and liver extract contained a small amount of choline and methionine. An injection of vitamin B_{12} concentrate, containing 1 gamma of vitamin B_{12}, supplied only 0.5 mg of choline, 0.19 mg of methionine, and 0.06 μg of folic acid. Based on 3 injections of the concentrate per week, each rat received only 0.21 mg of choline and 0.08 mg of methionine per day. These amounts of choline and methionine *per se* are ineffective in preventing the dietary induced fatty livers. The rats injected with 0.2 μg of

TABLE I.
Effect of Vitamin B_{12} Concentrate on Hepatic Changes of Rats Fed High-Fat Diet for 29 Days.

No. of rats	Avg wt change, g	Avg liver wt, g	Avg liver wt g/100 g rat	No. animals with hepatic fatty changes* 0	1+-2+	3+-4+	Avg liver fat, %
			High fat diet, untreated				
6	55 ± 6.12	17.1 ± 1.25	8.1 ± 0.47	0	0	6	31.5 ± 1.18
			High fat diet, + B_{12} conc. ≈ 0.2 μg B_{12} 3 times a wk				
5	48 ± 8.13	10.4 ± 0.66	5.1 ± 0.17	1	4	0	16.3 ± 1.47

* Grading of fatty change.
0 = None or only a rare large droplet.
1+ = Few large droplets in each lobule.
2+ = More than 1+, but less than half of lobule involved.
3+ = Over half of lobule involved, but some cells fat-free.
4+ = Almost no fat-free cells.

TABLE II.
Effect of Vitamin B_{12} Concentrate on Hepatic Changes of Rats Fed High-Fat Diet for 66 Days

No. of rats	Avg wt change, g	Avg liver wt, g	Avg liver wt g/100 g rat	No. animals with hepatic fatty changes* 0	1+-2+	3+-4+	Avg liver fat, %
			Normal diet, control				
4	160 ± 3.14	12.1 ± 1.13	4.0 ± 0.37	3	1	0	5.6 ± 1.08
			High fat diet, untreated				
8	43 ± 9.67	15.7 ± 1.07	8.5 ± 0.62	0	1	7	24.6 ± 0.59
			High fat diet, + B_{12} conc. ≈ 1.0 μg B_{12} 3 times a wk				
10	84.6 ± 8.44	10.7 ± 0.71	4.7 ± 0.22	8	1	1	7.8 ± 0.89

* Fatty changes graded as in Table I.

in untreated animals to 16.3% in treated rats (Table I). Histological examination also showed a partial effect of the treatment. The vitamin B_{12} concentrate also prevented the increase in liver weight that usually occurs in untreated rats fed a high-fat diet. There was no significant difference in the gain in weight between the two groups of animals.

Treatment with a higher amount of vitamin B_{12} concentrate (1 μg of vitamin B_{12} 3 times a week) over a period of 66 days demonstrated a marked lipotropic activity of the concentrate. The fat content of the liver of the treated animals was not significantly different from the amount present in rats fed a normal diet (Table II). Histological examinations of the liver also demonstrated the lipotropic activity of the vitamin B_{12} concentrates. The weight of the liver was similarly affected by the treatment and was in the range of the normal controls. Furthermore, there was a significantly greater weight gain in the treated group as compared with the animals fed a high-fat diet alone.

Discussion. A vitamin B_{12} concentrate administered over a period of 29 and 66 days to rats receiving a high-fat diet showed a significant lipotropic effect. This effect is comparable to the previous results obtained with liver extract.[1,2] Both vitamin B_{12} concentrate and liver extract contained a small amount of choline and methionine. An injection of vitamin B_{12} concentrate, containing 1 gamma of vitamin B_{12}, supplied only 0.5 mg of choline, 0.19 mg of methionine, and 0.06 μg of folic acid. Based on 3 injections of the concentrate per week, each rat received only 0.21 mg of choline and 0.08 mg of methionine per day. These amounts of choline and methionine *per se* are ineffective in preventing the dietary induced fatty livers. The rats injected with 0.2 μg of

vitamin B_{12} received only one-fifth of the amount of these added factors. The nature of the lipotropic action of the vitamin B_{12} concentrate is not known. It is possible that the effect of vitamin B_{12} and choline are interrelated, particularly as it has been reported that dietary choline has a specific sparing action on vitamin B_{12} as measured by the growth of the chick.[5] Further studies on the possible lipotropic action of crystalline vitamin B_{12} are in progress.

Conclusion. A concentrate of vitamin B_{12} exerted a marked lipotropic effect when injected into rats receiving a high-fat diet. This lipotropic effect was not due to the small amount of choline present in the concentrate.

5 Schaefer, A. E., Salmon, W. D., and Strength, D. R., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 202.

Received Sept. 26, 1949. P.S.E.B.M., 1949, 72.

A Micro Version of the Barcroft-Warburg Tissue Metabolism Apparatus.* (17443)

S. B. BARKER

From the Department of Physiology, State University of Iowa, Iowa City.

Often it is desirable to measure oxygen consumption or other gaseous exchange of amounts of tissue too small to yield adequate changes in the usual 15-17 ml Warburg vessel. Several different types of apparatus have been constructed for this purpose: Warburg mentioned one of 4 ml capacity,[1] the Fenn type[2] is well known, and in recent years more diversified models have appeared (cf. evaluation by Tobias[3]).

A miniature form of the standard Warburg type, shown in Fig. 1, was designed of 2.5 to 3.3 ml capacity which has proved so convenient that this brief report is being made to note its availability.† The actual size is about 20 mm bottom diameter and 35 mm height, with the main vessel orifice being standard taper 14/20 and the side-bulb opening 6/15. A lip is placed on the center well, facing the side-bulb, for ease in pouring if the barium hydroxide-hydrochloric acid procedure is used for measurement of CO_2 production in the same vessels in which O_2 consumption is determined. Four hooks, instead of the conventional two, are routinely placed on all of our flasks and manometers in order to avoid the need for repairs when one hook is accidentally knocked off.

The manometer has a graduated portion 150 mm long, made from tubing about 1 mm inner diameter, to increase the total capacity as little as possible. Calibration is carried out by the use of mercury, although it is desirable to

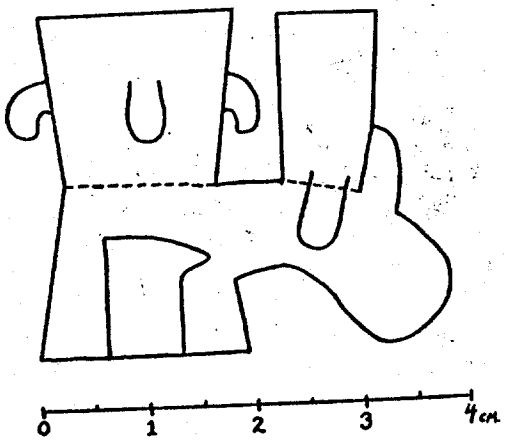


FIG. 1.
Diagram of micro respiration vessel.

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

1 Warburg, O., *Biochem. Z.*, 1923, **142**, 317.

2 Fenn, W. O., *Am. J. Physiol.*, 1928, **80**, 327.

3 Tobias, J. M., *Physiol. Rev.*, 1943, **23**, 51.

† The author wishes to thank the E. Machlett Company, 220 East 23rd St., New York City, for its cooperation in designing and fabricating these vessels.

Lyon Med. 221; 1165, 1969

French Dermatology and Syphilography Society
Meeting of January 16, 1969

Vitamin B_{12} Acne

by P. Dugois, P. Amblard, R. Imbert and
B. de Bignicourt
(Grenoble)

Among iatrogenic acnes, vitamin B_{12} acne seems to be quite frequent. During the last quarter of 1968, we encountered four cases of it at the Grenoble dermatology clinic.

Observations

--Mrs. As..., 32 years old, had not had acne since the age of 20. She came for treatment of acneiform eruptions on the face consisting of innumerable papulopustules on her forehead, cheeks and chin. These eruptions appeared after 11 injections of Maxi B (a combination of thiamine-pyridoxine and hydroxocobolamin 500 gamma).

--Mrs. Br..., 60 years old, presented a papulopustular eruption on the forehead after having received 12 intramuscular injections of a combination of vitamins B_1, B_6, B_{12}, 1000 gamma.

--Mr. Br..., 50 years old, was treated for lumbago with injections of Novobedouze (hydroxocobolamin 1000 gamma); after the tenth injection there appeared acneiform folliculitis, not very significant on the face, but much more marked on the back.

--Mrs. Cap..., 26 years old, usually had a slight outbreak of premenstrual acne made up of a few spots on the forehead. In the course of an attack of viral hepatitis, she had received tablets of a combination of vitamins B_1, B_6, B_{12} (in the form of cyanocobolamin). On the eighth day of treatment which coincided with the day before her menses, she presented a much more intense

outbreak of acne than usual, and this outbreak persisted after menstruation stopped.

Comments

The appearance of these outbreaks of acne can only be related to the taking of vitamin B_{12}:

—they appeared after 8 to 12 days of treatment, to the exclusion of all other factors, in particular to radiological iodine investigation or to treatment involving bromine;

--in three cases, outbreaks occurred at ages unusual for acne (32, 50 and 60 years);

--they rapidly improved after the stopping of vitamin B_{12}.

As in observations previously published, we noted, besides the female predominance, that the form of B_{12} responsible for the acne is most often hydroxocobolamin. Let us note finally, that for three cases out of four, vitamin B_{12} was associated with thiamine and pyridoxine which have, however, been proposed for the treatment of certain types of acne.

Lyon Méd. 221 : 1165-1167, 1969

SOCIETE FRANÇAISE DE DERMATOLOGIE ET DE SYPHILIGRAPHIE

Réunion de Lyon : CLINIQUE DERMATOLOGIQUE
Hôpital Edouard-Herriot - Pavillon R (Professeur THIERS)

Séance du 16 janvier 1969 (suite et fin).

Acnés à la vitamine B 12

P. DUGOIS, P. AMBLARD, R. IMBERT et B. DE BIGNICOURT (Grenoble).

Parmi les acnés iatrogènes, les acnés à la vitamine B 12 semblent assez fréquentes. Au cours du dernier trimestre 1968, nous en relevons quatre cas à la clinique dermatologique de Grenoble.

OBSERVATIONS.

— Mme As..., âgée de 32 ans, n'avait pas présenté d'acné depuis l'âge de 20 ans. Elle vient consulter pour une éruption acnéiforme du visage faite d'innombrables papulo-pustules sur le front, les pommettes et le menton. Cette éruption est apparue après onze injections de Maxi B (association de thiamine-pyridoxine et hydroxocobolamine 500 gamma).

— Mme Br..., âgée de 60 ans, a présenté une éruption papulo-pustuleuse sur le front après avoir reçu douze injections intramusculaires d'une association de vitamines B 1, B 6, B 12, 1.000 gamma.

— M. Br..., âgé de 50 ans, était traité pour un lumbago par des injections de Novobedouze (hydroxocobolamine, 1.000 gamma) ; après la dixième injection, apparaît une poussée de folliculite acnéiforme peu importante sur le visage, beaucoup plus marquée sur le dos.

— Mme Cap..., âgée de 26 ans, présentait habituellement une petite poussée d'acné prémenstruelle faite de quelques éléments sur le front. Au décours d'une hépatite virale, elle reçoit des comprimés d'une association de vitamines B 1, B 6, B 12 (sous forme de cyanocobolamine). Au huitième jour du traitement, qui coïncide avec la veille de ses règles, elle présente une poussée d'acné beaucoup plus intense que d'habitude, et cette poussée se maintient après l'arrêt des règles.

COMMENTAIRES.

L'apparition de ces poussées d'acné ne peut être rapportée qu'à la prise de vitamine B 12 :

— elles apparaissent après huit à douze jours de traitement, à l'exclusion de tout autre facteur, en particulier d'investigation radiologique iodée, ou de thérapeutique contenant du brome ;
— elles surviennent, pour trois cas, à un âge inhabituel pour l'acné (32, 50 et 60 ans) ;
— elles régressent rapidement après l'arrêt de la vitamine B 12.

Comme dans les observations publiées antérieurement, nous notons, outre la prédominance féminique, que la forme de B 12 responsable d'acné est plus souvent l'hydroxocobolamine. Signalons, enfin, que, pour trois des quatre cas, la vitamine B 12 était associée à de la thiamine et à de la pyridoxine qui a pourtant été proposée pour traiter certaines acnés.

Ulcère de jambe d'origine artérielle avec hyperplaquettose et gammopathie monoclonale IgG: Discussion pathogénique

P. DUGOIS, P. AMBLARD, R. IMBERT et B. DE BIGNICOURT (Grenoble).

Le diagnostic étiologique d'un ulcère de jambe est souvent aisé par la seule clinique. Cependant, la pratique systématique de certains examens paracliniques nous a montré qu'il peut exister des perturbations biologiques importantes et latentes. Le problème est alors de savoir si ces perturbations ont eu non un rapport pathogénique avec l'ulcère. L'observation que nous présentons illustre ces faits.

OBSERVATION.

Mme N... Rachel, 84 ans, est admise le 12 avril 1968 à la clinique dermatologique pour ulcère de jambe gauche.

Hormis une dysenterie amibienne en 1940 et une fracture humérale en 1953, cette malade n'a aucun antécédent particulier.

L'ulcère a débuté, quelques jours avant l'admission, par une plaque violacée puis noirâtre, siégeant à la face antéro-externe de la jambe gauche, à l'union des tiers moyen et inférieur ; les douleurs, sensiblement permanentes, étaient vives.

Lancet. Jan. 3, 1970: 35

08:66

Letters to the Editor

CYANOCOBALAMIN: A CASE FOR WITHDRAWAL

SIR,—We note the growing interest in clinical and laboratory studies of cyanide and vitamin-B_{12} metabolism in certain neuro-ophthalmological conditions. The use of hydroxocobalamin in the treatment of these conditions is based on the pharmacological effect of hydroxocobalamin as a powerful cyanide antagonist, with the formation of cyanocobalamin. Recent experience confirms the therapeutic superiority of hydroxocobalamin in the treatment of tobacco amblyopia.[1] Moreover, in the past few months one of us (J. W.) has seen four patients with early Leber's disease who had been treated with large doses of cyanocobalamin instead of hydroxocobalamin, contrary to recommendations.[2] All of these patients developed optic atrophy with a speed and severity which was unusual even in this condition, and this especially unfavourable outcome may have been directly due to the administration of cyanocobalamin.

In drawing attention to the superiority of hydroxocobalamin and the possible adverse effects of cyanocobalamin in these neuro-ophthalmological conditions, we wish to remind our colleagues that hydroxocobalamin, but not cyanocobalamin, is a physiologically active form of vitamin B_{12} which may have other therapeutic advantages in the treatment of vitamin-B_{12} deficiency.[3] To the best of our knowledge, the only circumstances in which the use of cyanocobalamin may have special advantages is in the performance of the Schilling test of vitamin-B_{12} absorption.

Because confusion is likely to persist in the profession over the differences between various forms of vitamin B_{12} commercially available, and over their possible adverse effects, we urge that it would be much safer, and no commercial disadvantage, if manufacturers withdrew cyanocobalamin in favour of hydroxocobalamin for therapeutic use.

A copy of this letter has been sent to the Committee on Safety of Drugs.

Tennent Institute of Ophthalmology, Glasgow. — W. S. FOULDS.
Princess Margaret Hospital, Swindon. — A. G. FREEMAN.
Department of Ophthalmology, Royal Eye Hospital, Manchester. — C. I. PHILLIPS.
M.R.C. Clinical Genetics Research Unit, National Hospital, Queen Square, London. — J. WILSON.

406

GASTROENTEROLOGY
Vol. 63, No. 1
Copyright © 1972 by The Williams & Wilkins Co.
Printed in U.S.A.

SPECIFICITY OF THE VITAMIN B_{12} TRANSPORT SYSTEM IN THE SMALL INTESTINE OF THE NEONATAL RAT

NEIL D. GALLAGHER, M.D., KAREN FOLEY, AND JANICE BROWN

Repatriation General Hospital, Concord, and Department of Medicine, University of Sydney, Sydney, Australia

The amount of vitamin B_{12} transported across the gut of 8- to 10-day-old rats was measured 5 hr after the delivery of 100 ng of $Co^{57}B_{12}$ into the stomach. The experiments were then repeated after the inclusion of 1 μg of a B_{12} analogue in the test dose. B_{12} analogues comprised substituted cobalamins, propionamides, and nucleotides. A total of 13 analogues were tested, eight of which interfered with B_{12} transport. The information which was obtained from these studies about the binding regions of the B_{12} molecule was compared with that obtained from in vitro studies of B_{12} binding in gastric juice and whey. Binding of the vitamin to the intestinal transport protein involved two of the propionamide side chains of the corrin nucleus and an unidentified portion of the 5,6-dimethylbenziminazole base. It bore a close resemblance to B_{12} binding in human gastric juice.

The distal small intestine of the neonatal rat has an infinite capacity to absorb vitamin B_{12}.[1] This phenomenon has been attributed to pinocytosis, which is a nonselective mechanism resulting in the ingestion of large molecules in early life.[2, 3] Once within the intestinal epithelium, the vitamin is located in a system of vacuoles which appears to be part of an intracellular transport system.[4, 5] However, only a small fraction of a large dose of the vitamin leaves the intestinal epithelium and passes to the circulation.[1]

Studies of the transport of the vitamin across neonatal gut epithelium have shown that it occurs independently of gastric intrinsic factor and the vitamin B_{12}-binding protein in milk. From the observations that the transport mechanism was saturable and that analogues interfered with the transport of the vitamin, it was deduced that the mucosa of the small intestine contained a specific B_{12} transport protein.[1] We now report the results of an investigation of the binding characteristics of this protein, using a variety of B_{12} analogues or derivatives. The effect of these compounds on B_{12} binding in human gastric juice and whey was also studied. The characteristics of B_{12} binding in the intestinal transport system were similar to those observed in human gastric juice.

Received October 18, 1971. Accepted February 7, 1972.

Address requests for reprints to: Dr. N. D. Gallagher, Department of Medicine, University of Sydney, Sydney, 2006, Australia.

Materials and Methods

Co^{57}-labeled vitamin B_{12} of specific activity 50 to 150 μc per μg was obtained from the Radiochemical Center, Amersham, Great Britain. Unlabeled vitamin B_{12} was purchased from Spierrel, Milan, Italy. Analogues of vitamin B_{12} were provided by Dr. L. Mervyn, Glaxo Research, Middlesex, Great Britain, and by Dr. J. J. Pfiffner, Wayne State University, Detroit, Michigan.

The spatial formula of the cyanocobalamin molecule, 5,6-dimethylbenziminazolyl cyanocobamide is shown in figure 1.[6] The molecule is divisible into two portions, a corrin and a nucleotide. The corrin ring resembles the structure of the porphyrins. It contains a cobalt atom centrally and acetamide and propion-

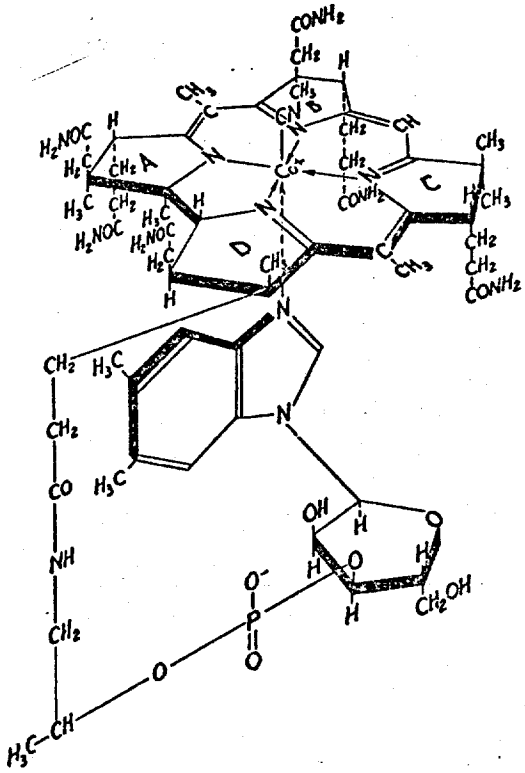


FIG. 1. Spatial formula of vitamin B_{12}, α-(5,6-dimethylbenziminazolyl) cobamide cyanide (Lester Smith, 1965).[8]

amide side chains. The nucleotide consisting of the base 5,6-dimethylbenziminazole, ribose, and phosphoric acid lies in a plane which is nearly at right angles to the corrin nucleus. The phosphate is esterified with 1-amino-2-propanol which is linked with the propionamide side chain of ring D of the corrin nucleus.

The ethylamide, methylamide, monobasic acid, and anilide analogues are formed by substitution of the amide of one of the propionamide side chains, $—CH_2·CH_2·CO·NH_2$, of the A, B, or C rings. The ethylamide monocarboxylic and the dibasic acids contain substitutions of two of the propionamide side chains. Analogues which differ from B_{12} in the nucleotide structure include 5-methylbenziminazole cyanocobamide and B_{12} carbanilide in which the carbanilide is joined to one of the ribose hydroxyl groups. Pseudovitamin B_{12} contains adenine in place of 5,6-dimethylbenziminazole. In hydroxocobalamin, sulfitocobalamin, methylcobalamin, ammonia cobalichrome, and 5'-deoxyadenosylcobalamin, or coenzyme B_{12}, the cyanide which is attached to the cobalt atom is replaced. Solutions of the light-sensitive compounds sulfitocobalamin, coenzyme B_{12}, and methylcobalamin were prepared on the day of the experiments. Precautions were taken to avoid their exposure to light. Stock solutions of the remainder of the analogues were stored in the dark at 4 C until required.

In vivo experiments with B_{12} analogues. Eight- to 10-day-old rats which had been fasted for 14 hr were fed 0.5 ml of a solution containing 100 ng of $Co^{57}B_{12}$. This quantity of the vitamin was chosen because it has previously been shown to saturate the intestinal transport mechanism. The analogue to be tested was included in the dose in a ratio of 10 parts by weight of analogue to 1 part of vitamin B_{12}. The dose was administered from a polyethylene tube which was passed without anesthesia into the stomach. Each animal received 30,000 to 50,000 counts per min of the radioactive vitamin.

The animals were killed by cervical dislocation 5 hr after feeding. The gastrointestinal tract was removed and divided into stomach, small intestine, and colon. The remainder of the animal (which is referred to as the carcass), the washings from the lumen of the small intestine, and the gut wall were assayed separately for radioactivity in a well counter. Details of these procedures have been provided in an earlier study.[1] Absorption refers to the amount of radioactive label which left the intestinal lumen and was recovered from the wall of the small intestine and carcass. Transport represents the amount of radioactivity which left the wall of the gut and was located in the carcass. In preliminary experiments, the absorption of the vitamin was shown to be unaffected by the simultaneous feeding of the analogues. Accordingly, the values for transport only are presented.

In vitro experiments with B_{12} analogues. Pooled human breast milk and neutralized human gastric juice were used as sources of vitamin B_{12}-binding proteins. The whey was collected after centrifugation of whole milk at 25,000 *g* for 1 hr at 5 C. Dilutions (1:5) of whey and gastric juice were prepared with 0.15 M sodium chloride—0.01 M phosphate buffer, *p*H 7.4. The B_{12} binding capacity of whey and gastric juice which was determined by a charcoal-coated albumin method was 25 ng per ml and 22 ng per ml, respectively.[7] After the addition of intrinsic factor-blocking antibody, it was found that 84% of the B_{12} binding in gastric juice was due to intrinsic factor. In

determining the effect of the analogues on B_{12} binding, 20 ng of $Co^{57}B_{12}$ and 200 ng of the analogue were added simultaneously to 0.2 ml of whey or gastric juice. Incubations were carried out at 37 C for 30 min. The degree of interference with B_{12} binding produced by each analogue is expressed as a percentage of the total binding in the sample.

Results

Effect of analogues on the intestinal transport of vitamin B_{12}

$Co^{57}B_{12}$ transport 5 hr after feeding 100 ng of vitamin and 1 μg of the analogues is shown in table 1.

Substituted amides. The ethylamide and methylamide derivatives interfered with B_{12} transport, whereas the anilide, ethylamide monocarboxylic, and monobasic and dibasic acids had no effect.

Cyanide-substituted compounds. The inclusion of hydroxocobalamin, methylcobalamin, 5'-deoxyadenosyl cobalamin, sulfitocobalamin, or ammonia cobalichrome in the test dose reduced B_{12} transport.

Alterations to the nucleotide. The B_{12} carbanilide and 5-methylbenziminazolyl cyanocobamide interfered with transport but the pseudovitamin was ineffective.

Effect of B_{12} analogues on $Co^{57}B_{12}$ binding to human whey and gastric juice

Each of the three classes of analogues interfered with B_{12} binding to whey proteins (table 2). The degree of interference produced by the individual analogues was similar, with the exception of the ethylamide monocarboxylic, monobasic, and dibasic acids, which were less effective. The substituted cobalamins and the ethylamide and methylamide derivatives also reduced B_{12} binding in gastric juice (table 2). However, the ethylamide monocarboxylic and dibasic acids did not alter binding, and the anilide and monobasic acids had little effect. B_{12} binding in gastric juice was slightly reduced in the presence of the pseudovitamin, in contrast to the results with the whey in which a moderate degree of interference was apparent.

TABLE 1. *Effect of analogues on B_{12} transport across neonatal rat intestine*[a]

B_{12} analogue or derivative	$Co^{57}B_{12}$ transport
	ng
Control	23.1 ± 2.9 (24)
Methylamide	8.8 ± 1.7 (6)
Ethylamide	8.8 ± 2.1 (5)
Ethylamide monocarboxylic acid	25.1 ± 4.8 (5)
Monobasic acid	22.6 ± 7.7 (7)
Dibasic acid	23.6 ± 3.4 (5)
Anilide	21.6 ± 1.2 (5)
Hydroxocobalamin	7.9 ± 2.1 (5)
Sulfitocobalamin	9.7 ± 2.5 (5)
Methylcobalamin	9.0 ± 2.9 (5)
Ammonia cobalichrome	10.0 ± 4.0 (4)
B_{12} carbanilide	12.5 ± 1.9 (5)
5-Methyl benziminazole cyanocobamide	10.1 ± 3.0 (4)
Pseudo-B_{12}	22.5 ± 3.2 (6)

[a] $Co^{57}B_{12}$ transport from gut to carcass was measured 5 hr after feeding 100 ng of vitamin B_{12} and 1 μg of the respective analogues. The results are shown as the mean ± standard deviation with the number of experiments in parentheses.

TABLE 2. *Effect of analogues on B_{12} binding in human whey and gastric juice*[a]

B_{12} analogue or derivative	Percentage reduction in B_{12} binding capacity	
	Whey	Gastric juice
Methylamide	79	61
Ethylamide	76	47
Ethylamide monocarboxylic acid	41	0
Monobasic acid	62	17
Dibasic acid	64	0
Anilide	78	9
B_{12} coenzyme	75	81
Methylcobalamin	73	80
B_{12} carbanilide	69	56
5-Methyl benziminazole cyanocobamide	70	58
Pseudo-B_{12}	79	18

[a] $Co^{57}B_{12}$ (20 ng) and 200 ng of analogue were added simultaneously to 0.2 ml of whey or gastric juice. Incubations were carried out for 30 min at 37 C. Each value is the mean of two experiments performed in duplicate.

Discussion

In an earlier account of the transport of vitamin B_{12} across the gut of the neonatal rat, it was shown that the kinetics of the transport mechanism were of the Michaelis-Menten type.[1] This observation led to the postulate that the transport of the vitamin required a specific binding protein. A high order of specificity was suggested by the finding of a binding or affinity constant of 0.4×10^{-10} M.[8] Additional support for the existence of a specific binding protein has been obtained in this study in which a large number of closely related molecules were found to interfere with the transport of the vitamin.

The presence of the analogues in the test dose did not invariably produce a reduction in the transport of the vitamin. The failure of certain analogues to influence B_{12} transport may therefore be regarded as indicating the regions of the B_{12} molecule which are required in binding to the transport protein. However, it is possible that groups of lesser importance in binding may have been revealed by varying the ratio of analogue to vitamin. Substitutions of the amide portion of one of the propionamide side chains, $—CH_2 \cdot CH_2 \cdot CO \cdot NH_2$, as in the formation of the monobasic acid, —OH, or anilide, $—NH \cdot C_6H_5$, were accompanied by loss of the ability to impede transport. Although one of the propionamide side chains is involved in binding, the terminal amide is not essential because both the ethylamide $—NH \cdot C_2H_5$ and the methylamide $—NH \cdot CH_2$ competed with the vitamin for transport. The involvement of a second propionamide group in binding is suggested by the observation that the ethylamide monocarboxylic and the dibasic acids did not affect the transport of the vitamin.

The experiments with the adenine-containing pseudovitamin B_{12} indicate that the 5,6-dimethylbenziminazole is also concerned in binding. The regions of the base which bind to protein are unknown, but at least one of the methyl groups does not appear to be involved because the 5-methylbenziminazole derivative retained the capacity to affect B_{12} transport. The presence of the carbanilide on one of the ribose hydroxyls also impaired the transport of the vitamin. The unimportance of the ligand attached to the cobalt atom was shown in the experiments in which each of the five substituted cobalamins including methylcobalamin, hydroxocobalamin, and coenzyme B_{12}, the naturally occurring forms of vitamin B_{12}, interfered with transport.

The results of the studies of the effect of the analogues on vitamin B_{12} binding in gastric juice and whey resembled that which was found in the experiments with rat small intestine. Substituted amides and cobalamins, and major alterations in the nucleotide structure, as in the pseudovitamin, produced similar effects in each system. Although gastric juice contains intrinsic factor and nonintrinsic factor binders, the degree of interference with B_{12} binding produced by the analogues was, in general, greater than could be explained by the presence of nonspecific binders. The results differ from those of another group who found that the effect of the analogues was restricted to nonspecific binders, when the ratio of the vitamin to analogue was unity.[9] The interference with intrinsic factor binding, which represented 84% of the total binding capacity, by substituted cobalamins, and the negligible effect of the pseudovitamin have been reported by others.[10-12] The difference in the extent to which the members of each class of analogue interfered with B_{12} binding to each of the human proteins confirms the observation that B_{12} binding to intrinsic factor is more specific.[10, 11]

The similarity between the effect of the analogues on binding in human gastric juice and milk and in neonatal rat epithelium suggests that there is a structural resemblance in the binding region of the B_{12} transport proteins. However, this region may have a definite specificity, because, although human intrinsic factor-blocking antibody cross-reacts with intrinsic factor from different species,[8, 13] it does not abolish B_{12} binding to other B_{12} transport proteins.

The observation that purified intrinsic

factor becomes more compact after combination with vitamin B_{12} has led to the proposal that the vitamin enters a pit or depression in the intrinsic factor molecule.[14] From the results of earlier studies with pseudo-B_{12} and other analogues, it was suggested that the nucleotide portion was directed toward intrinsic factor whereas the cyanide portion faced out.[14] Our results support this orientation of the B_{12} molecule and suggest that it may also be a feature of binding to the intestinal transport protein. Binding would require multipoint attachment, which has been proposed by others,[6] and would involve two of the propionamide side chains and a portion of the base.

REFERENCES

1. Gallagher ND, Foley K: Intrinsic factor independent transport of vitamin B_{12} across the gut of the neonatal rat. Gastroenterology 61:332-338, 1971
2. Clark SL: The ingestion of proteins and colloidal material by columnar absorptive cells of the small intestine in suckling rats and mice. J Biophys Biochem Cytol 5:41-50, 1959
3. Kraehenbuhl JP, Gloor E, Blanc B: Resorption intestinale de la ferritine chez deux especes animales aux possibilites d'absorption proteique neonatale differentes. Z Zellforsch Mikrosk Anat 76:170-186, 1967
4. Graney DL: The uptake of tritiated vitamin B_{12} by intestinal absorptive cells of suckling rats. An electron microscope autoradiographic study. Anat Rec 160:355-366, 1968
5. Cornell CA, Padykula HA: A cytological study of intestinal absorption in the suckling rat. Am J Anat 125:291-316, 1969
6. Smith EL: Vitamin B_{12}. New York, John Wiley, 1965, p 115-126
7. Gottlieb CW, Lau KS, Wasserman LR, et al: Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated binding capacity, antibody to IF, and serum unsaturated binding capacity. Blood 25:875-884, 1965
8. Gallagher ND, Foley K: Corticosteroids and the development of intrinsic factor-mediated vitamin B_{12} absorption in the rat. Gastroenterology 62: 247-254, 1972
9. Gottlieb CW, Retief FP, Herbert V: Blockade of vitamin B_{12} binding sites in gastric juice, serum and saliva by analogues and derivatives of vitamin B_{12} and by antibody to intrinsic factor. Biochim Biophys Acta 141:560-572, 1967
10. Bunge MD, Schilling RF: Intrinsic factor studies VI. Competition for vitamin B_{12} binding sites offered by analogues of the vitamin. Proc Soc Exp Biol Med 96:587-592, 1957
11. Gregory ME, Holdsworth ES: The binding of cyanocobalamin and its naturally occurring analogues by certain body fluids and tissue extracts. Biochim Biophys Acta 42:462-469, 1960
12. Chanarin I: The Megaloblastic Anaemias. Blackwell, Oxford, 1969, p 64-96
13. Frentz GD, Hansen HJ, Miller ON: Antigenic and chemical similarities between B_{12} binders of human gastric juice and B_{12} binders extracted from the fundus of the chimpanzee stomach (abstr). Fed Proc 25:429, 1966
14. Grasbeck R: Intrinsic factor and the transcobalamins with reflection on the general function and evolution of soluble transport proteins. Scand J Clin Lab Invest 19(suppl 95):7-18, 1967

Brit. J. Surg. 54(2) 123-131 (1967)

ABSORPTION OF VITAMIN B_{12} FROM THE SMALL INTESTINE

STUDY IN MAN, MONKEY, CAT, AND DOG*

J.-C. GAZET AND IAN McCOLL
GUY'S HOSPITAL, LONDON

THE ileum is thought to be the probable site of absorption of physiological amounts of vitamin B_{12} in man (Booth, 1957, 1961; Booth and Mollin, 1957; Mollin, Booth, and Baker, 1957; Doscherholmen, Finley, and Hagen, 1960; Allcock, 1961; Glass, 1963). This conclusion was based on the finding that disease of the ileum or resection of the ileum may cause impairment of vitamin-B_{12} uptake, whereas similar disease or resection of the jejunum does not. Furthermore, Booth and Mollin (1959) measured the Co^{58}-labelled vitamin B_{12} content of the small bowel at operation 3 hours after an oral dose of the vitamin and found that the maximum uptake occurred in the ileum. They suggested that only the ileum could absorb vitamin B_{12} because of the presence of a specific receptor mechanism in the ileum. Glass (1963) stated that the upper jejunum did not have any evident absorptive ability for physiological amounts of vitamin B_{12}, but some absorption from the jejunum has been recorded by Citrin, Derosa, and Halsted (1957a, b), Doscherholmen and others (1960), and Ronnov-Jessen and Hansen (1965).

* Paper read at the Surgical Research Society, London, January, 1966.

From a morphological point of view we found it difficult to believe that the ileum has an exclusive ability to absorb vitamin B_{12}. This paper records experiments designed to find out whether the jejunum, given the opportunity, can absorb physiological amounts of vitamin B_{12}. Intrinsic factor was not used. Absorption experiments were performed in surgical patients, monkeys, cats, and dogs.

METHOD

Absorption was studied by a method similar to that described by McColl and Nissim (1965). At laparotomy, under general anaesthesia, a loop of proximal jejunum and a loop of distal ileum were emptied by digital pressure and then isolated with non-crushing intestinal clamps which did not interfere with the mesenteric vessels. Normal saline, 50–100 ml., at 37° C. was then injected through the wall into the lumen with a syringe and needle. The loops were again emptied and this washing process repeated. This seems likely to have removed all particulate matter from the bowel as well as all detectable amounts of intrinsic factor (McColl, Gazet, Raven, Andrews, and Streek, 1966). Equivalent lengths of jejunum and ileum were measured and clamped off. The width of the bowel was taken into account to give segments of approximately equal surface area (150 sq. cm.). Into each isolated and washed segment of bowel a known volume of normal saline at 37° C., containing various concentrations of radioactive vitamin B_{12}, was injected. Either Co^{57}- or Co^{58}-labelled vitamin B_{12} (specific activity 1·1–7·0 c. per μg.) was used in solutions containing 0·002 to 0·011 μg. per ml. and this gave counts of 0–850 per sec. per 5 ml. In some experiments Na^{22} sodium chloride (specific activity 0·1 μc. per ml. normal saline) was used. The solutions were withdrawn after an interval of 15 min., their volume noted, and their radioactivity measured in a well scintillation counter. The percentage of vitamin B_{12} absorbed was calculated from the amount of vitamin B_{12} recovered compared with the total put in. In the interval between injection and aspiration of the test solutions, the surgeon was usually able to proceed with the operation. During this time segments of bowel under investigation were kept as near as possible to body temperature. The serum vitamin B_{12} level was estimated in most of the patients, monkeys, cats, and dogs.

The patients' permission was obtained after it had been fully explained to them that the proposed procedure extended the operation by 10 min. and was in no way therapeutic. The permission was obtained in accordance with the proposals laid down by the Medical Research Council (1962), but no written statement was recorded.

RESULTS

In preliminary experiments in 3 dogs and 5 monkeys the absorption of Co^{58}-labelled vitamin B_{12} and Na^{22}-labelled sodium chloride from jejunal and ileal segments was estimated over a period of 1 hour by taking serial samples from the solutions in the bowel lumen. The rate of absorption of normal saline was found to be approximately linear in both jejunum and ileum (*Fig.* 1). As the percentage absorption of vitamin B_{12} did not increase after the first reading at 15 min. (*Fig.* 2), this period of time was selected for all subsequent vitamin B_{12} absorption studies. This allowed a measurable amount of absorption to occur and prevented the clamps being left on the bowel for too long.

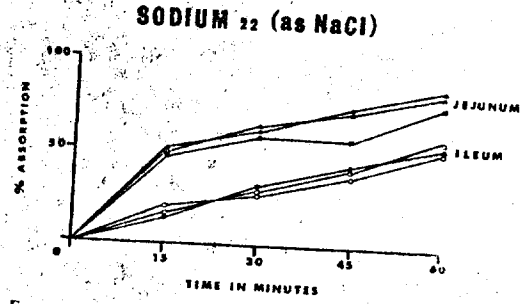


FIG. 1.—Graph showing the pattern of absorption of normal saline in jejunal and ileal segments in 3 dogs.

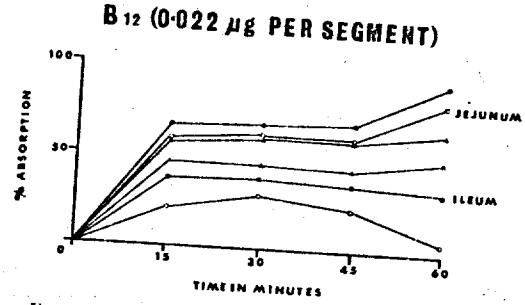


FIG. 2.—Graph showing the pattern of absorption of vitamin B_{12} (0·022 μg. in 10 ml. of normal saline) in jejunal and ileal segments of the 3 dogs in *Fig.* 1.

Table I.—ABSORPTION OF VITAMIN B_{12} IN MAN

Patient No.	Per Cent Absorption		Vitamin B_{12} injected into each segment		Serum Vitamin B_{12}
	Jejunum	Ileum	Volume (ml.)	Concentration (μg. per ml.)	(μg. per mg.)
1	20·0	16·6	50	0·0002	0·000370
2	16·7	11·2	50	0·0002	0·001100
3	12·6	8·4	25	0·0002	0·000280
4	28·0	11·1	25	0·0002	0·000390
5	20·2	7·2	25	0·0002	0·000440
6	16·0	13·0	25	0·001	0·000470
7	19·6	7·3	25	0·001	0·000950
8	17·8	16·7	25	0·001	0·000094
9	8·6	9·4	50	0·001	0·000440
10	13·5	16·5	50	0·001	0·000380
11	17·1	6·6	50	0·001	0·000120
12	15·0	25·0	50	0·001	0·000250
13	11·7	2·9	50	0·002	0·000025
14	33·3	28·8	50	0·002	0·000370
15	31·8	14·1	50	0·002	0·000430
16	20·7	18·1	50	0·002	0·000260
17	13·5	6·4	50	0·004	—
18	26·8	9·4	50	0·004	0·000160
19	30·5	32·9	25	0·004	—
20	25·9	17·4	25	0·004	0·000470

In a total of 326 segments studied in 20 patients, 10 monkeys, 9 cats, and 36 dogs, little difference was found between the absorptive ability of the jejunum and ileum.

The results of 20 human experiments are depicted in *Table I*. Regression lines were calculated for jejunal absorption ($b = 0{\cdot}194$, S.E. $(b) = \pm 0{\cdot}114$) and ileal absorption ($b = 0{\cdot}142$, S.E. $(b) = \pm 0{\cdot}129$). An analysis of covariance indicated that the jejunum absorbed more vitamin B_{12} than the ileum ($0{\cdot}01 < P < 0{\cdot}02$). It will be seen that 5 of the experiments involved a concentration of 0·0002 μg. per ml. of vitamin B_{12} in the bowel lumen which was less than the levels of vitamin B_{12} in the serum, and in another 7 the concentration of 0·001 μg. per ml. was at the upper limit of normal serum vitamin B_{12} levels (normal serum vitamin B_{12} = 0·0001—0·0011 μg. per ml.). Absorption in these cases must therefore involve active transport. As the concentration in the other 9 cases was either 0·002 or 0·004 μg. per ml., absorption could have involved passive diffusion or active transport.

Table II.—Absorption of Vitamin B_{12} in Monkeys

Animal No.	Per Cent Absorption		Serum Vitamin B_{12} (μg. per ml.)
	Jejunum	Ileum	
1	24 (27)	33 (25)	0·001200
2	10 (18)	19 (26)	0·000580
3	14 (38)	18 (21)	—
4	25 (18)	13 (20)	0·001800
5	39	31	0·000320
6	12	11	0·001000
7	14	55	—
8	14	2	—
9	35	39	—
10	14	17	0·001300

Each value is the mean of the percentage absorption of three segments. The figures in brackets are the values obtained when the tests were repeated in the same animals 3 months later. The concentration of vitamin B_{12} injected into the segments was 0·011 μg. per ml. There was no significant difference ($0{\cdot}4 < P < 0{\cdot}5$) between the absorption in the jejunum ($\bar{x} = 20{\cdot}1$, S.E. $\pm 3{\cdot}2$) and the ileum ($\bar{x} = 23{\cdot}8$, S.E. $\pm 4{\cdot}9$).

Table III.—Absorption of Vitamin B_{12} in Dogs

Animal No.	Per Cent Absorption		Serum Vitamin B_{12} (μg. per ml.)
	Jejunum	Ileum	
1	19	14	0·000080
2	23	50	0·000860
3	5	15	0·000190
4	12	14	0·000330
5	13	9	0·000080
6	12	6	0·000290
7	7	9	0·000320
8	19	24	0·000035
9	8	13	0·000010
10	10	15	0·000325
11	33	13	0·000380
12	9	14	0·000280
13	11	4	0·000840
14	14	7	0·000060
15	21	5	0·000120
16	11	15	0·000400
17	18	4	—
18	12	23	0·000200
19	12	6	0·000115
20	2	6	0·000480
21	4	20	0·000130
22	25	25	—
23	7	11	—
24	7	19	—
25	8	12	—
26	3	6	—
27	8	13	—
28	19	24	—

Each of the values in the first 7 dogs is the mean of the percentage absorption of three segments. The concentration of vitamin B_{12} injected into the segments was 0·011 μg. per ml. There was no significant difference ($0{\cdot}4 < P < 0{\cdot}5$) between the absorption in the jejunum ($\bar{x} = 12{\cdot}6$, S.E. $\pm 1{\cdot}4$) and the ileum ($\bar{x} = 14{\cdot}1$, S.E. $\pm 1{\cdot}8$).

Table IV.—Absorption of Differing Concentrations of Vitamin B_{12} in 8 Dogs

Concentration of Vitamin B_{12} Injected into Each Segment (μg. per ml.)	Per Cent Absorption	
	Jejunum	Ileum
0·011000	6·8	12·7
0·005500	9·6	6·5
0·002800	8·1	4·2
0·001400	0·5	4·5
0·000700	7·5	9·2
0·000350	7·8	12·7
0·000180	7·9	14·2
0·000090	14·6	24·6

Each value is the mean of the percentage absorption of four segments.

Table V.—Absorption of Vitamin B_{12} in Cats

Animal No.	Per Cent Absorption		Serum Vitamin B_{12} (μg. per ml.)
	Jejunum	Ileum	
1	14	11	0·000390
2	15	11	0·000430
3	13	16	0·000400
4	13	14	0·000270
5	15	7	0·000500
6	7	13	0·000190
7	6	14	0·000180
8	13	13	0·000325
9	14	9	0·000640

Each value is the mean of the percentage absorption of three segments. The concentration of vitamin B_{12} injected into the segments was 0·011 μg. per ml. There was no significant difference ($0{\cdot}8 < P < 0{\cdot}9$) between the absorption in the jejunum ($\bar{x} = 12{\cdot}2$, S.E. $\pm 3{\cdot}5$) and the ileum ($\bar{x} = 12{\cdot}0$, S.E. $\pm 0{\cdot}9$).

Table VI.—Liver Uptake of Vitamin B_{12} in Man

Patinet No.	Per Cent Uptake by Liver from Jejunum	Per Cent Absorption from Jejunum	Per Cent Uptake by Liver from Ileum	Per Cent Absorption from Ileum
1	2·0	20·0	1·26	16·6
2	1·4	16·7	0·5	11·2
12	2·4	15·0	1·6	25·0

Vitamin B_{12} absorption was studied in 10 rhesus monkeys, all of which provided 3 jejunal and 3 ileal segments. These results are shown in *Table II*. Student's *t* test revealed no significant difference between jejunal and ileal absorption ($0{\cdot}4 < P < 0{\cdot}5$). The tests were repeated in 4 of the monkeys after an interval of 3 months and no significant difference was found between the same segments of jejunum ($0{\cdot}3 < P < 0{\cdot}4$) and between the same segments of the ileum ($0{\cdot}5 < P < 0{\cdot}6$). Barnard's sequential *t* testing failed to establish a significant difference.

The results of the absorption experiments in the 28 dogs are presented in *Table III*. No significant difference was found between jejunal and ileal

absorption ($0{\cdot}4<P<0{\cdot}5$). The effect of varying the concentration of vitamin B_{12} in 64 segments in 8 dogs is shown in *Table IV*; 54 segments were studied in 9 cats (*Table V*). The difference in ileal and jejunal absorption was not significant ($0{\cdot}8<P<0{\cdot}9$) and Barnard's sequential *t* test failed to establish a significant difference.

Finally, experiments were carried out in 3 patients to try to determine whether the method of calculating the percentage absorption was valid. It had been assumed that the amount of vitamin B_{12} absorbed was the difference between the amount put into a segment and the amount retrieved. To be sure that this difference represents the amount absorbed, it would be necessary to demonstrate the vitamin B_{12} in the blood, or to estimate the vitamin B_{12} uptake of the liver. Therefore, equal amounts of Co^{57} vitamin B_{12} and Co^{58} vitamin B_{12} were placed in segments of jejunum and ileum respectively. The percentage absorption from the jejunal and ileal segments was calculated from the amounts of vitamin B_{12} aspirated from the segments. After 3 days the uptake of Co^{57} and Co^{58} vitamin B_{12} by the liver was measured by means of a 2-in. well scintillation counter (sodium iodide crystals). The two isotopes were differentiated by pulse-height analysis. In all 3 cases both isotopes were detected in the liver. They were present in quantities which approximately corresponded to those absorbed from the jejunal and ileal segments in 2 cases (*Table VI*).

DISCUSSION

The results of this investigation have shown that in the normal alimentary tract, given the opportunity, the upper segment of jejunum is capable of absorbing vitamin B_{12} in the absence of intrinsic factor to the same extent as the distal ileum. Under these circumstances this would suggest that there is no specialized or exclusive site for the absorption of vitamin B_{12} in man, monkey, cat, and dog.

Our experiments were designed to exclude four factors, which are being studied currently: (1) abnormality of the alimentary tract; (2) contents of the bowel; (3) transit time of bowel content; and (4) presence of intrinsic factor. All experiments were performed in normal animals or patients with apparently normal small bowel.

Clinical observations have shown that there is impairment of vitamin B_{12} absorption with regional enteritis limited to the ileum, but not when the disease is confined to the duodenum or jejunum (Cooke, Cox, Meynell, and Gaddie, 1957). The pathogenesis of this disease, however, is poorly understood and unknown factors may be present which impair ileal or jejunal absorption.

The presence of *Escherichia coli* in the small intestine can impair vitamin B_{12} absorption (Booth and Heath, 1962) and this may be improved by the administration of antibiotics (Booth, 1957).

Intestinal motility is a factor which has received little attention and may account for some of the conflicting results in vitamin B_{12} studies. Normally, the contents of the small bowel pass rapidly through the jejunum but linger in the ileum, and this may account for the reports that vitamin B_{12} is absorbed by the ileum. Booth and Mollin (1959) do not subscribe to this view. With massive resection of the small bowel, leaving only duodenum and 3–4 ft. of jejunum, no absorption of vitamin B_{12} occurs (Mollin and others, 1957). After this form of surgery, however, intestinal hurry is an important factor in reducing absorption (Baldwin-Price, Copp, and Singleton, 1965).

The effect of intrinsic factor will not be discussed here as it is under investigation (McColl and others, 1966). If current concepts of vitamin-B_{12} absorption are true, it is surprising that we obtained consistent amounts of absorption in the apparent absence of intrinsic factor.

SUMMARY

A series of vitamin B_{12} absorption studies were carried out in man, monkey, cat, and dog. The ability of isolated intestinal segments of jejunum and ileum to absorb vitamin B_{12} *in vivo* was compared and little difference between these sites was found. These results do not support the contention that vitamin B_{12} can only be absorbed by the ileum by an exclusive mechanism.

Acknowledgements.—We are greatly indebted to the consultant surgeons, registrars, and theatre staff of Guy's Hospital for their helpful co-operation, without which this work would have been impossible. We are very grateful to Dr. J. Raven for the serum vitamin B_{12} estimations, to Dr. K. Taylor and Mrs. D. Andrews for their assistance with the radioisotope techniques, to Mr. Phelan and the staff in the animal laboratory, and to Mrs. Streek for her invaluable technical assistance. Our thanks are due to Professor Hedley Atkins and Dr. I. MacDonald for their helpful suggestions in the preparation of this paper.

REFERENCES

ALLCOCK, E. (1961), *Gastroenterology*, **40**, 81.
BALDWIN-PRICE, H. K., COPP, D., and SINGLETON, A. O., jun. (1965), *Ann. Surg.*, **161**, 225.
BOOTH, C. C. (1957), M.D. Thesis, St. Andrews University.
— — (1961), *Post-grad. med. J.*, **37**, 725.
— — and HEATH, J. (1962), *Gut*, **3**, 70.
— — and MOLLIN, D. L. (1957), *Lancet*, **2**, 1001.
— — — — (1959), *Ibid.*, **1**, 18.
CITRIN, Y., DEROSA, C., and HALSTED, J. A. (1957a), *J. Lab. clin. Med.*, **50**, 667.
— — — — — — (1957b), *Fedn Proc. Fedn Am. Socs exp. Biol.*, **16**, 353.
COOKE, N. T., COX, E. V., MEYNELL, M. J., and GADDIE, R. (1957), *Lancet*, **2**, 1231.
DOSCHERHOLMEN, A., FINLEY, P. R., and HAGEN, P. S. (1960), *J. Lab. clin. Med.*, **56**, 547.
GLASS, G. B. J. (1963), *Physiol. Rev.* **43**, 529.
MCCOLL, I., GAZET, J.-C., RAVEN, J., ANDREWS, D., and STREEK, S. (1966), in the press.
— — and NISSIM, J. A. (1965), *Nature, Lond.*, **207**, 949.
MEDICAL RESEARCH COUNCIL (1962), Cmnd. 2382, 21.
MOLLIN, D. L., BOOTH, C. C., and BAKER, S. J. (1957), *Br. J. Haemat.*, **3**, 412.
RONNOV-JESSEN, V., and HANSEN, J. (1965), *Blood*, **25**, 224.

The effect of Vitamin B-12 on the limits of resistance in hypo-nutrition and on the daily percentage loss of body Weight.

By O. Giacalone
(Of the Institute for Human Physiology of the University of Palermo)

Palermo section -- Session of 30 May 1955.

In a preceding note (Giacalone, 1) we have referred to the effect of aneurin in rats subjected to caloric hypo-nutrition that is slow and progressive. The experimental results obtained by us allowed us to conclude that the administration of vitamin B-12 in excess of ~~with respect to~~ the daily requirement accentuates the daily percentage loss of body weight in under-nourished rats so that the greatest loss of body weight ~~(body)~~ compatible with life is ~~xxxxxxxx~~ reached earlier, but ~~however~~, with values that are almost identical with those for under-nourished rats that did not receive Vitamin B-12.

Sure and collaborators (2), Sure (3), Graham and Griffith, (4) Whipple and Church (5), McHenry (6-7) and others referred to the favorable effect of aneurin on body weight; in our research as well, (Rubino and Giacalone, 8,9; Giacalone and Monaco,10) on the influence of the vitamins of group B on the reconstructive processes in re-nutrition after ~~xxxxxxxxxxxxxxxx~~ food deprivation, we have demonstrated a clear effect of the vitamin B-12 understood to favor the development of reconstructive phenomena under discussion so that the rats treated with this vitamin regained the weight loss caused by previous food deprivation more ~~xxxxxx~~

quickly than the control rats, but in the two cases the amount of food consumed daily remained almost the same. It can therefore be seen from the results referred to above that under conditions of hypo-nutrition, the favorable effect of vitamin B-12 is lacking when the food is quantitatively and qualitatively adequate; in under-nourished rats, on the other hand, a greater intake of Vitamin B-12 than the normal daily requirements accentuates the development of the destructive processes.

Ciancimino and Rubino (11), recently studied the influence of Vitamin B-2 and of pantothenic acid on the ~~development~~ course of under-nutrition.

On the basis of their experimental results, they conclude that the administration of Vitamin B-2 in excess of the daily requirement does not influence the course of under-nutrition, but the administration of pantothenic acid in excess of the daily requirement causes, in under-nourished rats, an attenuation of the daily percentage loss of body weight and a later attainment of the maximum percentage loss of weight compatible with life, that still yields values almost equal to those of the controls.

Continuing this research, we studied the influence of Vitamin B-12 on the course of slow and progressive caloric hypo-nutrition in the rat, we being able also to compare these results with the data obtained by us (10) studying the influence of Vitamin B-12 on the re-

constructive processes in re-nutrition after food deprivation.

The research was carried out in the following manner: White, male, adult rats coming from the same litter, after having been under observation for a certain period of time during which they were able to eat at will (milk, meat, bread, greens) were selected; the animals chosen (on the basis of their generally good condition) were divided into two groups of five rats each. The animals of the two groups were kept separate one group from the other in suitable cages kept at an environmental temperature of 16-21°C. All the rats were subjected to slow and gradual under-nutrition by the progressive decrease of the daily quantity of food offered, up to the point where they died. The food was composed of wheat starch - 75% - lactic casein - 10%, butter-10%, saline mixture - 5%. The animals received, furthermore, filter paper and a sufficient quantity of vitamins to cover the daily requirements. Water was furnished in any amount they wished. All the animals received daily a quantity of food corresponding to 0.215 calories on an average per gram of body weight.

On alternate days the rats of the second group received hypodermically 8 μgr. of vitamin B-12; those of the first group, the control, always received on alternate days only 0.5 ml of physiological solution.

The results obtained summarized in the table annexed, permit the demonstration of the following facts:

The rats that were undernourished and subjected to the administration of Vitamin B-12 reached more quickly (52.0 ± 0.47 days on the average compared with 54.4 days ± 0.26 on the average for the control rats) the limit of resistance compatible with life expressed by the maximum total percentage loss of body weight (48.6 ± 0.26 on the average for rats treated with Vitamin B-12 and 47.4 ± 0.36 on the average for the control rats), that did not yield perceptively different results in the two groups of rats. This fact agrees with the greater daily percentage loss of body weight shown by the rats treated with Vitamin B-12 (0.93 ± 0.0008 on the average compared with 0.86 ± 0.0003 on the average for the control rats).

The differences with respect to the days of survival and the daily loss of body weight for rats treated with Vitamin B-12, $t = 3.108$ ($P < 0.02$) and $t = 6.754$ ($P < 0.001$) are statistically significant in comparison with those that were not treated.

From the above results, it can therefore be concluded that the administration of Vitamin B-12 in excess of the daily requirement accentuates the daily percentage loss of body weight so that the maximum loss of body weight compatible with life in undernourished rats treated with Vitamin B-12 is reached earlier but with the values being almost identical in the two cases.

Table I

TABELLA I.

Average values of

Valori medi di

Lotto dei ratti (Group of rats)	Condizione sperimentale (Experimental condition)	N. dei ratti sperimentali (No. of rats in experiment)	peso iniziale (initial weight) g	peso alla morte (weight at death) g	peso perduto (weight lost) g	alimento consumato in calorie pro g e pro die (food consumed in calories per gm. per day)	giorni di esperienza (days of experiment)	peso percentuale perduto (percentage weight lost)	peso percentuale perduto pro die (percentage of weight lost per day)
I	Ratti ipoalimentati di controllo (Under-nourished control rats)	5	188	99	89	0,215	54,4 ± 0,26	47,4 ± 0,36	0,86 ± 0,0003
II	Ratti ipoalimentati che ricevevano a giorni alterni μg 8 di vit. B_{12} durante la ipoalimentazione (Under-nourished rats that received 8 μg of vit. B_{12} on alternate days during period of hypo-nutrition)	5	183	97	91	0,215	52,0 ± 0,47	48,6 ± 0,26	0,93 ± 0,0008

Table II

TABELLA II.

Lotto dei ratti (Group of rats)	Condizione sperimentale (Experimental condition)	Lotti comparati (Groups compared)	Valori medi di giorni di esperienza (Average values of experiment days)	t =	P	Valori medi del peso percentuale perduto (Average values of weight percentage lost)	t =	P	Valori medi del peso percentuale perduto pro die (Average values of weight percentage lost per day)	t =	P
I	Ratti ipoalimentati di controllo (Control undernourished rats)	I – II	54,4 ± 0,26	3,108	< 0,02	47,4 ± 0,36	0,032	< 0,4	0,86 ± 0,0003	6,751	< 0,001
II	Ratti ipoalimentati che ricevevano a giorni alterni μg 8 di vit. B_{12} durante la ipoalimentazione (Undernourished rats that received on alternate days 8 μg of vit. B_{12} during period of hypo-nutrition)		52,0 ± 0,47			48,6 ± 0,26			0,93 ± 0,0008		

0948

AZIONE DELLA VITAMINA B_{12} SUI LIMITI DI RESISTENZA ALL'IPOALIMENTAZIONE E SULLA PERDITA PERCENTUALE GIORNALIERA DI PESO CORPOREO. — O. GIACALONE.

(Dall'Istituto di Fisiologia Umana dell'Università di Palermo).

Sezione di Palermo — Seduta del 30 maggio 1955.

In una precedente nota (Giacalone, 1) abbiamo riferito sull'azione svolta dalla aneurina nei ratti sottoposti ad ipoalimentazione calorica, lenta e progressiva. I risultati sperimentali da noi ottenuti ci hanno permesso di concludere che la somministrazione di vit. B_1, in eccesso rispetto al fabbisogno giornaliero, accentua la perdita percentuale giornaliera del peso corporeo dei ratti ipoalimentati, sicchè la massima perdita di peso corporeo compatibile con la vita è raggiunta più precocemente, pur rimanendo di entità pressochè identica a quella dei ratti ipoalimentati ma che non ricevono vit. B_1.

Sure e coll. (2), Sure (3), Graham e Griffth (4), Whipple e Church (5), McHenry (6-7) et alii, riferiscono sull'azione favorevole svolta dalla aneurina sul peso corporeo; anche con nostre ricerche (Rubino e Giacalone, 8, 9; Giacalone e Monaco, 10) sull'influenza delle vitamine del gruppo B sui processi ricostruttivi nella rialimentazione dopo digiuno, abbiamo messo in evidenza una spiccata azione della vit. B_1 intesa a favorire il decorso dei fenomeni ricostruttivi in parola, sicchè i ratti trattati con tale vitamina hanno recuperato il peso perduto per un precedente digiuno più prontamente rispetto ai ratti di controllo, pur restando nei due casi pressocchè eguale la quantità di alimento giornalmente assunta. Dai risultati sopra riferiti si può quindi rilevare che in condizione di ipoalimentazione viene a mancare quella azione favorevole svolta dalla vit. B_1 quando l'alimento è quantitativamente e qualitativamente sufficiente; nei ratti ipoalimentati, anzi, un maggiore apporto di vit. B_1, rispetto al fabbisogno normale, accentua il decorso dei processi demolitivi.

Ciancimino e Rubino (11), di recente, hanno studiato l'influenza svolta dalla vit. B_2 e dall'ac. pantotenico sul decorso della ipoalimentazione.

Sulla base dei loro risultati sperimentali essi concludono che la somministrazione di vit. B_2, in eccesso rispetto al fabbisogno giornaliero,

421

0948

non influenza il decorso della ipoalimentazione, la somministrazione invece di ac. pantotenico, in eccesso rispetto al fabbisogno giornaliero, determina nei ratti ipoalimentati una attenuazione della perdita percentuale giornaliera del peso corporeo ed un più tardivo raggiungimento della massima perdita percentuale del peso compatibile con la vita, che tuttavia risulta di entità pressocchè uguale a quella dei controlli.

Continuando tali ricerche abbiamo studiato l'influenza della vit. B_{12} sul decorso della ipoalimentazione calorica, lenta e progressiva, del ratto, potendo altresì mettere a confronto detti risultati con i dati da noi ottenuti (10) studiando l'influenza esercitata dalla stessa vit. B_{12} sui processi ricostruttivi nella rialimentazione dopo digiuno.

Le ricerche sono state eseguite nel seguente modo: ratti albini, maschi, adulti, provenienti dallo stesso allevamento, dopo essere stati in osservazione per un certo periodo di tempo durante il quale assumevano l'alimento (latte, carne, pane, verdura) *ad libitum*, venivano selezionati; gli animali prescelti (in base alle loro buone condizioni generali) venivano divisi in 2 gruppi di 5 ratti ciascuno. Gli animali dei 2 gruppi venivano tenuti separati l'uno dall'altro in adatte gabbie, poste alla temperatura ambiente di 16-21° C. Tutti i ratti venivano sottoposti a lenta e graduale ipoalimentazione per diminuzione progressiva della quantità giornaliera dell'alimento offerto, sino alla morte. L'alimento era composto da amido di frumento 75%, caseina lattica 10%, burro 10%, miscela salina 5%. Gli animali ricevevano inoltre carta da filtro ed una sufficiente quantità di vitamine atte a coprire il fabbisogno giornaliero. L'acqua era offerta *ad libitum*. Tutti gli animali ricevettero giornalmente una quantità di alimento corrispondente a calorie 0,215, in media, per g di peso corporeo.

A giorni alterni, per via ipodermica, i ratti del 2° gruppo ricevettero μg 8 di vit. B_{12}; quelli del 1° gruppo, di controllo, ricevettero, sempre a giorni alterni, soltanto ml 0,5 di soluzione fisiologica.

I risultati ottenuti, riassunti nella annessa tabella, permettono di mettere in evidenza i seguenti fatti: i ratti ipoalimentati e sottoposti a somministrazione di vit. B_{12} hanno raggiunto più precocemente (giorni 52.0 ± 0,47, in media, rispetto a giorni 54,4 ± 0,26, in media, dei ratti di controllo) il limite di resistenza compatibile con la vita, espresso dalla massima perdita percentuale complessiva del peso corporeo (48,6 ± 0,26, in media, nei ratti trattati con la vit. B_{12} e 47,4 ± 0,36, in media, nei ratti di controllo), che non è risultato peraltro sensibilmente diverso nei 2 gruppi di ratti. Tale fatto si accorda con la maggiore perdita percentuale di peso corporeo giornaliera presentata dai ratti trattati con vit. B_{12} (0,93 ± 0,0008, in media, rispetto a 0,86 ± 0,0003, in media, dei ratti di controllo).

Statisticamente sono significative le differenze riguardanti rispettivamente i giorni di sopravvivenza e la perdita del peso corporeo giornaliera dei ratti trattati con vit. B_{12}, $t = 3.108$ ($P < 0,02$) e $t = 6,754$ ($P < 0,001$) in confronto con quelli non trattati.

Dai risultati sopra riferiti, si può pertanto concludere che la somministrazione di vit. B_{12}, in eccesso rispetto al fabbisogno giornaliero, accentua la perdita percentuale giornaliera del peso corporeo, sicchè la massima perdita del peso corporeo compatibile con la vita, nei ratti ad

422

TABELLA I.

Lotto dei ratti	Condizione sperimentale	N. dei ratti sperimentati	Valori medii di: peso iniziale g	peso alla morte g	peso perduto g	alimento consumato in calorie pro g e pro die	giorni di esperienza	peso percentuale perduto	peso percentuale perduto pro die
I	Ratti ipoalimentati di controllo	5	188	99	89	0,215	54,4 ± 0,26	47,4 ± 0,36	0,86 ± 0,0003
II	Ratti ipoalimentati che ricevevano a giorni alterni µg 8 di vit. B_{12} durante la ipoalimentazione	5	183	97	91	0,215	52,0 ± 0,47	48,6 ± 0,26	0,93 ± 0,0008

TABELLA II.

Lotto dei ratti	Condizione sperimentale	Lotti comparati	Valori medi dei giorni di esperienza	t =	P	Valori medi del peso percentuale perduto	t =	P	Valori medi del peso percentuale perduto pro die	t =	P
I	Ratti ipoalimentati di controllo	I – II	54,4 ± 0,26	3,108	< 0,02	47,4 ± 0,36	0,932	< 0,4	0,86 ± 0,0003	6,754	< 0,001
II	Ratti ipoalimentati che ricevevano a giorni alterni µg 8 di vit. B_{12} durante la ipoalimentazione		52,0 ± 0.47			48,6 ± 0,26			0,93 ± 0,0008		

423

0948

ipoalimentazione trattati con vit. B_{12}, è raggiunta più precocemente pur rimanendo di entità pressocchè identica nei due casi.

(1) *Arch. Fisiol.*, 1955, 55, 67.—(2) *Science*, 1931, 73, 285.—(3) *J. Biol. Chem.*, 1932, 97, 133. — (4) *J. Nutrition*, 1933. 6, 195. — (5) *J. Biol. Chem.*, 1937, 119, ctii. — (6) *J. Physiol.*, 1937, 89, 287. — (7) *Science*, 1937, 86, 200. — (8) Questo *Bollettino*, 1953, 29, 975. — (9) *Ibidem*, p. 978. — (10) *Ibidem*, p. 1570. — (11) *Quad. Nutriz.*, in stampa.

Home. The age of the patients ranged from 48 to 92, mean 71; 34 patients were over 65 years of age. The group was composed of 24 men and 26 women. They gave no evidence of circulatory or renal failure. Their red blood cell (RBC) counts and hemoglobin were either within normal limits, or only slightly decreased before treatment. No systematic study of the effect of hydroxo- and cyanocobalamin treatment on the blood status was carried on during this study. Most of the subjects were diagnosed as having chronic arteriosclerotic cerebrovascular disease, chronic osteoarthritis, post-traumatic bone and joint disease, or as requiring custodial care.

For the purpose of better statistical evaluation, the cases were divided into two groups. One group received treatment for ten weeks, the other for a 14-week period. Each group was subdivided into two equal subgroups, of which one received weekly intramuscular injections of 500 μg of aqueous cyanocobalamin, and the other, 500 μg of aqueous hydroxocobalamin weekly. During the entire observation period, patients received no other vitamins, ie, none including B_{12} or folic acid, and no hematinics or blood transfusions. They remained on the same mixed regular hospital diet and on the same wards for the entire duration of the study, which, in some cases, extended to one year.

Before treatment, vitamin B_{12} blood levels were assayed twice at intervals of several weeks, and during treatment, every two weeks, by *L. leichmannii* method.[2] All glassware had been pretreated to prevent contamination; it was used only once and then disposed of. Blood for the assays was drawn immediately preceding the cobalamin injection, so that the interval between the B_{12} blood assays and the preceding hydroxocobalamin or cyanocobalamin injection was always seven days.

After the injection treatments were concluded, the patients in all groups remained on the same regimen, in the same wards, without any other hematinic or vitamin treatment. The B_{12} blood level assays were continued, first bimonthly, and later at monthly intervals.

Results

Group of Patients Treated with Ten Weekly Injections of Hydroxocobalamin or Cyanocobalamin.—The results obtained are drawn in a semilogarithmic plot in Figure 1. The means and ranges of values are drawn separately for the group of 25 individuals treated with hydroxocobalamin, and for the group of 22 individuals treated with cyanocobalamin, both for a ten-week period. Several patients from both these groups had to be eliminated from this study, temporarily or permanently, because of intercurrent disease or death.

In Table 1, the means with standard deviations are listed, and the two pretreatment values are listed as O_1 and O_2. During the cyanocobalamin treatment, B_{12} blood levels exceeded the upper limit of the normal (800 μμg/ml) after three weekly

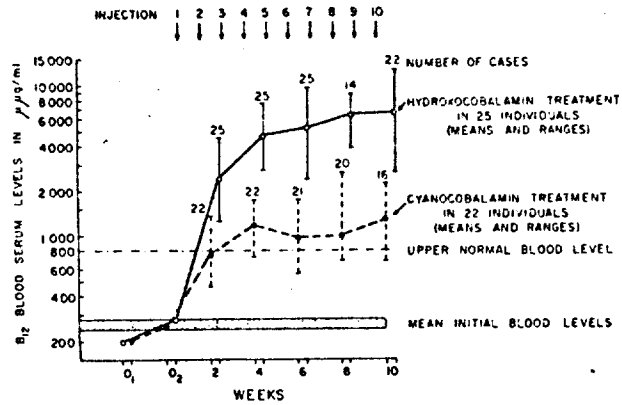


Fig 1.—Vitamin B_{12} blood serum levels during ten weekly intramuscular injections of 500 μg serum hydroxocobalamin or cyanocobalamin in 47 individuals with initial B_{12} blood levels below 250 μμg/ml.

injections. They remained at the level of about 1,000 to 1,500 μμg/ml for the rest of the ten-week treatment, the maximal value not exceeding 2,000 μμg/ml (Fig 1). During hydroxocobalamin treatment, however, the B_{12} blood levels exceeded the upper limit of normal as early as after the second weekly injection, and kept climbing steadily with further treatment. At the conclusion of eight hydroxocobalamin injections, the blood B_{12} levels averaged about 5,000 μμg/ml. This was more than four times higher, on the average, than at the completion of the cyanocobalamin treatment with the same doses. As shown by statistical evaluation of the data in Table 1, the B_{12} levels were three to five times higher, during the entire treatment period, on hydroxocobalamin, than on cyanocobalamin therapy, this difference being statistically highly significant ('p'<0.05-0.001).

Treatment was discontinued in 23 individuals after ten weekly injections of 500 μg hydroxocobalamin or cyanocobalamin, and the B_{12} blood levels were studied for a period of eight months without any further treatment. The results (means and ranges) are drawn in a semilogarithmic plot in

Table 1.—Statistical Evaluation of Vitamin B_{12} Blood Serum Levels During 10 to 14 Weekly Intramuscular Injections of 500 μg Hydroxocobalamin or Cyanocobalamin

	Weeks of Treatment	Blood Serum B_{12} Levels in μμg/ml (Means with Standard Deviations): Hydroxocobalamin Treatment	Cyanocobalamin Treatment	Statistical Evaluation of Differences in the Means: 'n'	't'	'p'
Group treated with 10 weekly intramuscular injections of hydroxo- or cyanocobalamin	O_1	193±45	167±53	27	1.3	>.2
	O_2	281±236	226±86	28	0.8	>.4
	2	2,208±914	769±219	28	5.4	<.001
	4	3,905±939	1,135±280	28	10.2	<.001
	6	4,919±1,912	887±242	27	7.3	<.001
	8	4,974±1,573	1,055±339	14	2.5	<.05
	10	4,385±1,868	1,076±281	20	5.6	<.001
Group treated with 14 weekly intramuscular injections of hydroxo- or cyanocobalamin	O_1	213±133	210±64	15	0.7	>.5
	O_2	276±193	306±112	15	0.4	>.7
	2	2,677±1,100	721±160	15	5.7	<.001
	4	4,655±1,613	1,358±318	15	6.3	<.001
	6	5,272±1,613	1,216±336	15	7.8	<.001
	8	6,368±1,502	1,478±690	15	8.6	<.001
	10	8,826±2,304	1,702±323	14	8.8	<.001
	12	6,450±2,383	1,705±773	13	5.3	<.001
	14	7,594±2,931	1,745±749	15	5.9	<.001

098

Table 2.—Statistical Evaluation of Vitamin B_{12} Blood Serum Levels After Discontinuation of 10 to 14 Weekly Intramuscular injections of 500 μg Hydroxocobalamin or Cyanocobalamin

	Weeks After Discontinuation of Treatment	Blood Serum B_{12} Levels in μμg/ml (Means with Standard Deviations)		Statistical Evaluation of Differences in the Means		
		Hydroxocobalamin Treatment	Cyanocobalamin Treatment	'n'	't'	'p'
Group treated with 10 weekly intramuscular injections of hydroxo- or cyanocobalamin	1	3,977±1,114	944±292	18	8.3	<.001
	3	2,160±775	631±192	20	6.0	<.001
	5	1,401±366	601±312	19	7.1	<.001
	7	1,439±678	552±137	18	3.8	<.01
	9	1,145±712	472±221	18	2.6	<.02
	11	839±229	479±142	18	3.9	<.001
	13	761±206	472±108	16	2.4	<.05
	17	982±376	421±55	17	3.9	<.001
Group treated with 14 weekly intramuscular injections of hydroxo- or cyanocobalamin	1	6,947±2,083	1,749±748	12	6.3	<.001
	3	2,687±942	1,290±418	10	3.1	<.01
	5	1,982±664	795±319	10	3.5	<.01
	7	1,186±371	869±286	12	1.6	>.1
	9	1,115±221	680±206	11	3.3	<.01
	11	1,037±141	626±72	11	6.4	<.001
	13	1,065±265	661±208	12	2.9	<.02
	17	959±230	673±93	11	3.0	<.02

Figure 2, and listed in Table 2 as means with standards deviations.

The B_{12} blood levels reverted to the normal range in 11 patients treated with ten weekly injections of 500 μg cyanocobalamin, four weeks after discontinuing treatment. They remained within the normal range thereafter, but for eight months they were about twice as high as they had been before treatment. Twelve patients treated previously with ten weekly intramuscular injections of 500 μg hydroxocobalamin, maintained their high B_{12} levels much longer than the cyanocobalamin group. After only 11 weeks, the blood levels came down to the upper limit of normal, which was about four to five times the initial preinjection level, where they remained for the next ten weeks. After eight months, the mean B_{12} blood levels were still about three times higher than before hydroxocobalamin treatment, and more than twice as high as after cyanocobalamin (Fig 2). The data are statistically evaluated in Table 2. For four months, the mean B_{12} blood levels were significantly higher after discontinuation of hydroxocobalamin than of cyanocobalamin, the difference being statistically highly significant. The 'p' value was less than 0.001 after one and four months, less than 0.02 after two months, and less than 0.05 after three months. (The differences in samples' size account for differences in 'p' values.)

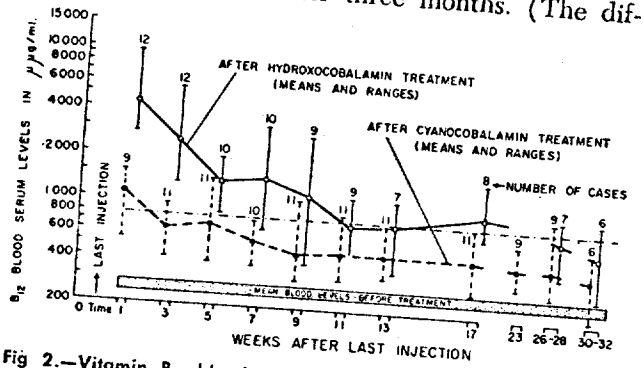


Fig 2.—Vitamin B_{12} blood serum levels after ten weekly intramuscular injections of 500 μg hydroxocobalamin or cyanocobalamin in 23 individuals, determined for duration of 8 mo.

Group Treated with 14 Weekly Intramuscular Injections of Hydroxocobalamin or Cyanocobalamin.—Therapy in 14 individuals, initially treated for ten weeks with intramuscular injections of hydroxocobalamin or cyanocobalamin, was extended up to 14 weeks. The means and ranges of B_{12} blood levels obtained are drawn in a semilogarithmic plot in Figure 3, and standard deviations are listed in Table 1.

Again, much higher B_{12} blood levels were obtained after hydroxocobalamin treatment than after cyanocobalamin injections. During cyanocobalamin treatment, the B_{12} blood levels leveled off after the 10th to 11th injection, and remained so in the range of 1,200 to 1,700 μμg/ml for the remaining treatment period. In the group treated with hydroxocobalamin, the mean B_{12} blood levels, ten weeks after the conclusion of treatment, were over 8,000 μμg/ml. In some cases, levels as high as 15,000 μμg/ml were reached (Fig 3).

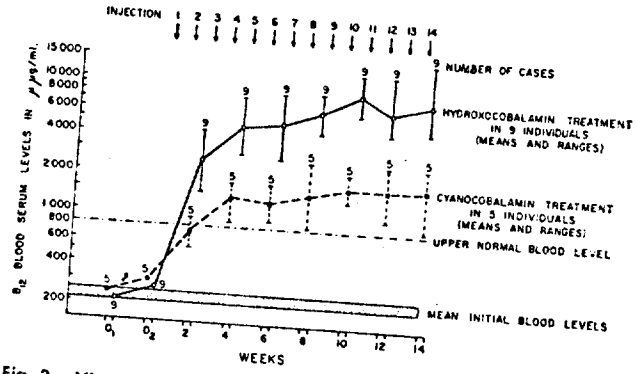


Fig 3.—Vitamin B^{12} blood serum levels during 14 weekly intramuscular injections of 500 μg hydroxocobalamin or cyanocobalamin in 14 individuals with initial B_{12} blood levels below 250 μμg/ml.

Here, also, there was some evidence of leveling off after 10 to 11 weekly injections. This phenomenon was similar to that observed after cyanocobalamin. For the entire duration of treatment, the B_{12} blood levels remained about four to five times higher than after cyanocobalamin, this difference being statistically highly significant at all times ('p'=<0.001).

In this group of cases, B_{12} blood levels were studied for eight months after discontinuation of the treatment, bringing the total observation period to one year. As shown in Figure 4, only one month was required for the B_{12} blood levels in the group of patients treated with cyanocobalamin to revert back to the upper limit of normal. They remained within the normal range for the rest of the eight months' observation period. After similar hydroxocobalamin treatment, however, the abnormally high B_{12} blood levels were maintained for more than four months, returning to the upper limit of normal

426

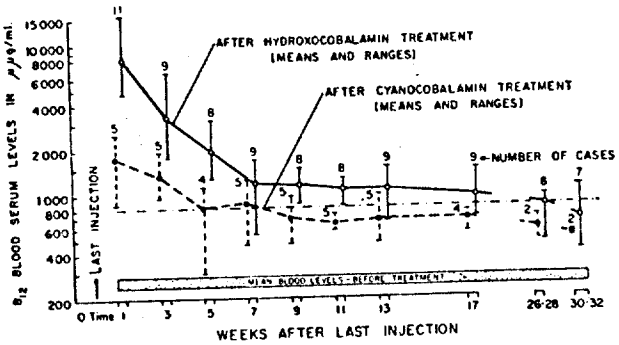


Fig 4.—Vitamin B_{12} blood serum levels after 14 weekly intramuscular injections of 500 μg hydroxocobalamin or cyanocobalamin in 16 individuals, determined for duration of 8 mo.

only after six to seven months, but still remaining three times higher than prior to treatment (Table 2). The statistical evaluation of the differences in B_{12} blood levels after hydroxo- and cyanocobalamin treatment in Table 2, shows that they were statistically significant at all times ('p'=<0.02). The only exceptions were data recorded at the seven-week interval where, because of the inadequate size of the sample, the 'p' value was not significant.

Comment

This study offered clear-cut evidence that long-range treatment with large intramuscular doses of hydroxocobalamin builds up much higher and more prolonged B_{12} blood levels than a similar treatment schedule with cyanocobalamin. Furthermore, the high B_{12} blood levels after hydroxocobalamin treatment persist for a much longer time. This effect cannot be obtained with similar doses of cyanocobalamin. The differences between B_{12} blood levels obtained with both treatments are statistically significant at any point of the study and have definite practical implications.

One of the difficulties in the long-range management of the B_{12} deficiencies, including pernicious anemia, has been that routine cyanocobalamin treatment is unable to replenish adequately the B_{12} depletion.[12-14] The usual parenteral B_{12} doses (30 to 60 μg/mo) in the majority of cases kept the blood status under control, but the B_{12} blood levels usually remained much below the levels of the normal population.[12-14] This indicated persistence of B_{12} deficiency. The recent, and as yet unpublished, work from Sweden by Ernstsson, Reizenstein, and Sellberger, shows the persistence of high mean corpuscular hemoglobin in pernicious anemia patients in remission on conventional treatment, thus pointing in the same direction.

The basic aim of the treatment of any nutritional deficiency is not the correction of a symptom, such as anemia, but the correction of the deficiency itself. The basic therapeutic goal has not been satisfactorily met with the conventional B_{12} treatment schedule, as shown by the frequent persistence of low B_{12} blood levels during this treatment,[12-14] and occasional development of signs of combined degeneration of the cord, as we have seen at times. Many investigators have contended, therefore, that the depleted B_{12} stores should be replenished at the onset of the treatment with massive B_{12} doses.[12-14] Only then should the maintenance treatment with conventional bimonthly doses be continued.

However, the difficulty with parenteral administration of large doses of cyanocobalamin has been that massive doses unavoidably result in massive urinary losses. Under some circumstances, these may reach or exceed even 80% of the dose injected.[2] This makes it difficult to evaluate the amount of B_{12} really retained in the body; hence there may be inadequate replenishment of depleted B_{12} stores. Since marked urinary losses amounting to as much as 30% to 40%[15] are also observed after intramuscular injections of relatively moderate doses of cyanocobalamin in the 60- to 100-μg dose range, the efficiency of the conventional B_{12} treatment is also impaired.

Attempts were made, therefore, to develop various depot preparations with the thought that they might cause a more efficient retention of cyanocobalamin in the body.[2] The blood levels obtained with these preparations, however, were not strikingly different from those achieved with aqueous cyanocobalamin. They were far below those attainable with aqueous hydroxocobalamin.[2,5] Moreover, the results were somewhat unpredictable[2] because of varying rates of absorption of artificially prepared depot materials from the site of injection.

The hydroxocobalamin treatment did satisfy all aims of therapy of B_{12} deficiency, and provided exceedingly high B_{12} blood levels unattainable with other cobalamin materials.[1-3] The urinary losses of hydroxocobalamin at the 500 μg intramuscular dose are on the average of only 16.6 ± 4.2%, while those of cyanocobalamin amounted to 69.4 ± 16.0% within 72 hours.[2] The urinary excretion after hydroxocobalamin injection at a later date is negligible, as shown by random sampling of urines for the duration of four weeks.[2] One may therefore calculate that, after ten injections of 500 μg hydroxocobalamin, about 4,000 μg B_{12} will remain in the body, which is in excess of the total metabolic needs of man.[12-14] The fecal losses of B_{12} administered parenterally are notoriously low.[12,14] They are derived from the excretion of small amounts of B_{12} through the bile into the intestinal lumen, from which one part escapes into the feces, the rest being reabsorbed. This is less than one per cent of the dose injected into humans in the case of cyanocobalamin, as has been noted by Uchino, from our laboratory, in unpublished observations. It is improbable that hydroxocobalamin, which is known to form much stronger bonds with the blood and tissue proteins than cyanocobalamin,[6,16,17] will be excreted into the intestine at a higher concentration.

The exact calculation of the amount of hydroxocobalamin retained in the body during the long-range treatment is not yet available, and would require balance studies. That this retention in man[1,2,4,5] and dog[6] is higher than that of cyanocobalamin is beyond doubt. The compartment which participates in this retention is under current investigation. The better retention of hydroxocobalamin in the body is due to its forming firmer bonds with body proteins and peptides which bind B_{12}.[16] This is true for muscles (at the site of injection),[1,4,5,7,8] blood proteins,[16,17] and other tissues, including the liver.[17] The close relation of hydroxocobalamin to coenzyme B_{12} may be one of the causes of better retention of hydroxocobalamin.[18] It should also be remembered that hydroxocobalamin, and not the artificially produced cyanocobalamin, is the physiological form of food B_{12} metabolized in the human body.[18]

From the practical point of view, a new schedule of parenteral treatment of B_{12} deficiencies will probably evolve from hydroxocobalamin studies. It is the contention of the authors that hydroxocobalamin treatment may make it unnecessary to inject patients at weekly, biweekly, or monthly intervals. Instead, there is a good possibility that it may be feasible to give these patients, including those with pernicious anemia, a short but massive course of eight to ten injections of 1,000 μg of hydroxocobalamin within two to three weeks once a year only. This will saturate the body B_{12} stores and keep them saturated with B_{12} for the rest of the year. Similar thinking has been expressed by other workers in this field,[5] and further work along these lines is in progress. The final evaluation of these possibilities should be withheld, however, pending a longer trial period.

New York Medical College, Flower & Fifth Ave Hospitals, Fifth Ave & E 105th St, New York City 29 (Dr. Jerzy Glass).

Generic and Trade Names of Drug

Cyanocobalamin—*Berubigen, Bevantine, Bevidox, Bexii, Crystwel, Dodecabee, Dodex, Hemomin, Rametin, Redisol, Rubramin, Sytobex, Vitamin B_{12} Crystalline.*

References

1. Glass, G. B. J., et al: Clinical Applicability of Hydroxocobalamin as Long-Acting Vitamin B_{12} in Treatment of B_{12} Deficiencies, 8th Int Congress of Hemat, Tokyo, Sept 4-10, 1960, abstr, Pp 253–254; *Nature (Lond)* 189: 138-140 (Jan) 1961; *Fed Proc* 20:449 (March) 1961.

2. Glass, G. B. J., et al: Hydroxocobalamin. I. Blood Levels and Urinary Excretion of Vitamin B_{12} in Man After Single Parenteral Dose of Aqueous Hydroxocobalamin, Aqueous Cyanocobalamin, and Cyanocobalamin Zinc-Tannate Complex, *Blood* 18:511-521 (Nov) 1961.

3. Boger, W. P., and Gavin, J. J.: 5th Int Congress Nutrition, Washington, D.C., Sept 1-7, 1960, abstr, no 287, p 62.

4. Killander, A., and Schilling, R. F.: Studies on Hydroxocobalamin I. Excretion and Retention of Massive Doses in Control Subjects, *J Lab Clin Med* 57:553-561 (April) 1961.

5. Heinrich, H. C., and Gabbe, E. E.: Intravitale Retention und Excretion des Aquocobalamids, einer für die Therapie geeigneten natürlichen Vitamin B_{12} Depot Form, *Klin Wschr* 39:689-691 (July) 1961; *Radiometrische Testung des Stoffwechsel-Verhaltens von Vitamin B_{12} Depot Präparationen. "Vitamin B_{12} und Intrinsic Factor. 2nd Europ Symposion,"* Hamburg, Aug 2-5, 1961, H. C. Heinrich, ed, Stuttgart: F. Enke, 1962, Pp 638-663.

6. Skeggs, H. R., et al: Hydroxocobalamin: Physiological Retention in Dog, *Proc Soc Exp Biol Med* 105:518-521 (Dec) 1960.

7. Glass, G. B. J.; Lee, D. H.; and Hardy, W. W.: Hydroxocobalamin. II. Absorption from Site of Injection and Uptake by Liver and Calf Muscle in Man, *Blood* 18:522-526 (Nov) 1961.

8. Milhaud, G.: Mecanisme de l'Effet Retard de l'Hydroxocobalamin, *Rev Franc Etud Clin Biol* 6:922-924, 1961.

9. Lichtman, H., et al: Vitamin B_{12a}: Some Properties and Its Therapeutic Use, *Proc Soc Exp Biol Med* 72:643-645 (Dec) 1949.

10. Schilling, R. F.; Harris, J. W.; and Castle, W. B.: Observations on Etiologic Relationship of Achylia Gastrica to Pernicious Anemia. XIII. Hematopoietic Activity of Vitamin B_{12a} (Vitamin B_{12b}), *Blood* 6:228-232 (March) 1951.

11. Glass, G. B. J., et al: *Applicability of Hydroxocobalamin as a Long-Acting Vitamin B_{12} in Man. "Vitamin B_{12} und Intrinsic Factor. 2nd Europ Symposion,"* Hamburg, Aug 2-5, 1961, H. C. Heinrich, ed, Stuttgart; F. Enke, 1962, Pp 673-685.

12. Gräsbeck, R.: *Physiology and Pathology of Vitamin B_{12}: Absorption, Distribution and Excretion, Advances in Clinical Chemistry,* New York: Academic Press, 1960, Pp 299-366.

13. Adams, J. F.: *Considerations Governing the Maintenance Treatment of Patients with Pernicious Anemia. "Vitamin B_{12} und Intrinsic Factor. 2nd Europ Symposion,"* Hamburg, Aug 2-5, 1961, H. C. Heinrich, ed, Stuttgart: F. Enke, 1962, Pp 628-637.

14. Reizenstein, P. G.: Vitamin B_{12} Metabolism: Some Studies on Absorption, Excretion, Enterohepatic Circulation, Turnover Rate, Body Distribution and Tissue Binding of B_{12}, *Acta Med Scand* 165:1-31, suppl no 347, 1959.

15. Glass, G. B. J., et al: Metabolic Interrelations Between Gastric Intrinsic Hemapoietic Factor and Vitamin B_{12}, *Bull NY Med Coll* 15:17-41 (Dec) 1952.

16. Bauriedel, W. R.; Picken, J. C., Jr.; and Underkoffer, D. A.: Reactions of Cyanocobalamin and Aquocobalamin with Proteins, *Proc Soc Exp Biol Med* 91:377-381 (March) 1956.

17. Chosy, J. J.; Killander, A.; and Schilling, R. F.: *Studies on Hydroxocobalamin. "Vitamin B_{12} und Intrinsic Factor. 2nd Europ Symposion,"* Hamburg, Aug 2-5, 1961, H. C. Heinrich, ed, Stuttgart: F. Enke, 1962, Pp 668-672.

18. Bernhauer, K., et al: *Zum Mechanismus der Biosynthese des Vitamin B_{12} Molekules. "Vitamin B_{12} und Intrinsic Factor. 2nd Europ Symposion,"* Hamburg, Aug 2-5, 1961, H. C. Heinrich, ed, Stuttgart: F. Enke, 1962, Pp 37-58; Discussion, Vitamin B_{12} Symposium, 7th Congress Int Soc Hemat, Rome, 1958, *Haemat Lat* 2:322 (April-June) 1959.

BLOOD J HEMATOL 27(2) 234-241 (1966)

Hydroxocobalamin

V. Prolonged Maintenance of High Vitamin B_{12} Blood Levels following a Short Course of Hydroxocobalamin Injections

By George B. Jerzy Glass, Helen R. Skeggs and Duk Ho Lee

It has recently become clear that the pathology and symptomatology of vitamin B_{12} deficiency diseases is the result of depletion of vitamin B_{12} stores from the body, and that hematologic disturbances are only one manifestation of underlying vitamin deficiency. The therapeutic emphasis has, therefore, shifted from correcting abnormal blood count and bone marrow to the repletion of vitamin B_{12} stores in the body, and the maintenance of vitamin B_{12} levels in the blood. The physiologic requirements of vitamin B_{12}, its metabolic turnover in the body, and its biological half-life in various body compartments had to be taken into consideration in devising modern treatment of vitamin B_{12} deficiency. With a better understanding of the absorption, utilization and excretion of vitamin B_{12} in normal and vitamin B_{12}-depleted individuals, the shortcomings of conventional therapy with commercially available vitamin B_{12} cyanocobalamin have been placed in their proper perspective. This has resulted in a need for preparation which could be better retained in the body than cyanocobalamin.

In the search for this long-acting vitamin B_{12}, we have screened cobalamins in various media and modifications and have compared them with aqueous cyanocobalamin. Of the many materials screened, only aqueous hydroxocobalamin (vitamin B_{12a}) gave the most striking performance;[1,2] it was singled out as the preparation possessing the best features of a long-acting vitamin B_{12}. The initial results of this work were published in abstract form in September 1960,[1] and then in more detail in January 1961.[2] Hydroxocobalamin was absorbed more slowly from the site of injection, eliminated more slowly in the urine, and built up higher and longer-lasting blood levels than cyanocobalamin.[1,2] These findings were corroborated and extended by other investigators: Skeggs et al., on dogs,[3] Boger and Gavin,[4] Killander and Schilling,[5] Heinrich and Gabbe,[6,7] Tauber et al.,[8] and Herbert et al.,[9] on man, as well as further work of our group.[10-14]

Since equivalence of the hematopoietic effects of hydroxocobalamin to that

From Department of Medicine, New York Medical College—Metropolitan Medical Center, New York, New York, and Merck Sharp and Dohme Research Laboratories, West Point, Pa.

Supported by Research Grant-in-Aid #AM-00068-12 and 13 from the National Institute of Arthritis and Metabolic Diseases, USPHS, Bethesda, Md.

First submitted Mar. 9, 1965; accepted for publication Apr. 9, 1965.

George B. Jerzy Glass, M.D.: *Professor of Medicine and Chief, Section of Gastroenterology, New York Medical College.* Helen R. Skeggs: *Research Associate, Merck Sharp and Dohme Research Laboratories, Westport, Pa.* Duk Ho Lee, M.D.: *Former Research Fellow, Gastroenterology Research Laboratory, New York Medical College.*

of cyanocobalamin had been long established,[15,16] and since the superior retention of hydroxocobalamin over cyanocobalamin has been demonstrated,[1-14] one could expect a definite advantage from using hydroxocobalamin in treating vitamin B_{12} deficiency and pernicious anemia.

This led us to explore the long-range effects of hydroxocobalamin upon the vitamin B_{12} blood levels in man. Preliminary reports of these studies extending over a period of 8 months were published in 1962[12] and 1963.[14] We concluded at that time that hydroxocobalamin was the drug of choice in treatment of vitamin B_{12} deficiency. Similar convictions have been voiced lately by Heinrich,[17] and Withey and Kilpatrick.[18]

In this current work, we bring the evidence that 6 intramuscular injections of 1000 μg. hydroxocobalamin given over a period of 10 days to individuals with relatively low vitamin B_{12} levels are capable of maintaining vitamin B_{12} blood concentration much above starting vitamin B_{12} level for a period of one year or more, without any further treatment. The practical significance of this finding will be discussed.

Material of Cases

Out of a group of over 100 cases from Bird S. Coler Memorial Hospital and Home for Chronic Diseases, in whom vitamin B_{12} blood levels were determined, 20 were selected for this study with blood levels extending from 64 to 250 μμg. (mean 185 μμg.)/ml. Patients ranged in age from 60 to 70 years and demonstrated no liver or kidney disease and no heart decompensation. Some had subtotal gastrectomy; others had atrophic gastritis; still others had multiple sclerosis. Some patients had macrocytic anemia and many had macrocytosis. In 5 of these patients, follow-up had to be interrupted at an earlier date for various reasons. In 15, the study was continued for 8 months; in 12, for 10 months; and in 11 of these, for 15 months. Four additional patients with PA in remission and having pretreatment vitamin B_{12} blood levels ranging from 200–800 μμg./ml. have also been included in this study.

Method

Of 20 non-PA patients, each received 6 intramuscular injections of 1000 μg. hydroxocobalamin every other day for the duration of 10 days. Blood was drawn prior to injection and at monthly time intervals, starting one month after the last injection and continuing to the tenth month. Five to 6 months later—i.e., 15 to 16 months after the conclusion of injections—blood was drawn for the last time. Vitamin B_{12} serum levels were assayed by means of *Lactobacillus leichmannii* method, as described by Skeggs et al.[19] Care was taken that no one received additional vitamin B_{12} or polyvitamin treatment and that the patients remained on the same hospital diet for the entire period of the study.

Each of the 4 PA subjects received 8 to 12 weekly intramuscular injections of 500 μg. hydroxocobalamin during the 2–3 month period, following which their vitamin B_{12} levels were followed up for an additional 14 to 16 months without any further treatment.

Results

Results obtained in 4 PA patients are listed in Table 1. The total amount of hydroxocobalamin administered during the course of treatment in one case was 7 mg.; in another, 6 mg.; and in 2 others, 4 mg. One month after conclusion of treatment, vitamin B_{12} serum levels ranged from 1536 to 3900 μμg./ml., and 6 months after injections they ranged from 298 to 1394, with a mean of

Table 1.—*Serum Vitamin B_{12} Levels in μμg./ml. in 4 Patients with Pernicious Anemia prior to and following 12 Weekly Intramuscular Injections of 500 μg. Hydroxocobalamin*

		Prior to Injections		Months after Last Injection											
#	Initials	O_1	O_2	1	2	3	4	5	6	7	8	9	10	11	14–16
1	M. P.	159	261	3900	3190	2300	1675	1239	1075	616	536	382			[illegible]
2	M. M.	666	646		2270	1700	1260	1264	1394	1244	1256	686	612	629	[illegible]
3	P. M.	807	966	1536	1210	1460	950	682	709	593	649	521	331	411	[illegible]
4	V. S.	554	335	1540	713	408	379	295	298	301	216	402	182		[illegible]
Mean		547	552		1845	1467	1066	870	869	688	664	499			[illegible]
St. deviation		241	290		958	683	473	405	401	344	376	121			[illegible]

869 μμg./ml. This was about 60 per cent higher than prior to treatment. Nine months after conclusion of hydroxocobalamin injections, in only one patient the vitamin B_{12} concentration dropped below the pretreatment baseline, while in 3 others it remained above or at the preinjection levels. Neither of these patients has shown megaloblastic changes in the bone marrow nor neurologic signs of combined degeneration of the cord throughout the entire observation period of 9 months.

Thus, a course of 8–12 weekly intramuscular hydroxocobalamin 500 μg. injections given to pernicious anemia patients for 2–3 months and totalling 4–6 mg. was adequate to maintain vitamin B_{12} serum levels for additional 9 months at the preinjection level in 3 of these 4 patients.

However, 14–16 months after conclusion of therapy, the vitamin B_{12} serum levels remained at the pretreatment level in only one patient, while in 3 others they decreased below preinjection values. In one case, vitamin B_{12} serum concentration came down as low as 53 μμg./ml. at that time—i.e. within the range of frank vitamin B_{12} depletion—and bone marrow has shown megaloblastic changes.

Results obtained in the second and larger group of nonpernicious anemia cases are listed in Tables 2 and 3 and Figure 1. These initially had mostly low normal blood levels, ranging from 81 to 242 μμg./ml. (mean with st. dev. 185 ± 44 μμg./ml.) and were treated by a schedule consisting of 6 intramuscular injections of 1000 μg. hydroxocobalamin every other day for only 10 days.

One month after conclusion of hydroxocobalamin injections, the mean serum vitamin B_{12} blood level was 2634 μμg. (14 times preinjection level). One month later, it was 1136 μμg./ml. (6 times higher than prior to injection), and 4 months after the last injection, it was on the average still more than 4 times higher than before treatment (mean value 828 μμg./ml.). Half a year after the last injection, serum levels were still more than 3 times higher than prior to treatment.

In 12 cases which were studied for 10 months after discontinuation of injections (cases 1–12, Table 2), the mean serum levels were more than double the pretreatment levels. This increase in serum levels was highly significant statistically (Table 3), with a "p" value of 0.001.

In 11 cases which were studied 15 months after the last injection, vitamin

Table 2.—*Serum Vitamin B_{12} Levels in μμg./ml. in 18 Individuals prior and following 6 Intramuscular Injections of 1000 μg. Hydroxocobalamin Given Every Other Day*

#	Initials	Prior to Injections	Months after Last Injections 1	2	3	4	5	6	7	8	9	10	15
1	A. G.	209	1800	890	739	1010	687	684	720	541	476	313	394
2	C. S.	215	1810	874	597	698	702	771	667	422	372	423	305
3	J. C.	81	2519	937	541	518	498	382	350	351	257	240	183
4	H. McC.	242	3360	1210	1000	926	851	658	700	604	637	491	452
5	B. J.	142	7000	2650	975	827	889	872	520	474	412	320	223
6	J. S.	223	1580	845	654	689	604	923	758			272	220
7	M. M.	204	5150	1540	985	665	602	650	716	546	438	371	375
8	T. K.	206	3412	1788	1059	1213	1038	1059	1172		1051	858	259
9	B. K.	230	2633	1060	611	615	587	421	430	365	418	390	196
10	E. B.	238	1440	648	517	649	650	573	529	442	431	390	163
11	G. M.	148	1746	744	719	868	641	429	513	291	281	276	120
12	A. A.	208	1410	837	642	550	699	718	648	627	520	537	‡
13	J. O.	233	2640	disch.						707	468	disch.	
14	T. A.	90	1342	659	1217	617		557	632	377	362	disch.	
15	P. K.	220	3400	1170	829	548	499	547	748	430	‡		
16	M. A.	97	2120	1480	664	2030†	‡						
17	E. V.	195		8417*	5183*	‡							
18	G. S.	137	1420	850	‡								
19	G. S.	232	disch.										
20	D. K.	144	disch.										
Means		185	2634	1136	783	828	688	660	650	475	471	407	255
St. deviation		44	1470	503	207	212	114	191	188	118	106	106	93

*Patient developed inf. v. cava thrombosis, with renal insufficiency; the asterisk-marked figures are not included into calculations.

†Patient developed cardiovascular failure with pulmonary embolism; the asterisk-marked figure is not included into calculation.

‡Died. (Cases 12, 15, and 18 died of coronary thrombosis or cerebral vascular accident.)

Table 3.—*Statistical Evaluation of the Significance of Differences in Serum Vitamin B_{12} Levels prior and 10 and 15 Months after 6 Intramuscular Injections of 1000 μg. Hydroxocobalamin*

A. 10-Month Follow-up in 12 Cases (1–12)	
Serum vitamin B_{12} level prior to injections μμg./ml. (mean and st. dev.)	194.0 ± 45.7
Serum vitamin B_{12} level 10 months after last injection (mean and st. dev.)	407.0 ± 160.4
Difference of the means	213.0
St. error of the difference of the means	48.3
t	4.41
n	12
p	0.001
B. 15-Month Follow-up in 11 Cases (1–11)	
Serum vitamin B_{12} level prior to injections (mean and st. dev.)	195.0 ± 45.6
Serum vitamin B_{12} level 15 months after last injection (mean and st. dev.)	255.0 ± 93.0
Difference of the means	61.0
St. error of the difference of the means	32.8
t	1.86
n	11
p	$< 0.1 > 0.05$

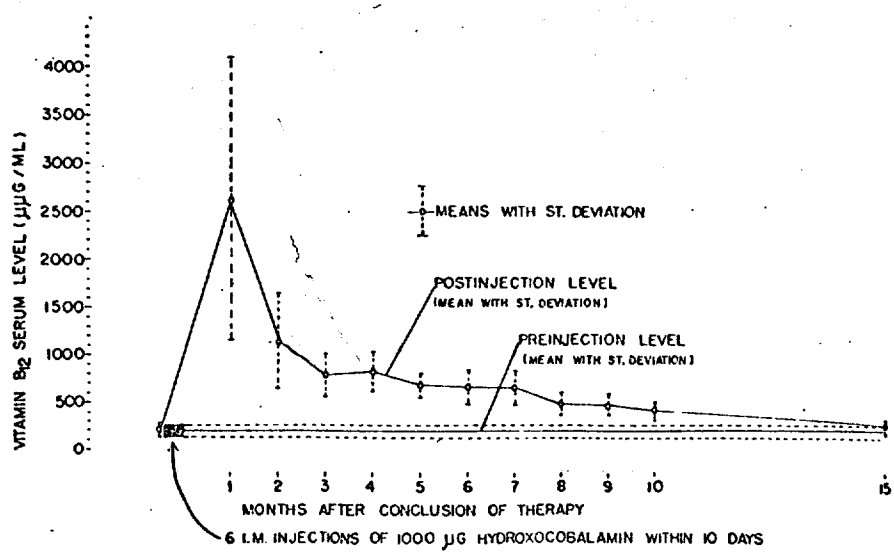


Fig. 1.—Mean serum vitamin B_{12} levels in 18 individuals prior and following 6 intramuscular injections of 1000 μg. hydroxocobalamin given within 10 days.

B_{12} serum levels ranged from 120 to 375, with a mean and standard deviation of 255 ± 93 μμg./ml. In 7 cases, they were higher than before treatment. The statistical evaluation of these cases is listed in Table 3, showing a "p" value between 0.05 and 0.1.

Discussion

The 2–3 month-long treatment schedule used here for pernicious anemia patients in remission was adequate to maintain vitamin B_{12} serum concentration at the preinjection level for additional 4–9 months. It would seem that the schedule used of 8–12 intramuscular injections of 500 μg. hydroxocobalamin administered over a period of 2–3 months was not optimal for this purpose. Addition of a few more injections at the onset of treatment, or use of the schedule which is described below would perhaps provide better results and maintain higher and more satisfactory vitamin B_{12} serum levels in these patients.

In a group of 15 patients with low normal vitamin B_{12} levels (mean with st. dev. 185 ± 44 μμg./ml.), results obtained with 6 intramuscular injections of 1000 μg. hydroxocobalamin every other day for 10 days were very satisfactory. Administration of 6000 μg. hydroxocobalamin during the 10-day period enabled 15 individuals to maintain vitamin B_{12} serum concentration half a year later at levels exceeding 3 times the pretreatment values. Furthermore, in all 11 patients who were studied for more than one year after conclusion of injections, vitamin B_{12} concentration in serum at the time was double the initial pretreatment level. It should be noted that 2 of these subjects had vitamin B_{12} serum levels in nontreated PA range: 81 and 90 μμg./ml. prior to injections.

Results of this long-term study on patients with relatively low vitamin B_{12} serum levels suggest that a similar treatment schedule will perhaps also be

adequate for patients with frank vitamin B_{12} deficiency and pernicious anemia. Administration of 6–8 intramuscular injections of 1000 μg. hydroxocobalamin given every other day over a period of 10–14 days, should be adequate to maintain normal vitamin B_{12} serum levels in these patients for at least a one-year period.

No comparable results were ever reported with massive cyanocobalamin therapy. The mechanism of this prolonged hydroxocobalamin effect is probably related to its higher ability to bind to body proteins, as compared to cyanocobalamin. This was demonstrated some time ago by Bauriedel et al.[20] and Miller and Sullivan,[21] and more recently by Skeggs et al.,[9] using dog sera, and Chosy et al.,[22] with normal human sera, using the dialysis technic. Withey and Kilpatrick[18] have lately compared the binding capacity of serum for cyanocobalamin and hydroxocobalamin using the gel filtration on Sephadex columns method.[23] The binding capacity of serum for hydroxocobalamin at a higher dose level was about 3 times higher than that for cyanocobalamin. Killander and Schilling[4] suggested that a better retention of hydroxocobalamin than cyanocobalamin in the body was due to hydroxocobalamin's being closely related to the final form under which vitamin B_{12} is stored in the body.

The whole body counting studies of Schiffer et al.[24] indicated that the biological half-life of hydroxocobalamin was similar to that of cyanocobalamin. In our laboratory, using the double-label technic and surface counting of the liver, we found that the hepatic biological half-life of hydroxocobalamin was somewhat longer than that of cyanocobalamin,[25] but only at a later phase of its turnover. If one takes into account these results, hydroxocobalamin is perhaps more tenaciously stored in the liver and plasma than in other tissues, and may be less readily available from these sources to the rest of the body than cyanocobalamin. This may explain[26] why the hydroxocobalamin requirements, as evidenced in this study, are somewhat higher than those theoretically calculated by Heinrich[27] on the basis of the vitamin B_{12} turnover data obtained with cyanocobalamin.

It should be emphasized, however, that although it is generally considered that with some approximation the vitamin B_{12} serum levels are the reflection of the size of the vitamin B_{12} body's stores, this has not been proved yet in regard to patients treated with massive hydroxocobalamin injections. Because of this consideration, further work is now in progress to determine the concentration of vitamin B_{12} in some of the tissues of our patients, prior, during and following hydroxocobalamin therapy.

Summary and Conclusions

Administration of 6 intramuscular injections of 1000 μg. hydroxocobalamin every other day for 10 days enabled 15 individuals with initial vitamin B_{12} serum levels of 81 to 242 (mean 185 ± 44 μμg./ml.) to maintain vitamin B_{12} concentration of the blood 6 months later at levels exceeding 3 times the pretreatment values. In 12 of these individuals who were studied for at least a 10-month period, vitamin B_{12} concentration in the serum 10 months after conclusion of injections was still double the initial pretreatment levels. This difference was highly significant statistically ($p = 0.001$). Of 11 patients fol-

lowed for 15 months after conclusion of injections, in 7 the serum levels were still higher than before treatment.

No comparable results were ever reported with cyanocobalamin therapy.

Summario in Interlingua

Le administration de 6 injectiones intramuscular de 1000 μg de hydroxocobalamina a intervallos de 2 dies resultava in 15 subjectos con initial nivellos seral de vitamina B_{12} de inter 81 e 242 μμg./ml. (al media 185 ± 44) in le mantenentia, usque a 6 menses plus tarde, de un concentration sanguinee de vitamina B_{12} in excesso de 3 vices le valores pretractamental. In 12 del subjectos, le quales esseva studiate durante periodos de al minus 10 menses, le concentration seral de vitamina B_{12} 10 menses post le conclusion del cursos de injectiones esseva ancora 2 vices le valor pretractamental. Iste differentia esseva statisticamente significativissime ($p = 0.001$). Inter le 11 patientes tenite sub observation usque a 15 menses post le conclusion del cursos de injectiones, 7 monstrava ancora a ille tempore nivellos seral de vitamina B_{12} plus alte que illos constatate ante le tractamento.

Resultatos comparabilemente notabile ha nunquam essite reportate pro le therapia con cyanocobalamina.

REFERENCES

1. Glass, G. B. J., Skeggs, H. R., Lee, D. H., Jones, E. L., and Hardy, W. W.: Clinical applicability of hydroxocobalamin as a long-acting vitamin B_{12} in the treatment of B_{12} deficiencies (Abstract). Proc. VIII Internat. Congress of Haematology, Tokyo, Japan, September 4–10, 1960.
2. —, —, —, —, and —: Applicability of hydroxocobalamin as a long-acting vitamin B_{12}. Nature 189:138, 1961.
3. Skeggs, H. R., Hanus, E. J., McCauley, A. B., and Rizzo, V. J.: Hydroxocobalamin: physiological retention in the dog. Proc. Soc. Exper. Biol. Med. 105:518, 1960.
4. Boger, P., and Gavin, J. J.: Hydroxocobalamin: Comparison with coenzyme B_{12}, cyanocobalamin and cyanocobalamin zinc-tannate (Abstract). 5th Internat. Congr. Nutrition, Washington, D. C., Sept. 1–7, 1960.
5. Killander, A., and Schilling, R. F.: Studies on hydroxocobalamin. I. Excretion and retention of massive doses in control subjects. J. Lab. Clin. Med. 57:553, 1961.
6. Heinrich, H. C., and Gabbe, E. E.: Intravitale Retention und Exkretion des Aquocobamids, einer für die Therapie geeigneten natürlichen Vitamin B_{12}-Depotform. Klin. Wschr. 13:68, 1961.
7. Heinrich, H. C., and Gabbe, E. E.: Radiometrische Testung des Stoffwechsel-Verhaltens von Vitamin B_{12}-Depot-Präparationen. *In* Vitamin B_{12} and Intrinsic Factor (H. C. Heinrich, Ed.). 2 Europ. Symposium, Hamburg 1961. Stuttgart, Ferdinand Enke, 1962, p. 638. *Also:* Arzneimittel Forsch. 11:1087, 1961.
8. Tauber, S. A., Skeggs, H. R., Itkin, A., and Stanley, J. L.: Parenteral studies with vitamin B_{12} complexes. Amer. J. Clin. Nutr. 10:480, 1962.
9. Herbert, V., Zalusky, R., and Skeggs, H. R.: Retention of injected hydroxocobalamin versus cyanocobalamin versus liver extract-bound cobalamin. Amer. J. Clin. Nutr. 12:145, 1963.
10. Glass, G. B. J., Skeggs, H. R., Lee, D. H., Jones, E. L., and Hardy, W. W.: Hydroxocobalamin. I. Blood levels and urinary excretion of vitamin B_{12} in man after a single parenteral dose of aqueous hydroxocobalamin, aqueous cyanocobalamin and cyanocobalamin zinc-tannate complex. Blood 18:511, 1961.

1. Glass, G. B. J., Lee, D. H., and Hardy, W. W.: Hydroxocobalamin. II. Absorption from the site of injection and uptake by the liver and calf muscle in man. Blood 18:522, 1961.
2. Glass, G. B. J., Skeggs, H. R., Lee, D. H., Jones, E. L., and Hardy, W. W.: Applicability of hydroxocobalamin as a long-acting vitamin B_{12} in man. *In* Vitamin B_{12} and Intrinsic Factor (H. C. Heinrich, Ed.). 2 Europ. Symposium, Hamburg, 1961, Stuttgart, Ferdinand Enke, 1962, p. 673.
13. Glass, G. B. J., Skeggs, H. R., Lee, D. H., Jones, E. L., and Hardy, W. W.: Hydroxocobalamin: Its absorption, deposition, excretion and applicability as a long-acting vitamin B_{12} to the treatment of B_{12} deficiencies and pernicious anemia. Proc. VIII Internat. Congr. Haematology, Sept. 4–10, Tokyo, 1960, Pan Pacific Press, Tokyo, Japan, 1961, 882–891.
14. Glass, G. B. J., Lee, D. H., Skeggs, H. R., and Stanley, J. L.: Hydroxocobalamin. III. Long-acting effects of massive parenteral doses on vitamin B_{12} levels in man. J. Amer. Med. Ass. 183:425, 1963.
15. Schilling, R. F., Harris, J. W., and Castle, W. B.: Observations on the etiologic relationshp of achylia gastrica to pernicious anemia. XIII. Hematopoietic activity of vitamin B_{12a} (vitamin B_{12b}). Blood 6:228, 1951.
16. Meyer, L. M., Sawitsky, A., Ritz, M. D., and Brabin, C.: Treatment of pernicious anemia with crystalline vitamin B_{12b}. Blood 8:358, 1953.
17. Heinrich, H. C.: Modern vitamin-B_{12}-therapie: oral, rektal oder parenteral? Med. Welt 762, 1963.
18. Withey, J. L., and Kilpatrick, G. S.: Hydroxocobalamin and cyanocobalamin in Addisonian anaemia. Lancet 1:16, 1964.
19. Skeggs, H. R., Nepple, K. M., Valentik, K. A., Huff, J. W., and Wright, L. D.: Observations on the use of Lactobacillus leichmannii in the estimation of vitamin B_{12} activity. J. Biol. Chem. 184:211, 1950.
20. Bauriedel, W. R., Picken, J. C., and Underkofler, L. A.: Reactions of cyanocobalamin and aquocobalamin with proteins. Proc. Soc. Exper. Biol. Med. 91:377, 1956.
21. Miller, A., and Sullivan, J. F.: Increased binding of vitamin B_{12} by serum mucoproteins in chronic myelogenous leukemia (Abstract). J. Clin. Invest. 37:917, 1958.
22. Chosy, J. J., Killander, A., and Schilling, R. F.: Studies on hydroxocobalamin. II. Estimates of absorption from the gut and binding to serum and liver. *In* Vitamin B_{12} and Intrinsic Factor (H. C. Heinrich, Ed.). 2 Europ. Symposium, Hamburg, 1961. Stuttgart, Ferdinand Enke, 1962, p. 668.
23. Kakei, M., and Glass, G. B. J.: Separation of bound and free vitamin B_{12} on Sephadex G-25 column (Abstract). Fed. Proc. 21:469, 1962; Proc. Soc. Exper. Biol. Med. 111:270, 1962.
24. Schiffer, L. M., Price, D. C., Cohn, S. H., and Cronkite, E. P.: Body retention of hydroxocobalamin and cyanocobalamin (Abstract). Clin. Res. 11: 199, 1963.
25. Glass, G. B. J., and Lee, D. H.: Hydroxocobalamin. IV. Biological half-life of hydroxocobalamin in the human liver. Blood 27:227–233, 1966.
26. —: Hydroxocobalamin and its long-acting effects in treatment of vitamin B_{12} deficiency. Pharmakotherapia 2: 21–58, 1964 (Review).
27. Heinrich, H. C.: Die Umsatzräte des Vitamin B_{12} als Grundlage der Errechnung des Vitamin-B_{12}-Bedarfes und der Vitamin-B_{12}-Dosierung in der Therapie. Verh. Dtsch. Ges. Inn. Med. 67:861, 1961.

Goodhart, R.S., and M.E. Shile (Eds.). 1973

V. Herbert, Folic Acid Vitamin B_{12}

Modern Nutrition in Health and Disease Dietotherapy, 5th Edition

Lea and Febiger, Philadelphia
pp. 221-244

Placental Transport of Vitamin B_{12} in the Pregnant Rat

STANLEY E. GRABER, URSULA SCHEFFEL, BARBARA HODKINSON, and PATRICIA A. MCINTYRE

From the Divisions of Hematology and Nuclear Medicine, The Johns Hopkins Medical Institutions, Baltimore, Maryland 21205

ABSTRACT Placental transport of vitamin B_{12} was studied in the pregnant rat in two series of experiments. In the first series animals were given cyanocobalamin-^{57}Co intravenously at various stages of gestation. High specific activity tracer was used and doses of B_{12} were 1–2 ng per animal. The rats were killed from 15 min to 24 hr after injection and the fetuses, placentas, and serum were assayed for radioactivity. In the second series using uninjected animals, absolute amounts of vitamin B_{12} in fetuses and placentas were measured at stages of gestation from day 12 through day 20.

There was a progressive increase in B_{12} transferred to the fetus during gestation. Although the quantity of vitamin B_{12} transported per 24 hr was proportional to fetal weight, the amount transported per gram of placenta increased tenfold from day 10 through day 19. Uptake of tracer B_{12} by placenta was initially rapid; however, no radioactivity appeared in the fetus until 2 hr after injection. The actual amount of B_{12} in placenta increased throughout gestation, and the placental concentration of B_{12} was greater than maternal plasma and fetal tissue concentrations at all times measured.

These data suggest that the ability of placenta to transport B_{12} increased throughout gestation, and that the rate-limiting step in the transport process was either the passage of B_{12} from the maternal to the fetal side of placenta or the transfer from placenta into fetal plasma.

INTRODUCTION

Several investigators have shown that radioactive cyanocobalamin[1] crosses placenta from mother to fetus (1–3). This transfer can occur against a concentration gradient, since maternal plasma vitamin B_{12} levels late in pregnancy are much lower than corresponding fetal levels (4, 5). Luhby, Cooperman, Stone, and Slobody have suggested that labeled vitamin B_{12} accumulates in the human placenta before passing to the fetus (6). In their work, substantial amounts of radioactivity were not transferred to the fetus until 24 hr after administration (7). Ullberg, Kristoffersson, Flodh, and Hamngren (8) injected mice with supra-physiologic doses of cyanocobalamin-^{58}Co and also showed a buildup of radioactivity in placenta with relatively slow passage to fetus. Other than the above, little is known about the transport process. The experiments reported here were undertaken to characterize further the kinetics and mechanism of vitamin B_{12} placental transfer.

METHODS

Animal experiments. Pregnant Sprague-Dawley descendant rats[2] were used as an animal model. Total gestation in this species is 21 days and the number of fetuses per litter ranges from 8 to 16. Onset of pregnancy was timed by the observation of a copulation plug. All animals were fed a standard commercial laboratory rat diet.[3] Two separate methods of study were employed. In the first method, each rat was given 1–2 ng of high specific activity (165 μCi/μg) cyanocobalamin-^{57}Co[4] intravenously through a tail vein. At selected time intervals the rats were killed and the fetuses, placentas, and maternal serum were assayed for ^{57}Co radioactivity. Animals from 10 to 19 days of gestation were studied 24 hr after injection, and 16- and 18-day pregnant rats were studied from 15 min to 24 hr postinjection. In several other animals, whole body and urine radioactivity was monitored for a period of 48 hr after injection. During this time whole body radioactivity did not change and less than 1% of the injected dose appeared in the urine.

In the second method, rats which had not been given tracer vitamin B_{12} were sacrificed at days 12, 14, 16, 18, and 20 of gestation. The placentas and fetuses from each animal were separated and pooled and the vitamin B_{12} was extracted and measured by a competitive binding assay.

Tissue extraction of vitamin B_{12}. Vitamin B_{12} was extracted from fetuses and placentas by a modification of the

This work was presented in part to the meeting of the Society of Nuclear Medicine, 12 July 1970, Washington, D. C. 1970 *J. Nucl. Med.* 11: 322. (Abstr.)

Dr. Graber's present address is the Veterans Administration Hospital, Department of Medicine, 1310 24th Avenue South, Nashville, Tenn. 37203

Received for publication 6 October 1970.

[1] The terms cyanocobalamin, vitamin B_{12} and B_{12} are used interchangeably throughout the text and refer to cyanocobalamin.

[2] Charles River Breeding Laboratories, Wilmington, Mass.

[3] Ralston Purina Company, St. Louis, Mo.

[4] Amersham-Searle Corp., Arlington Heights, Ill.

method of Bacher, Boley, and Shonk (9). Tissues were homogenized in deionized water and transferred quantitatively to a boiling flask. A known amount of tracer cyanocobalamin-^{58}Co was then added. Sufficient volumes of 2% sodium nitrite and 0.8% potassium cyanide were placed in the flask to give a final concentration of 0.5 and 0.2% respectively. The pH was adjusted to 4.5 with concentrated acetic acid and the mixture was boiled vigorously for 7–8 min. After cooling, the slurry was filtered through a Büchner funnel. A 10 ml sample of this filtrate was passed through a millipore filter (pore diameter 0.45 μ) and then placed on an ion exchange column consisting of 10 ml of Amberlite[5] IR 120 overlaid with 10 ml of IRA 400. The first 10 ml of eluate was discarded (void volume) and the second 10 ml containing most of the radioactivity was collected. The ^{58}Co radioactivity in 1.0 ml of this material was measured and compared to the total counts added to determine the recovery of vitamin B_{12}. A sample of this column eluate was then used in the competitive binding assay of B_{12}.

Assay of nonradioactive vitamin B_{12}. Vitamin B_{12} was measured by a modification of the competitive binding assay described by Friedner, Josephson, and Levin (10). Saliva (11) was used as the B_{12} binder and cyanocobalamin-^{57}Co as the assay tracer. This B_{12} binder has an estimated molecular weight of 60,000 (11), while cyanocobalamin's molecular weight is 1357. Free B_{12} was separated from bound B_{12} by ultrafiltration through Centriflo[6] membrane cones, which retain molecules above 50,000 molecular weight and have essentially no retention for molecules below 5000. The ^{57}Co radioactivity in an aliquot of the ultrafiltrate was used as a measure of free B_{12}. For each run a standard curve was constructed by treating tubes containing from 0 to 1500 pg of cyanocobalamin exactly as the unknowns. The standard curves were sigmoid in shape and most sensitive in the middle two-thirds. Therefore, a dilution of the column eluates of the unknowns which gave values within this portion of the curve was used.

Radioactivity measurement. All specimens were measured for radioactivity in a well-type NaI(Tl) scintillation detector coupled to a standard spectrometer. Sufficient counts were obtained to ensure counting errors of less than 2%. Dual isotope counting for separation of ^{57}Co from ^{58}Co activity was performed by the two window method (12). The per cent injected dose or per cent recovery was determined by comparison with the appropriate standard prepared in an equal volume for identical counting geometry.

RESULTS

Fig. 1 shows the radioactivity in fetuses and placentas at various times during gestation in animals killed 24 hr after injection of vitamin B_{12}-^{57}Co. The rate of transfer of label to the fetuses per 24 hr period was found to increase throughout pregnancy from 0.5% of the injected dose at day 10 to 55% at day 19. This increase was particularly rapid during the latter part of gestation, rising from 33% on day 18 to 55% on day 19. Accumulation of label in placentas followed a similar pattern to fetuses through day 14, then plateaued at about 12% of the dose, a level considerably less than the corresponding fetal values. In Fig. 2, the same fetal data shown in Fig. 1, are plotted on a per gram basis. The concentration of tracer in the fetus did not change appreciably during gestation. However, the amount of label transported to the fetuses per gram of placenta per 24 hr period steadily increased from 0.5% on day 10 to 5.4% on day 19.

Fig. 3 shows the accumulation of labeled B_{12} in fetuses and placentas of 16-day animals at various times after injection. Initially there was very rapid uptake of tracer by placenta, which increased until 2 hr after injection. The radioactivity then leveled off at 17% of the injected dose and finally began to slowly decrease after 16 hr.

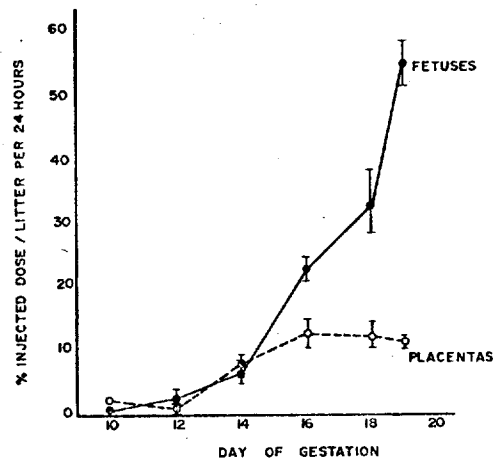


FIGURE 1 Fetal and placental vitamin B_{12}-^{57}Co radioactivity 24 hr after injection at various stages of gestation. Values equal the mean of three or four animals ±1 SD except the 10-day points, which represent a single animal.

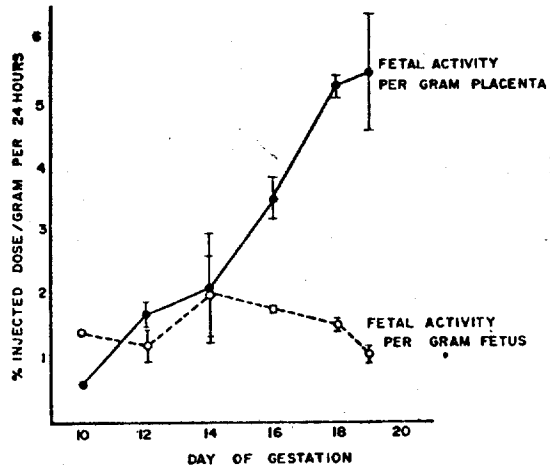


FIGURE 2 Fetal data from Fig. 1 expressed as radioactivity per gram fetus and per gram placenta. Values equal the mean of three or four animals ±1 SD except the 10-day points, which represent a single animal.

[5] Mallinckrodt Chemical Works, St. Louis, Mo.
[6] Amicon Corporation, Lexington, Mass.

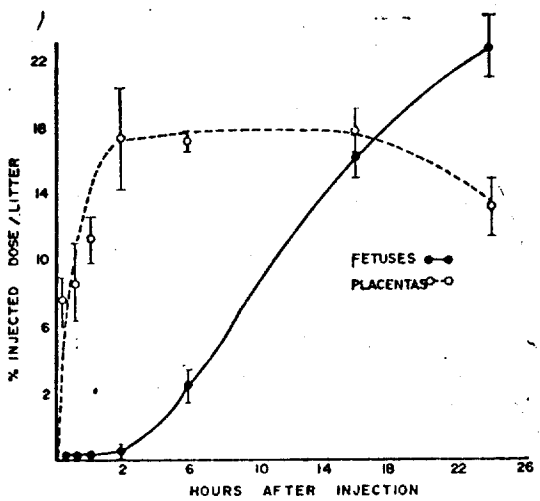


FIGURE 3 Fetal and placental vitamin B_{12}-^{57}Co radioactivity 15 min to 24 hr after injection in the 16th day of pregnancy. Values equal the mean of three or four animals ±1 SD.

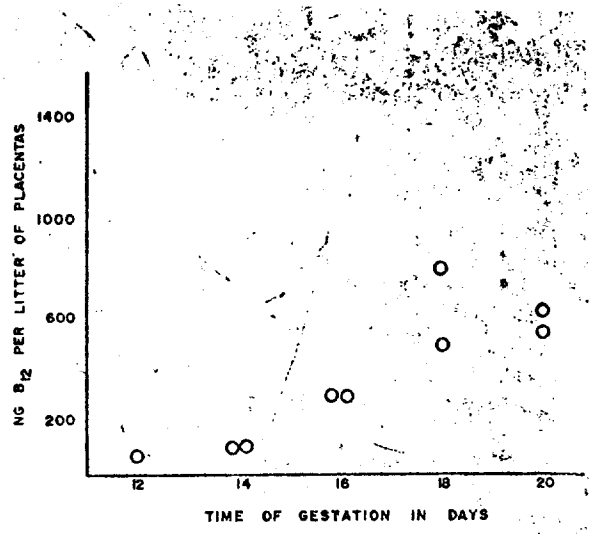


FIGURE 5 Vitamin B_{12} content of placentas on various days of gestation. Each point represents the pooled placentas from a single animal.

In contrast, no activity was seen in the fetuses until 2 hr postinjection when only 0.3% of the injected dose was present. Subsequently, there was progressive fetal uptake of label reaching 22.5% of the dose at 24 hr. This same type of data obtained in 18-day rats is shown in Fig. 4. Total uptake by the placentas and fetuses was significantly greater than in 16-day animals, but the pattern of uptake was similar. In both the 16- and 18-day animals the plasma clearance of radioactive B_{12} was initially very rapid, with less than 0.55% of the dose per ml of serum present 15 min after injection at both stages of gestation. The serum activity then decreased slowly over the next 24 hr to 0.043 and 0.039% per ml.

Figs. 5 and 6 show the total amount of actual vitamin B_{12} in placenta and fetus at various stages of gestation from day 12 through day 20. The quantity of vitamin in the placenta appeared to increase throughout gestation, as the total content per litter rose from 75 ng on day 12 to over 550 ng on day 20. The quantity of B_{12} in the fetuses also increased progressively, rising from 40 ng on day 12 to 1400 ng on day 20. The amount of vitamin B_{12} per gram of fetus or placenta at the various stages of gestation is plotted in Fig. 7. The fetal concentration was relatively constant throughout pregnancy ranging from 22 to 39 ng per gram of tissue, but the

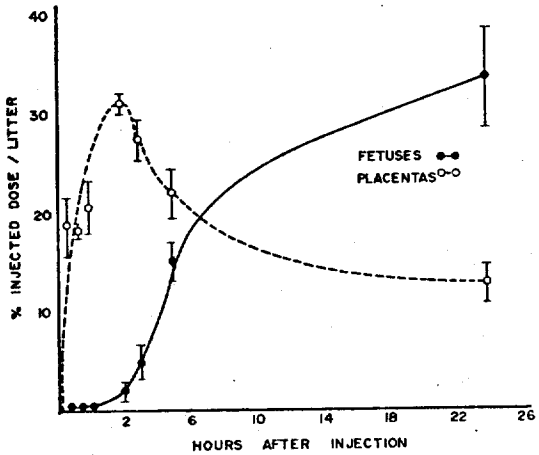


FIGURE 4 Fetal and placental vitamin B_{12}-^{57}Co radioactivity from 15 min to 24 hr after injection in the 18th day of pregnancy. Values equal the mean of three or four animals ±1 SD.

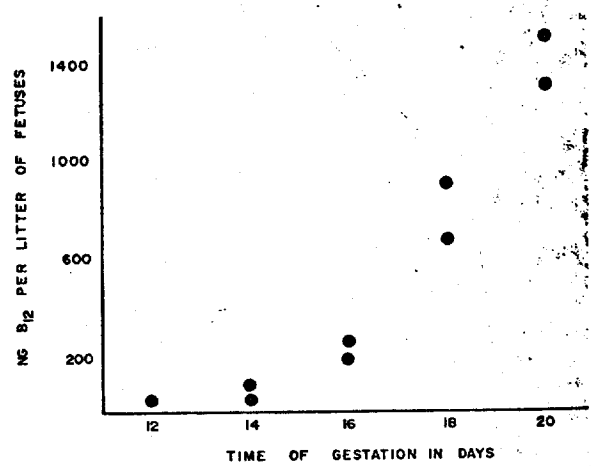


FIGURE 6 Vitamin B_{12} content of fetuses on various days of gestation. Each point represents the pooled fetuses from a single animal.

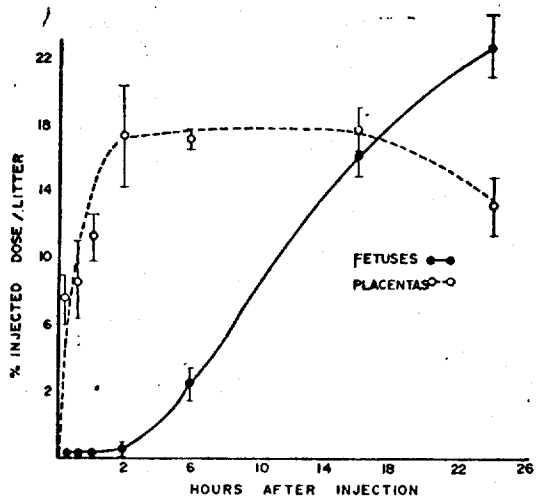


FIGURE 3 Fetal and placental vitamin B_{12}-^{57}Co radioactivity 15 min to 24 hr after injection in the 16th day of pregnancy. Values equal the mean of three or four animals ±1 SD.

In contrast, no activity was seen in the fetuses until 2 hr postinjection when only 0.3% of the injected dose was present. Subsequently, there was progressive fetal uptake of label reaching 22.5% of the dose at 24 hr. This same type of data obtained in 18-day rats is shown in Fig. 4. Total uptake by the placentas and fetuses was significantly greater than in 16-day animals, but the pattern of uptake was similar. In both the 16- and 18-day animals the plasma clearance of radioactive B_{12} was initially very rapid, with less than 0.55% of the dose per ml of serum present 15 min after injection at both

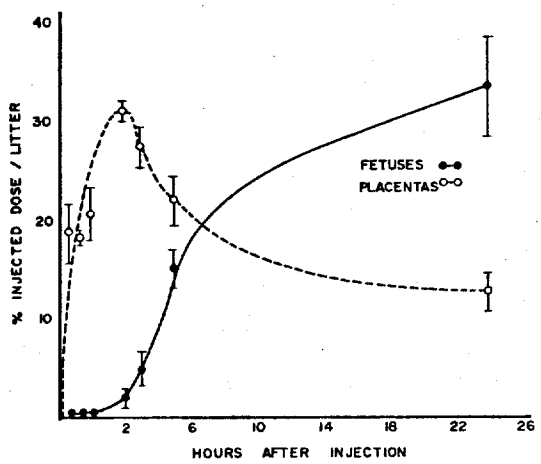


FIGURE 4 Fetal and placental vitamin B_{12}-^{57}Co radioactivity from 15 min to 24 hr after injection in the 18th day of pregnancy. Values equal the mean of three or four animals ±1 SD.

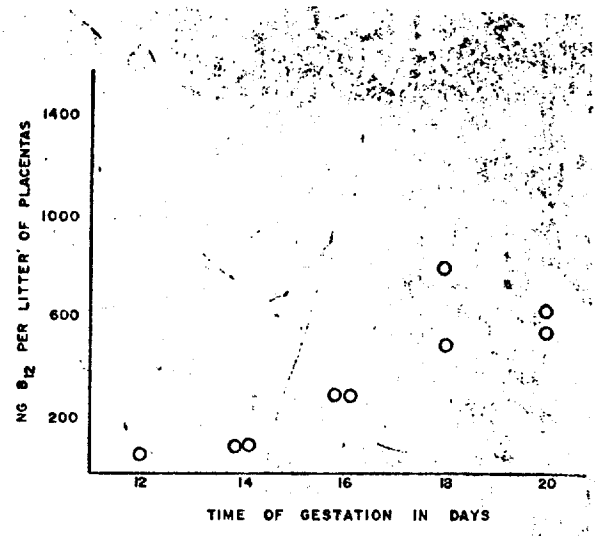


FIGURE 5 Vitamin B_{12} content of placentas on various days of gestation. Each point represents the pooled placentas from a single animal.

stages of gestation. The serum activity then decreased slowly over the next 24 hr to 0.043 and 0.039% per ml.

Figs. 5 and 6 show the total amount of actual vitamin B_{12} in placenta and fetus at various stages of gestation from day 12 through day 20. The quantity of vitamin in the placenta appeared to increase throughout gestation, as the total content per litter rose from 75 ng on day 12 to over 550 ng on day 20. The quantity of B_{12} in the fetuses also increased progressively, rising from 40 ng on day 12 to 1400 ng on day 20. The amount of vitamin B_{12} per gram of fetus or placenta at the various stages of gestation is plotted in Fig. 7. The fetal concentration was relatively constant throughout pregnancy ranging from 22 to 39 ng per gram of tissue, but the

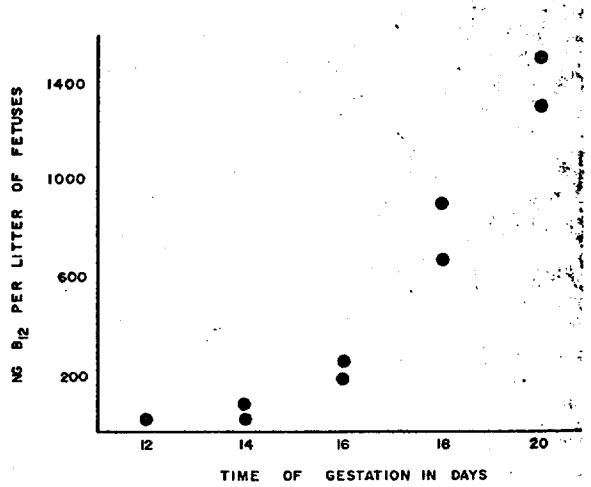


FIGURE 6 Vitamin B_{12} content of fetuses on various days of gestation. Each point represents the pooled fetuses from a single animal.

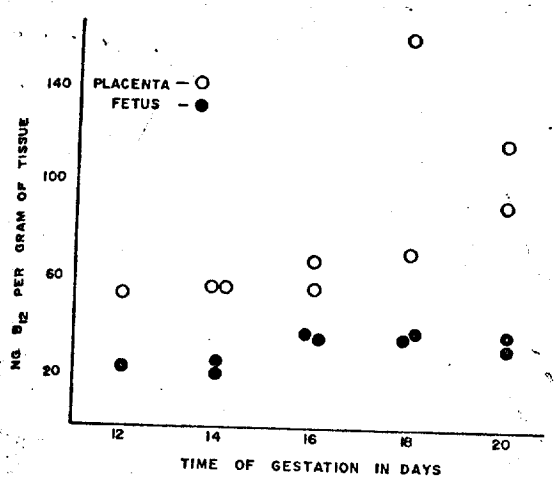


FIGURE 7 Vitamin B_{12} content per gram fetus and per gram placenta on various days of gestation. Each point represents the pooled fetuses or placentas from a single animal.

placental concentration increased during the latter 4 days of pregnancy, and was much greater than the fetal concentration at all times measured during gestation.

DISCUSSION

Previous investigators (6–8) have shown that late in pregnancy, radioactive cyanocobalamin accumulates in the placenta and only slowly passes to the fetus. Our work has confirmed these findings. In addition, we have used very small doses of radioactive B_{12} to study the rate of transfer of tracer to the fetus throughout gestation, and have measured the total vitamin B_{12} content of the fetuses and placentas periodically during pregnancy.

In our experiments the 24 hr rate of placental transfer of tracer B_{12} increased progressively throughout gestation, as did the fetal content of vitamin B_{12}. These increases were most rapid during the latter 3–4 days of gestation, and appeared to be proportional to fetal growth. The concentration per gram of fetus of radioactivity and of total B_{12} remained relatively constant throughout pregnancy. On the other hand the amount of tracer vitamin B_{12} transferred daily to fetus per gram of placenta steadily rose some ten-fold from day 10 to day 19. This suggests that either the ability of placenta to transport B_{12} increased during gestation or alternatively, that fetal growth was associated with removal of some block in the transport process. Possibly a feedback mechanism exists which is related to fetal tissue vitamin B_{12} concentration.

The rat chorioallantoic placenta consists of three layers of trophoblast, a basement membrane, and one layer of fetal endothelium (13). It is perfused on one side by maternal blood and on the other by fetal blood. Transport of vitamin B_{12} from mother to fetus can thus be divided arbitrarily into three stages: (*a*) transfer of vitamin from maternal plasma across the maternal-placenta interface into placenta, (*b*) passage from the maternal to the fetal side of placenta, and (*c*) transfer across the placental-fetal interface into the fetal plasma. In the tracer experiments in which rats at one stage of gestation were killed at various times after injection, there was rapid uptake of labeled vitamin B_{12} by placenta and approximately 2 hr elapsed before label was found in the fetus. These results are in agreement with those of Ullberg et al. (8), who used much larger doses of vitamin B_{12}, and indicate that stage (*a*) of the transport process is rapid and not rate limiting. Furthermore, since the concentration of B_{12} in placenta, which ranged from 55 to 163 ng per g, was much greater than maternal plasma levels (4, 5, 14), this initial uptake of vitamin B_{12} by placenta may represent an active transport process. Alternatively, placental tissue might contain a binder with a much higher affinity for vitamin B_{12} than maternal plasma. The 2 hr lag before appearance of radioactivity in the fetus and the accumulation of B_{12} in the placentas during gestation suggest that either stage (*b*) or (*c*) represents the rate-limiting step of the transport process. This concept would be in keeping with the observed downhill concentration gradient from placenta to fetus throughout pregnancy, which was greatest during the latter part of gestation when B_{12} transport was increasing rapidly.

The 1–2 ng of tracer cyanocobalamin given each animal apparently did not exceed the capacity of its plasma B_{12}-binding proteins, since virtually no label appeared in the urine, and whole body radioactivity was unchanged for the 48 hr following injection. Rosenblum, Reizenstein, Cronkite, and Meriwether have shown identical tissue distribution of oral and intravenously administered radioactive cyanocobalamin (15). The small amounts of tracer B_{12} given in our studies thus presumably behaved in a similar fashion to vitamin B_{12} absorbed from the mother's gastrointestinal tract.

The time pattern of the placental transport of B_{12} may be analogous to the transfer of intrinsic factor bound B_{12} across ileal mucosa into blood. In this case in man there is slow passage of labeled B_{12} across the mucosal cell and blood levels do not peak until 6–10 hr after oral ingestion (16). Booth, Chanarin, Anderson, and Mollin used 2.5 ng oral doses of B_{12}-^{60}Co and demonstrated in the rat that 40% of the ingested B_{12} was bound to the wall of the small intestine within 15 min (17). No radioactivity was found in the blood until 1.5 hr after ingestion and peak levels were not seen until 4 hr after ingestion.

In contrast, the pattern of placental transport of vitamin B_{12} appears to be distinctly different from that of

iron, a nutrient which like vitamin B_{12} is necessary for normal erythropoesis, is bound to a specific plasma protein, and is present in lower concentrations in maternal than fetal plasma (18). When tracer ^{59}Fe was bound to transferrin and injected into 18-day pregnant rats, significant differences in placental transport from tracer B_{12} were observed (19, 20). Transfer of ^{59}Fe to the fetus began immediately and rapidly reached a plateau, while B_{12}-^{57}Co did not appear in the fetus until 2 hr after injection and continued to increase for the period of observation. In addition, significant placental accumulation of radioactivity was not observed when ^{59}Fe was injected, but did occur after injection of B_{12}-^{57}Co.

ACKNOWLEDGMENTS

These studies were supported by U. S. Public Health Service grants GM 10548, AM 43549, AM 05260, and 5 ROI-A1-09077-02.

REFERENCES

1. Chow, B. F., L. Barrows, and C. T. Ling. 1951. The distribution of radioactivity in the organs of the fetus or of young rats born by mothers injected with vitamin B_{12} containing Co^{60}. *Arch. Biochem. Biophys.* **34**: 151.
2. Luhby, A. L., J. M. Cooperman, and A. M. Donnenfeld. 1959. Placental transfer and biological half-life of radioactive vitamin B_{12} in the dog. *Proc. Soc. Exp. Biol. Med.* **100**: 214.
3. Woods, W. D., W. B. Hawkins, and G. H. Whipple. 1960. Vitamin B_{12} Co^{60} readily passes the placenta into fetal organs and nursing provides B_{12} from mother to pup. A record of its distribution. *J. Exp. Med.* **112**: 431.
4. Luhby, A. L., R. Feldman, J. F. Marley, O. Odang, and J. M. Cooperman. 1961. Serum vitamin B_{12} in infancy. (Abstr.) *Fed. Proc.* **20**: 451.
5. Lowenstein, L., M. Lalonde, E. B. Deschenes, and L. Shapiro. 1960. Vitamin B_{12} in pregnancy and the puerperium. *Amer. J. Clin. Nutr.* **8**: 265.
6. Luhby, A. L., J. M. Cooperman, M. L. Stone, and L. B. Slobody. 1961. Physiology of vitamin B_{12} in pregnancy, the placenta, and the newborn. *Amer. J. Dis. Child.* **102**: 753. (Abstr.)
7. Luhby, A. L., J. M. Cooperman, A. M. Donnenfeld, J. M. Herrero, D. N. Teller, and J. B. Wenig. 1958. Observations on transfer of vitamin B_{12} from mother to fetus and newborn. *Amer. J. Dis. Child.* **96**: 532. (Abstr.)
8. Ullberg, S., H. Kristoffersson, H. Flodh, and A. Hamgren. 1967. Placental passage and fetal accumulation of labeled vitamin B_{12} in the mouse. *Arch. Int. Pharmacodyn. Ther.* **167**: 431.
9. Bacher, F. A., A. E. Boley, and C. E. Shonk. 1954. Radioactive tracer assay for vitamin B_{12} and other cobalamins in complex mixtures. *Anal. Chem.* **26**: 1146.
10. Friedner, S., B. Josephson, and K. Levin. 1969. Vitamin B_{12} determination by means of radioisotope dilution and ultrafiltration. *Clin. Chim. Acta.* **24**: 171.
11. Carmel, R., and C. A. Coltman, Jr. 1969. Radioassay for serum vitamin B_{12} with the use of saliva as the vitamin B_{12} binder. *J. Lab. Clin. Med.* **74**: 967.
12. Ross, D. A., and C. C. Harris. 1968. Measurement of Radioactivity. *In* Principles of Nuclear Medicine. Henry N. Wagner, Jr., editor. W. B. Saunders Company, Philadelphia. 130.
13. Tillack, T. W. 1966. The transport of ferritin across the placenta of the rat. *Lab. Invest.* **15**: 896.
14. Hellegers, A., K. Okuda, R. E. L. Nesbitt, Jr., D. W. Smith, and B. F. Chow. 1957. Vitamin B_{12} absorption in pregnancy and in the newborn. *Amer. J. Clin. Nutr.* **5**: 327.
15. Rosenblum, C., P. G. Reizenstein, E. P. Cronkite, and H. T. Meriwether. 1963. Tissue distribution and storage forms of vitamin B_{12} injected and orally administered to the dog. *Proc. Soc. Exp. Biol. Med.* **112**: 262.
16. Doscherholmen, A., and P. S. Hagen. 1957. A dual mechanism of vitamin B_{12} plasma absorption. *J. Clin. Invest.* **36**: 1551.
17. Booth, C. C., I. Chanarin, B. B. Anderson, and D. L. Mollin. 1957. The site of absorption and tissue distribution of orally administered ^{60}Co-labelled vitamin B_{12} in the rat. *Brit. J. Haematol.* **3**: 253.
18. Morgan, E. H. 1964. Passage of transferrin, albumin and gamma globulin from maternal plasma to foetus in the rat and rabbit. *J. Physiol.* (London). **171**: 26.
19. Graber, S. E., P. J. Hurley, R. M. Heyssel, and P. A. Mc Intyre. 1970. Behavior of iron-, indium-, and iodine-labeled transferrin in the pregnant rat. *Proc. Soc. Exp. Biol. Med.* **133**: 1093.
20. Bothwell, T. H., W. F. Pribilla, W. Mebust, and C. A. Finch. 1958. Iron metabolism in the pregnant rabbit; iron transport across the placenta. *Amer. J. Physiol.* **193**: 615.

1033

J. Lab. Clin. Med. 419-429

Lognormal distribution of serum vitamin B_{12} levels and dependence of blood values on the B_{12} level in a large population heavily infected with *Diphyllobothrium latum*

RALPH GRÄSBECK, M.D., WOLMAR NYBERG, M.D., MATTI SAARNI, M.D., and BERTEL VON BONSDORFF, M.D.
Helsingfors and Vasa, Finland

In a field study in eastern Finland, 1,345 persons in a "tapeworm district" were subjected to various examinations, including a search for tapeworm ova in the feces, microbiologic assay of the serum vitamin B_{12} level, and determination of a number of hematologic values. In 27.5 per cent Diphyllobothrium latum ova were found, and the mean serum vitamin B_{12} level was significantly lower in the tapeworm carrier group than in the subjects having no ova in the feces. Further analysis of the material revealed that in both groups the serum vitamin B_{12} values were lognormally distributed. Analysis of the distribution in the group with no tapeworm ova in the feces indicated that not more than 5 to 10 per cent of this group were worm carriers.

The material being unique in respect to the high incidence of low serum vitamin B_{12} levels, it was possible to elucidate the dependence of the blood values on the vitamin B_{12} concentration.

When the serum vitamin B_{12} level decreased below 60 to 90 μμg per milliliter, the red cell count began to decrease and the mean corpuscular hemoglobin and mean corpuscular volume indices and the erythrocyte diameter to increase. No similar dependence on the vitamin B_{12} level of the hemoglobin concentration and the volume of packed red cells could be detected. The reason for the lognormal distribution and the relative merits of various hematologic examinations used to diagnose pernicious anemia were discussed. It was concluded that in Finland D. latum deprives its host of vitamin B_{12} and frequently causes hematologic abnormalities.

In order to elucidate the incidence of *Diphyllobothrium latum* infection, tapeworm anemia, and low vitamin B_{12} concentration in the blood serum, a popu-

From the Fourth Department of Medicine, Helsingfors University (address: Maria Hospital), Helsingfors, and the Department of Medicine, Vasa Central Hospital, Vasa, Finland.

Supported by Grant No. E-1693 of the United States Public Health Service, National Institutes of Health.
Received for publication May 13, 1961.

444

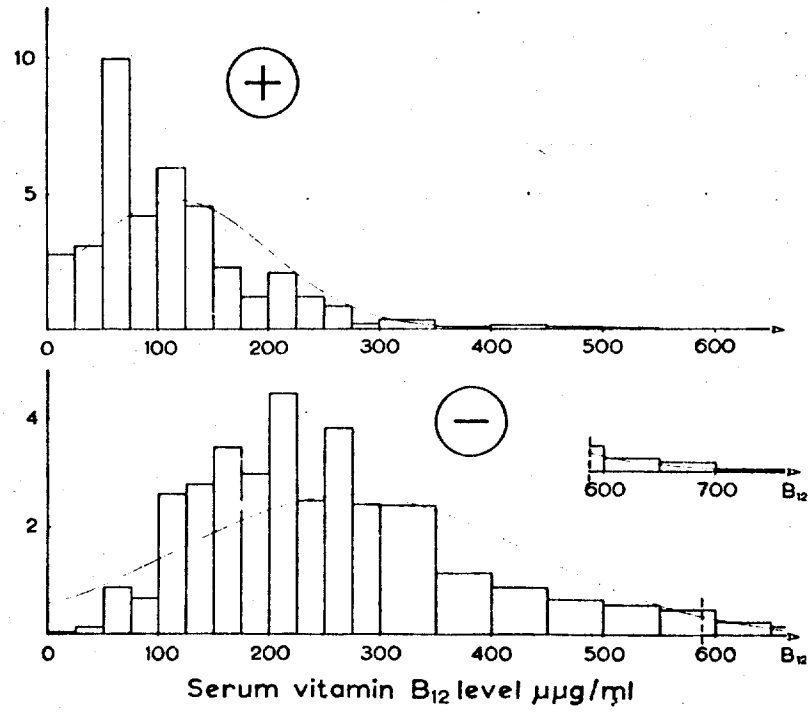


Fig. 1. Distribution of serum vitamin B_{12} values in tapeworm carriers (+) and subjects with no tapeworm ova in the feces (−). Ordinate: arbitrary units describing the number of cases. Note skewness.

lation in eastern Finland was subjected to a mass examination. This included a search for tapeworm ova in the feces, determination of the serum vitamin B_{12} level, and a number of hematologic examinations. The details of the investigation procedure and the findings regarding the effect of tapeworm infection on the appearance of anemia and low serum vitamin B_{12} levels have been reported.[1]

The data obtained during this mass examination provided us with an opportunity to study the statistical distribution of serum vitamin B_{12} levels in a large population. Also, as the material was unique in respect to the high incidence of low vitamin B_{12} values, it was possible to elucidate the dependence of the hematologic findings on the serum vitamin B_{12} concentration.

Materials and methods

The data were obtained from 1,345 subjects who, alerted by a local press and school campaign in the Lieksa region in eastern Finland, visited temporary reception offices installed in schools, etc. The patients brought with them samples of feces, which were examined for the presence of tapeworm ova. A blood sample was taken into a thoroughly washed centrifuged tube, from which the serum was obtained after clotting by centrifugation. The serum was frozen in boxes containing dry ice, transported to the laboratory, and stored in a deep freeze until assayed for its vitamin B_{12} concentration with *Euglena gracilis* of the *z* strain, according to the method of Hutner and associates.[2]

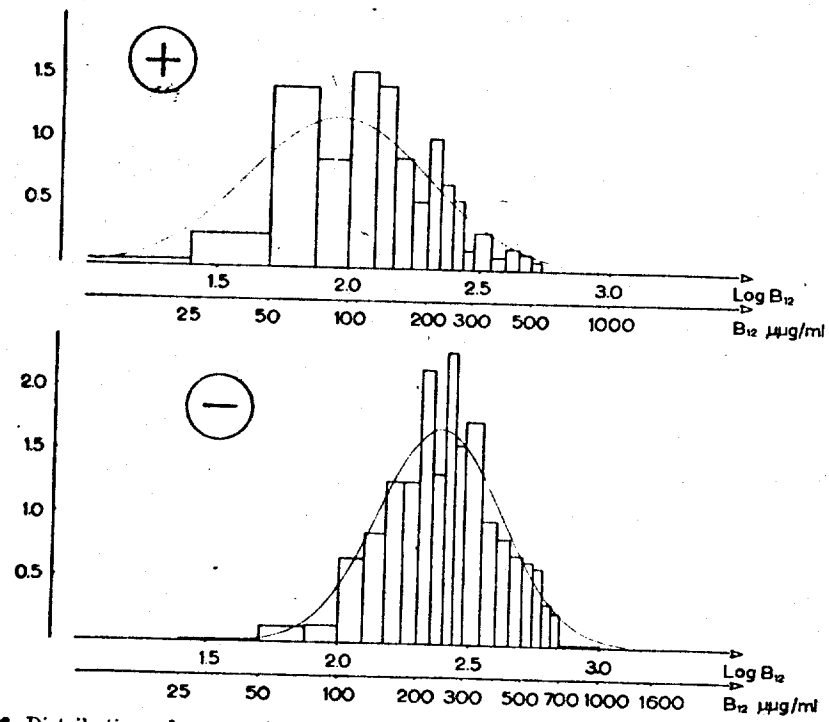


Fig. 2. Distribution of serum vitamin B_{12} values in tapeworm carriers (+) and control subjects (−). The serum vitamin B_{12} value is plotted on a logarithmic scale. The distribution curve assumes a gaussian appearance.

In addition, a number of hematologic examinations were performed: Determination of the erythrocyte count (visual counting); hemoglobin concentration (alkaline oxyhemoglobin method with photoelectric colorimeter); volume of packed red cells (a disposable tube method); and erythrocyte diameter (halometrically from smears). Fingertip blood was used for all determinations. In addition, 2 indices were calculated from these data: Mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV). If clearly abnormal blood was found, the patient was persuaded to enter a hospital, or other arrangements were made to obtain a complete hematologic examination. This included bone marrow biopsy and radiovitamin B_{12} absorption tests.

Every subject was assigned a number, and the persons performing the laboratory examinations knew neither the name of the patients nor the results of the other examinations. When all the specimens had been examined, the data were transferred to punched cards. An exception to this rule was made in the case of the clearly anemic patients, who were examined more carefully during the field study.

Some characteristics of the material are given in Table I. Other data can be found in the article by Nyberg and colleagues.[1]

A substantial part of the statistical work was performed with the aid of electronic computers.

Results

Gaussian distribution. The number of samples being large, it was possible to study the statistical distribution of the serum vitamin B_{12} concentration. First,

histograms representing the distribution were drawn separately for the tapeworm ova positive and negative groups (Fig. 1). No attempt was made to differentiate between the sexes, since there appeared to be no significant difference between them in respect to the serum B_{12} level or the frequency of tapeworm infection (Table I). Also, there are no definite indications that in a normal series the serum vitamin B_{12} level is different in different age groups, and, in the tapeworm ova positive group, significant differences in this respect were noted only when the extreme age groups were compared with each other.[1] The distribution curves are seen to be extremely skew. Also, when the cumulative frequencies were plotted on probability paper, a straight line could not be constructed.

The appearance of the histograms suggested that a lognormal distribution curve would describe the actual distribution better. When the vitamin B_{12} concentration was substituted by its logarithm, the distribution curve assumed a gaussian appearance (Fig. 2). Also, straight lines could be discerned when the cumulative frequences were plotted on probability paper (Fig. 3).

These results indicate that when results of serum vitamin B_{12} assays are subjected to statistical analysis ("t" test, analysis of variance, correlation analysis, etc.) the logarithm of the vitamin B_{12} concentration should be used rather than the primary values for the vitamin B_{12} concentration.

Finally, a quantitative examination was performed. A χ^2 test was carried out to elucidate how well the modes of distribution fit the observed data. The results are given in Table II. Even though all the test results indicate that the

Table I. Some characteristics of the material which was analyzed statistically*

Sex and age group (years)	*No. of subjects examined*	*Worm carriers (per cent)*	*Mean of serum B_{12} level of tapeworm + and − groups† (μμg/ml.)*	
M, 9-15	208	15.4	149	284
F, 9-15	305	20.0	146	290
M, 16-30	63	27.0	127	274
F, 16-30	111	26.1	97	272
M, 31-40	44	29.5	98	235
F, 31-40	121	29.8	113	275
M, 41-50	83	44.6	113	233
F, 41-50	135	29.6	101	278
M, 51-60	61	41.0	99	256
F, 51-60	74	31.1	94	263
M, 61-70	24	41.7	113	304
F, 61-70	25	32.0	90	245
M, 71-89	24	58.3	80	208
F, 71-89	40	42.5	138	202
All men	507	29.2	116	268
All women	811	26.4	117	278
Both sexes	1,318	27.5	116	274

*From this table are omitted 27 cases in which the serum of vitamin B_{12} assay was void due to loss of sample, etc.

†The difference in the serum vitamin B_{12} levels of the positive and negative groups is highly significant ($p < 0.005$).[1]

1083

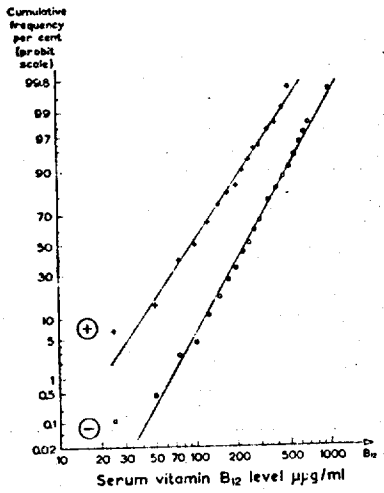


Fig. 3. Cumulative frequencies plotted on probability paper. Ordinate: percentage of cases having a vitamin B_{12} level $\leq$ corresponding abscissa. The serum vitamin B_{12} value is plotted on a logarithmic scale. (+) Indicates worm carriers, (−) indicates subjects with no tapeworm ova. Straight lines can be discerned.

observed data differ significantly from the theoretical distribution, they still support the conclusions drawn from the graphic analysis. The greater the χ^2 obtained, the greater is the difference from the suggested mode of distribution. In the light of these figures, a lognormal distribution must be considered to describe the actual distribution much better than does an ordinary normal distribution. In assessing the results of the test one should pay attention to the nonrandom systematic experimental errors which always occur in a large body of data and also to the fact that the χ^2 method is only approximate. It is also worth noting that the logarithmic distribution fits the tapeworm ova negative group much better than it fits the positive group.

Homogeneity of the tapeworm ova negative group. The finding of tapeworm ova proves that the subject examined is a worm carrier (unless the worms were expelled only a few days before examination). On the other hand, a negative finding does not prove that the worm is not present. Therefore it may be suspected that the tapeworm ova negative group contains worm carriers. Attempts were made to discover by statistical analysis how many worm carriers were present in this group. The analysis was based on the observation that in carriers of *D. latum* the serum vitamin B_{12} levels are highly significantly lower than in control subjects.

The most suitable hypothetical distribution which would give the results just described for the negative group seemed to be a mixture of 2 lognormal distributions. In fitting the theoretical to the observed distribution the conventional χ^2 criterion was used. Thus a distribution pattern was sought in which the result of the χ^2 test for the difference between the observed and the theoretical distribution would be as small as possible. The results obtained were a minimum value of the χ^2, the best possible values for the logarithmic means and the variances of the 2 contributing distributions and the ratio of the 2 distributions (presence and absence of worm) which were contained in the tapeworm material.

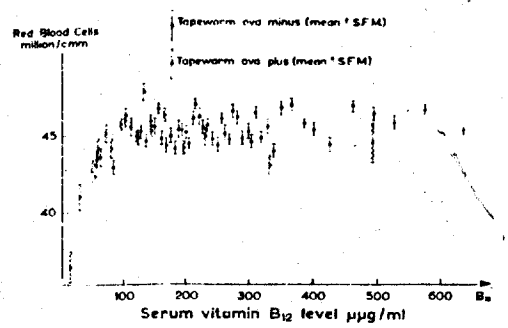


Fig. 4. Mean erythrocyte count (± standard error of the mean) of groups consisting of 20 subjects plotted against their mean serum vitamin B_{12} level. Note decrease in R.B.C. below 90 $\mu\mu g$ per milliliter.

From the start it was known that the percentage of worm carriers in the tapeworm ova negative group was likely to be small. Because of this the results had to be interpreted with caution, and in order to check the results the calculation was performed by grouping the material in different ways. This procedure was made possible by having an electronic computer perform the calculation. The computer sought the minimum value empirically by changing in small steps the 5 parameters of theoretical distribution, viz., the means for the 2 contributing distributions, the standard deviations, and the ratio of the 2.

The results of the different calculations varied considerably. However, the over-all impression was that not more than 5 to 10 per cent of the tapeworm ova negative group were worm carriers.

Dependence of the hematologic data on the serum vitamin B_{12} concentration. The correlation between the hemoglobin concentration, erythrocyte count, volume of packed red cells, erythrocyte diameter, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) on the one hand, and the serum vitamin B_{12} level on the other, was studied in the following manner: The data were divided into tapeworm ova positive and negative groups, and these were divided further into groups consisting of 20 subjects. The latter were found by tabulating the serum vitamin B_{12} levels in order of size and dividing the data into groups of 20 subjects, starting from the lowest figures. For each of the groups a group mean was computed for every hematologic value, and the means were compared in the usual manner, using one-dimensional analysis of variance.

As a result of the analysis of variance, the significance levels given in Table III were calculated and used to determine whether a true difference existed between the means.

As the number of means to be compared was great (in the tapeworm ova positive group their number was 18 and in the negative group 48), mere over-all comparison of the means is not sufficient. A detailed graphical comparison was made, the abscissa in the graph being the mean of the vitamin B_{12} group, the ordinate being the mean of the corresponding hematologic value (Figs. 4 through 7). The graphs offer very natural explanations for the observed differences between the means. It is evident that differences are produced only when the serum vitamin B_{12} level is low. This explains why significant differences between the means are found in the tapeworm carrier group only;

Table II. Chi-square test to elucidate how well the suggested modes of distribution fit the observed data

Subject groups	Test result: Distribution assumed normal	Distribution assumed lognormal	Limit for 99.95% significance
Tapeworm ova positive (362 observations)	χ^2 (10) = 160.1	χ^2 (10) = 53.0	χ^2 (10) = 31.4
Tapeworm ova negative (956 observations)	χ^2 (16) = 227.7	χ^2 (16) = 46.8	χ^2 (16) = 41.3

in the tapeworm ova negative group low serum vitamin B_{12} levels were very rare. From the graphs it is possible to estimate with fair accuracy the critical vitamin B_{12} level. The borderline values, estimated by our statistician from the tapeworm carrier material, are given in Table IV.

During this study no attempt was made to divide the data into male and female groups, though the sexes are known to have different normal values for hemoglobin concentration and red blood cell count. In Fig. 4 the standard errors of the means would certainly have become smaller if the sexes had been separated into different groups. However, within the new groups the serum

Table III. Dependence of hematologic values on the serum vitamin B_{12} level (analysis of variance)

	Test result: Ova positive	Ova negative	Significance level: Ova positive	Ova negative
Red blood cells	F(18,339) = 4.05	F(47,905) = 1.23	99.9 %	not significant
Hemoglobin	F(18,338) = 1.38	F(47,907) = 1.17	not significant	not significant
Hematocrit	F(18,314) = 1.23	F(47,641) = 1.14	not significant	not significant
Red cell diameter	F(18,329) = 3.28	F(47,872) = 0.93	99.9 %	not significant
Mean corpuscular volume	F(18,314) = 3.91	F(47,637) = 1.36	99.9 %	not significant
Mean corpuscular hemoglobin	F(18,338) = 3.89	F(47,904) = 1.23	99.9 %	not significant

Table IV. Borderline serum vitamin B_{12} levels below which blood changes occur; the estimation is based solely on the data for tapeworm carriers

Hematologic value	Borderline serum vitamin B_{12} value ($\mu\mu g$/ml.)
Red blood cells	90
Hemoglobin	no detectable borderline value
Hematocrit	no detectable borderline value
Red cell diameter	80
Mean corpuscular volume	90
Mean corpuscular hemoglobin	60

vitamin B_{12} values would have varied more, and therefore the sharpness with which the borderline values were detected would have suffered.

Hematologic values in tapeworm carriers. Examination of the plots in Figs. 4 through 7 reveals that low mean erythrocyte counts and high mean red cell diameters and MCV and MCH indices occurred almost exclusively in the tapeworm ova positive groups. Doubtless this is due to their lower serum vitamin B_{12} levels.

Discussion

The distribution of the serum vitamin B_{12} level was lognormal. A similar distribution has been reported for other substances contained in blood plasma.[3] To our knowledge no explanations for this phenomenon have been advanced in the literature. However, when the mean normal level is small compared to the standard deviation, asymmetric distributions of this kind must necessarily occur, since a symmetric distribution would imply negative values.[4] Second, many biologic effects are connected logarithmically with the concentration, e.g., that of drugs. It may be that most biometric measurements are really lognormally distributed, but when the mean is large compared to the standard deviation, the logarithmic and normal curves are so similar that they are difficult to distinguish.[5]

It is possible also to suggest explanations more closely connected with vitamin B_{12}. It is well known that the serum vitamin B_{12} activity is protein-bound, and that "free" vitamin is rapidly excreted with the urine.[6] The

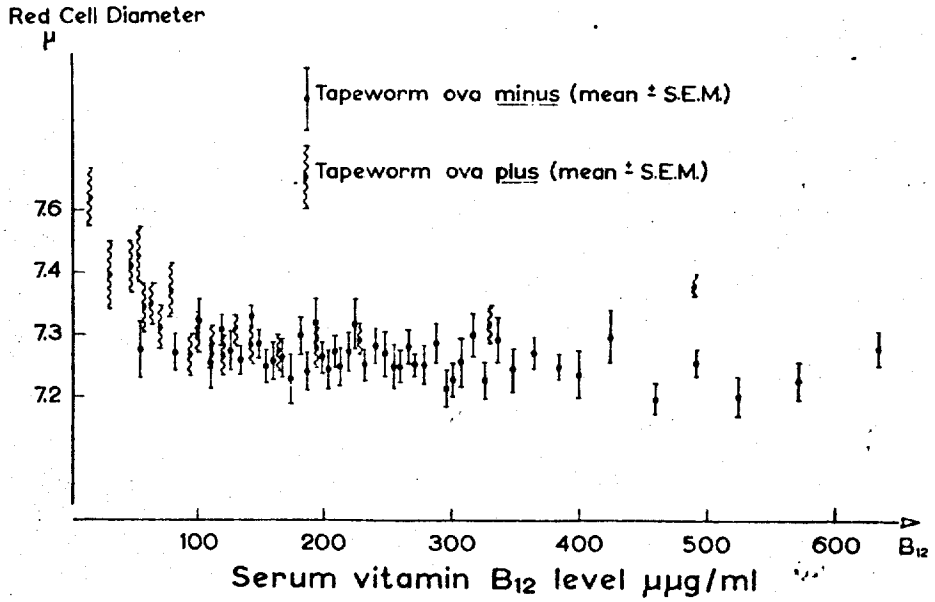


Fig. 5. Red cell diameter plotted against the serum vitamin B_{12} level. Note increase in erythrocyte diameter below 80 $\mu\mu g$ per milliliter.

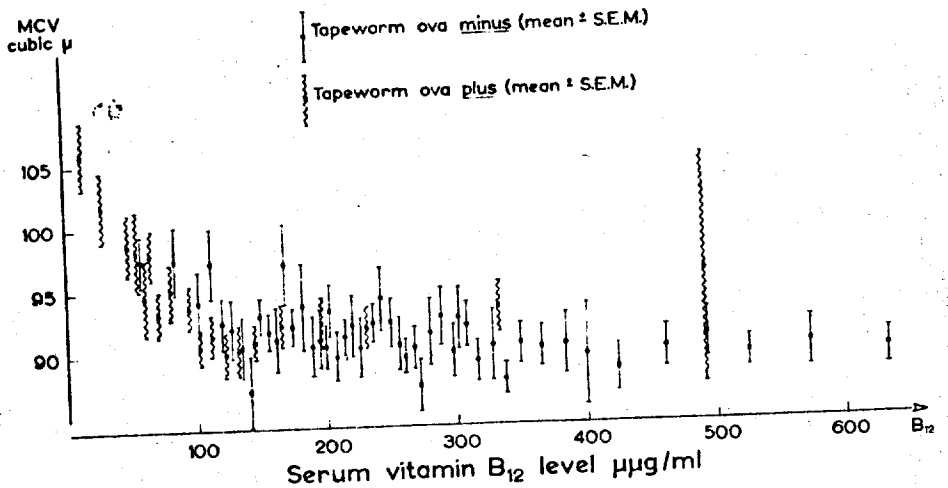


Fig. 6. Mean corpuscular volume plotted against the serum vitamin B_{12} level. Note increase in MCV below 90 $\mu\mu g$ per milliliter.

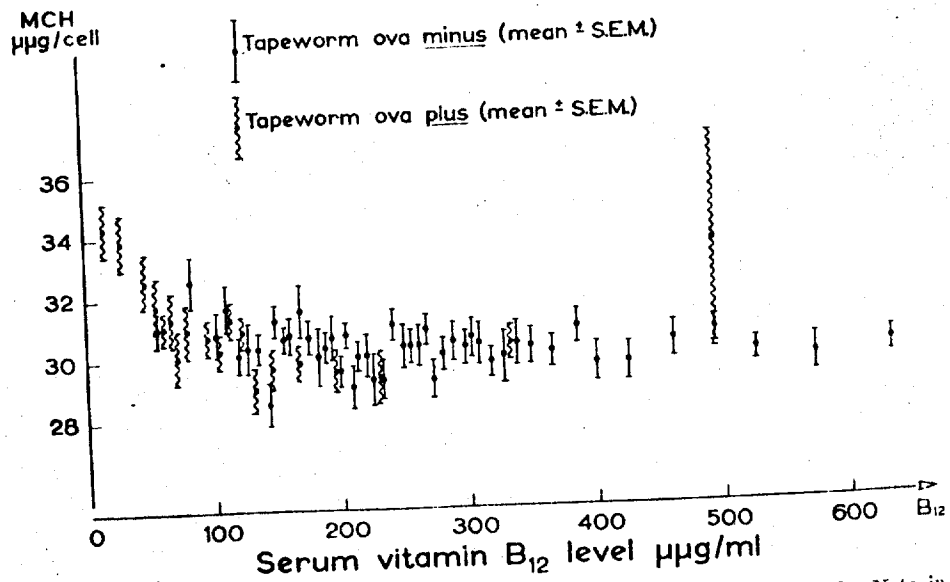


Fig. 7. Mean corpuscular hemoglobin plotted against the serum vitamin B_{12} level. Note increase in MCH below 60 $\mu\mu g$ per milliliter.

dissociation of the vitamin B_{12} protein complex—probably a unimolecular reaction—should obey first-order kinetics. If the cases are randomly distributed according to the gaussian distribution law around a mean located on an exponential scale (due to the dissociation phenomenon), the distribution of the serum vitamin B_{12} values would be lognormal.

Another less likely possibility is that the mean body vitamin B_{12} pool of our entire material was decreasing. This idea gains some support from the general experience in Finland that new cases of genuine and tapeworm pernicious anemia are most frequent in the late spring, and our data were collected in April. Such a decrease may be assumed to obey first-order kinetics.[7]

It has been assumed generally that there are borderline vitamin B_{12} levels, below which hematologic alterations occur. This study adds considerable weight of evidence in favor of this view, and indicates that, statistically, the borderline values lie in the region of 60 to 90 $\mu\mu$g per milliliter. Naturally, in an individual case the borderline value may be quite different. Our data also offer an opportunity to judge the relative sensitivities of different hematologic examinations used to detect "prepernicious anemia" states. The erythrocyte count and the mean corpuscular volume seem to react in a fairly sensitive manner to a decrease in the serum vitamin B_{12} value; the erythrocyte diameter and the mean corpuscular hemoglobin and volume react too, whereas the hemoglobin concentration and the hematocrit value seem to be very insensitive to vitamin B_{12} deficiency. This finding seems to indicate that vitamin B_{12} does not play a decisive role in the biosynthesis of hemoglobin. On the other hand, the formation of the red blood cells appears to be dependent on vitamin B_{12}, since all the hematologic values describing the number and size of the erythrocytes react. The hematocrit value is insensitive to vitamin B_{12} deficiency, apparently because this value depends on 2 factors, viz., the number and size of the red cells, a change in one of which tends to neutralize the effect on the hematocrit value of the other.

This study adds considerable evidence in favor of the view that in Finland *D. latum* deprives its host of vitamin B_{12} and very frequently causes hematologic changes. These range from mild macrocytosis to full-blown megaloblastic anemia. It has been stated that *D. latum* rarely, if ever, produces such anemia in other parts of the world.[8] This may be due to specific differences in the worms known under the name of *D. latum* in different countries.[9] However, it is also possible that the explanation lies in the fact that in Finland, where the fish are heavily infected with *D. latum* plerocercoids, it is common for worm carriers to harbor a large number and mass of worms for a time, whereas in less heavily infected regions the patients carry smaller numbers and mass of worms.

The statistical work was skillfully carried out by Dr. Martti Tienari, at the Computer Center of the Finnish Cable Works Co., Ltd.

REFERENCES

1. Nyberg, W., Gräsbeck, R., Saarni, M., and von Bonsdorff, B.: Serum Vitamin B_{12} Levels and Incidence of Tapeworm Anemia in a Population Heavily Infected With Diphyllobothrium latum, Am. J. Clin. Nutrition. (In press.)

2. Hutner, S. H., Bach, M. K., and Ross, G. I. M.: A Sugar-Containing Basal Medium for Vitamin B_{12}-Assay With Euglena; Applications to Body Fluids, J. Protozool. 3: 101, 1956.
3. Wootton, I. D. P., King, E. J., and Maclean Smith, J.: The Quantitative Approach to Hospital Biochemistry, Brit. M. Bull. 7: 307, 1951.
4. Gaddum, J. H.: Lognormal Distributions, Nature 156: 463, 1945.
5. Wootton, I. D. P.: Personal communication.
6. Gräsbeck, R.: Physiology and Pathology of Vitamin B_{12} Absorption, Distribution, and Excretion, Adv. Clin. Chem. 3: 299, 1960.
7. Gräsbeck, R.: Calculations on Vitamin B_{12} Turnover in Man. With a Note on the Maintenance Treatment in Pernicious Anemia and the Radiation Dose Received by Patients Ingesting Radiovitamin B_{12}, Scandinav. J. Clin. & Lab. Invest. 11: 250, 1959.
8. Scudamore, H. H., Thompson, J. H., Jr., and Owen, C. A., Jr.: Absorption of Co^{60}-Labeled Vitamin B_{12} in Man and Uptake by Parasites, Including Diphyllobothrium latum, J. LAB. & CLIN. MED. 57: 240, 1961.
9. Nyberg, W., Gräsbeck, R., and Sippola, V.: Urinary Excretion of Radiovitamin B_{12} in Carriers of Diphyllobothrium latum, New England J. Med. 259: 216, 1958.

1035

Biochem. Biophys. Acta 252(1), 217-220

BBA Report

BBA 21316

Removal of R-type vitamin B_{12}-binding protein from hog intrinsic factor preparations

R. GRÄSBECK and H. ARO

Minerva Foundation Institute for Medical Research and Medix Co., Helsinki 10 (Finland)

(Received September 15th, 1971)

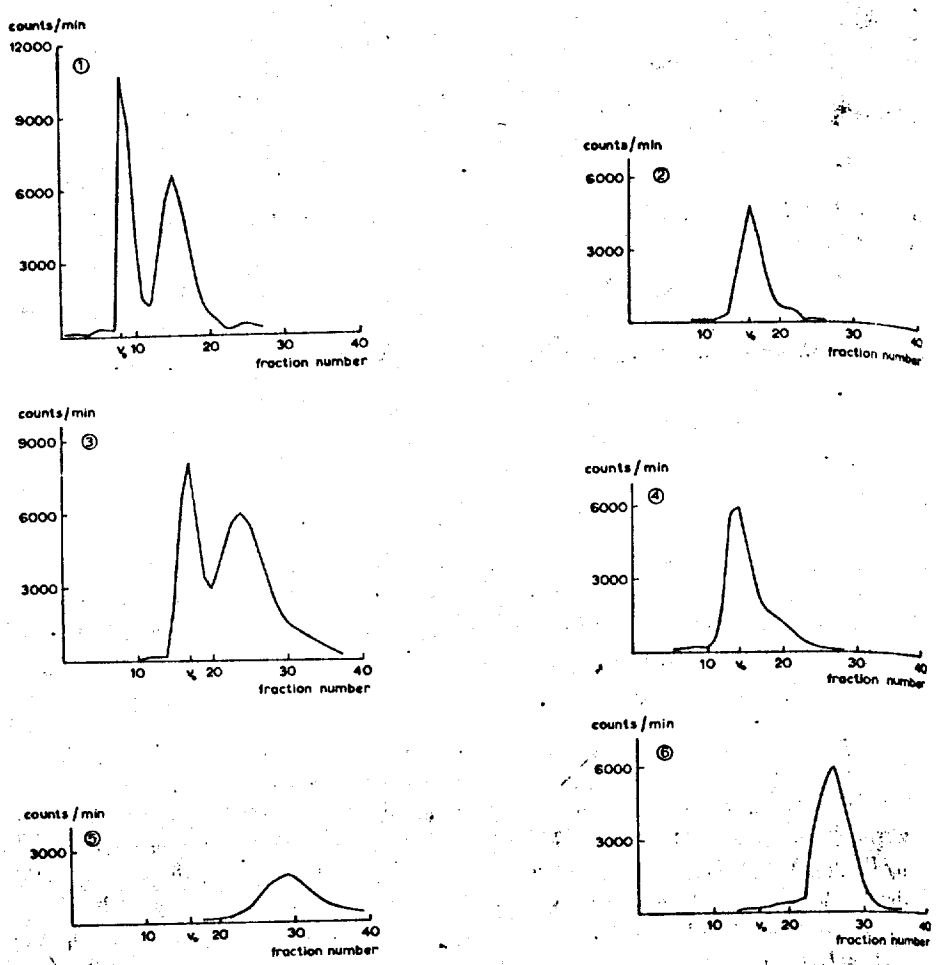
SUMMARY

The vitamin B_{12}-binding protein lacking intrinsic factor activity was removed from porcine intrinsic factor preparations by adding whole or fractionated antiserum from rabbits immunized with a hog leukocyte preparation. The antibody-bound non-intrinsic factor was separated from intrinsic factor using gel filtration.

Sources of intrinsic factor contain a vitamin B_{12} (B_{12})-binding protein lacking intrinsic factor activity. In man it has been called the R-binder[1], in hog non-intrinsic factor[2]. The human R-binder is immunologically identical with B_{12}-binding proteins occurring in saliva, bile, serum, leukocytes and other sources[1]. The molecular weight of many R-type proteins is around 120 000[1], which, incidentally, is almost the same as that of the dimerized B_{12}-intrinsic factor complex[2,3]. In isoelectric focusing hog intrinsic factor and non-intrinsic factor both resolve into several components with very similar pI's[4]. Fractionation methods based on differences in molecular size or charge are therefore inefficient in separating hog intrinsic factor and non-intrinsic factor. The present report demonstrates that such separation can be achieved by immunological means. Preliminary experiments revealed that also the hog non-intrinsic factor is immunologically identical with the B_{12}-binding protein in the leukocytes of the same species. Consequently, non-intrinsic factor was removed from the intrinsic factor preparations by treating the latter with an antiserum prepared against hog leukocytes.

The leukocytes contained in 1 l hog blood were isolated as described by Dioguardi *et al.*[5]. The leukocytes were frozen and lysed by sonication for 5 min and the debris was removed by centrifugation (10 000 × *g*, 10 min). The B_{12}-binding capacity of the leukocyte preparation was determined and found to be approximately 13 μg. The preparation was saturated with a small excess of nonradioactive B_{12} and used as antigen. 0.1 ml was mixed with an equal volume of Freund's complete ajuvant and injected subcutaneously into each of 15 rabbits. The injection was repeated 2, 6, 9 and 11 weeks after

455



Figs. 1–6. Elution profiles observed in gel filtration of B_{12}-binding proteins treated with antisera.

Fig. 1. A hog B_{12}-intrinsic factor preparation (containing B_{12}–non-intrinsic factor *plus* anti-leukocyte serum.

Fig. 2. The peak eluted later in Fig. 1 *plus* anti-intrinsic factor serum.

Fig. 3. Same intrinsic factor preparation as in Fig. 1 *plus* anti-intrinsic factor serum.

Fig. 4. The peak eluted later in Fig. 3 *plus* anti-leukocyte serum.

Fig. 5. The peak eluted later in Fig. 3 *plus* anti-intrinsic factor serum.

Fig. 6. The V_O peak in Fig. 2 dissociated from its antibody *plus* anti-leukocyte serum.

the first injection. 12 weeks after the start of the immunization 15 ml blood were taken from each rabbit and the serum was separated. The best antisera were able to bind about 18 μg intrinsic factor per ml.

Model experiments were performed with a highly purified hog intrinsic factor preparation produced as described by Aro *et al.*[6], the last purification step being DEAE-cellulose chromatography. About 60% of the preparation consisted of B_{12}-binding proteins. 40 μl of this preparation, containing in bound form 12 ng ^{57}Co-labeled B_{12} were mixed with 50 μl antiserum and 0.5 ml 0.9% NaCl. After 30 min of incubation at room temperature the sample mixed with a small amount of Blue Dextran 2000 (Pharmacia) as a marker was passed through a Sephadex G-150 column (2 cm × 35 cm) using 0.1 M sodium phosphate of pH 7.4 as the buffer. As shown in Fig. 1 the antibody-B_{12}-non-intrinsic factor complex was eluted with Blue Dextran in the totally excluded volume (V_0). B_{12}-intrinsic factor, from which B_{12}-non-intrinsic factor had been removed, was eluted later, its V_e/V_0 value being 1.8, corresponding to a mol. wt. of about 60 000[1].

The following experiments demonstrate that intrinsic and non-intrinsic factors were separated and that intrinsic factor remained native. The B_{12}-intrinsic factor complex eluted in the gel filtration was pooled and concentrated, anti-intrinsic factor serum obtained from a pernicious anaemia patient was added to it and the sample was passed through Sephadex as just described. All the B_{12}-binding protein was now eluted in V_0 (Fig. 2), indicating that intrinsic factor had reacted with the antibody. When the original preparation containing both intrinsic and non-intrinsic factor was treated with similar patient serum, the antibody-B_{12}-intrinsic factor complex was eluted in the V_0, whereas the B_{12}-non-intrinsic factor complex was eluted in free form (Fig. 3). The B_{12}-non-intrinsic factor eluted in the last-mentioned experiment was treated in the same way with both anti-hog leukocyte serum and anti-intrinsic factor serum. Figs. 4 and 5 show that B_{12}-non-intrinsic factor reacts with anti-leukocyte serum, but not with anti-intrinsic factor serum. The antibody-B_{12}-intrinsic factor complex shown in Fig. 2 was split using 0.1 M glycine-HCl buffer of pH 2.75[7] followed by gel filtration through Sephadex G-150 contained in the same buffer. This caused the separation of the antibody from the antigen. Addition of anti-leukocyte serum to the isolated neutralized antigen followed by gel filtration indicated that B_{12}-intrinsic factor was not bound to the anti-leukocyte immunoglobulins (Fig. 6).

Similar experiments have been performed using an otherwise identical hog intrinsic factor preparation prepared without saturation of its B_{12}-binding capacity. We have also employed the purified immunoglobulin fraction[8] of the antiserum instead of the unfractionated antiserum.

An example: 2 ml of the intrinsic factor preparation, having a B_{12}-binding capacity of 1.5 μg/ml, was saturated with [^{57}Co]cyanocobalamin with a specific activity of 0.065 μC/μg. The mixture was incubated at room temperature for 10 min, 2 ml of a solution containing the purified anti-leukocyte immunoglobulin was added, the mixture was incubated for another 30 min and the mixture was filtered through a Sephadex G-150 column equilibrated with 0.1 M sodium phosphate of pH 7.4. The B_{12}-non-intrinsic factor-antibody complex was eluted in the V_0 fraction and the B_{12}-intrinsic factor complex with a V_e/V_0 of 1.8. An amount of the latter protein material corresponding to 1 μg of bound radioactive B_{12} was administered to a patient with genuine pernicious

anaemia using the Schilling test procedure[9]. During 2 days after administration of this preparation the urinary excretion of radioactivity of the patient was 17.8% as compared to a basal excretion of 3.5% following the administration of 1 μg free radioactive B_{12}. With a standard preparation of hog intrinsic factor a 2-day excretion of 12.1% was observed. The antibody-treated intrinsic factor preparation was thus biologically active.

We have also bound the anti-leukocyte immunoglobulin to polymerized agarose (Sepharose 2B, Pharmacia AB) using the cyanogen bromide procedure[10]. This preparation also removes non-intrinsic factor from intrinsic factor preparations.

Supported by grants from the Sigrid Jusélius and E.J. Sariola Foundations and the Finnish Research Council for Medical Science.

REFERENCES

1 R. Gräsbeck, *Prog. Hematol.*, 6 (1969) 233.
2 L. Ellenbogen and D.R. Highley, *J. Biol. Chem.*, 242 (1967) 1004.
3 R. Gräsbeck, K. Simons and I. Sinkkonen, *Biochim. Biophys. Acta*, 127 (1966) 47.
4 H. Aro and R. Gräsbeck, *Scand. J. Clin. Lab. Invest. Suppl.*, 113 (1970) 65.
5 N. Dioguardi, A. Agostini, G. Fiorelli and B. Lomanto, *J. Lab. Clin. Med.*, 61 (1963) 713.
6 H. Aro, R. Gräsbeck and K. Visuri, *Scand. J. Clin. Lab. Invest. Suppl.*, 108 (1969) 28.
7 H.N. Eisen, N.-Aronsson and E.S. Simms, *Methods Med. Res.*, 10 (1964) 91.
8 D.M. Weir, *Handbook of Experimental Immunology*, Blackwell Scientific Publications, Oxford and Edinburgh, 1967, p. 116.
9 R. Gräsbeck, *Adv. Clin. Chem.*, 3 (1959) 88.
10 J. Porath, R. Axén and S. Ernback, *Nature*, 215 (1967) 1491.

1037

Biochem. Biophys. Acta 263(3): 721-733

BBA 36097

THE VITAMIN B_{12}-BINDING PROTEIN IN HUMAN SALIVA ISOLATION, CHARACTERIZATION AND COMPARISON WITH THE UNPURIFIED PROTEIN

RALPH GRÄSBECK, KALEVI VISURI AND ULF-HÅKAN STENMAN

Biochemical Section, Minerva Foundation Institute for Medical Research, SF-00100 Helsinki 10 (Finland)

(Received November 11th, 1971)

SUMMARY

The salivary vitamin B_{12}-binding substance was isolated from human saliva as its cyanocobalamin complex. The procedure consisted of a series of concentration, ion exchange and gel filtration steps. The isolated complex appeared to be a glycoprotein free from contaminating proteins, but was heterogeneous in some analytical procedures, notably electrofocusing. The molecular size of the end product was found to be smaller and less acid than the complex contained in unfractionated saliva. The differences in size and charge were attributed to progressive loss of carbohydrate, probably sialic acid, during purification. Analytical data of the original and isolated salivary vitamin B_{12} complexes are reported. Like other R-type vitamin B_{12}-binding proteins, the salivary vitamin B_{12}-binding substance is a mixture of "isoproteins" differing in isoelectric point.

INTRODUCTION

Saliva has long been known to form a large-molecular complex with vitamin B_{12} (ref. 1). It was shown in our laboratory that the salivary vitamin B_{12} binder is immunologically identical with a large number of "R-type" vitamin B_{12} binders occurring in various cells and body fluids such as leucocytes, gastric juice and blood plasma[2]. There is genetic evidence that the R-proteins are closely related[3]. To gain further insight into the chemical nature of these R-type proteins we have isolated and characterized the binding protein from human saliva. An abstract of the early phases of the present work has appeared[4].

MATERIALS AND ANALYTICAL METHODS

Most of our methods were identical with those previously described[5,6].

Saliva

Preliminary studies indicated that in several fractionation procedures the

459

vitamin B_{12}-binding protein in saliva was heterogeneous. In order to rule out microbial degradation as the cause, measures were taken to prevent bacterial action. The donors rinsed their mouths with a mouthwash containing dilute H_2O_2 (Oxygenol, Oxygenol Co., Helsinki), they were given pieces of paraffin to chew on and were asked to spit into a cup kept on an ice bath and containing penicillin–streptomycin granulate (kindly supplied by Kabi AB, Stockholm, Sweden, 400 I.U. benzylpenicillin and 0.4 mg dihydrostreptomycin per cup).

Analytical procedures

Amino acid composition[7]

The protein was hydrolysed with 6 M HCl for 24 h at 110 °C. The hydrolysate was analysed in the Beckman 120 C amino acid analyser. Tryptophan was not determined owing to limited amounts of material.

Hexose

Hexose was determined by the phenol–H_2SO_4 method[8] without previous hydrolysis. To improve the sensitivity, the volume of the reaction mixture was reduced to 1.4 ml. The standard solution contained equal amounts by weight of galactose and mannose. 0.5 μg of hexose was measurable with this method.

Sialic acid

Sialic acid was determined by the method of Warren[9] after hydrolysis in 0.05 M H_2SO_4 at 80 °C for 1 h. The reaction volume was reduced to achieve a sensitivity of 0.1 μg per sample.

Immunodiffusion

Rabbit antisera against whole saliva and leucocytes, a version of the Ouchterlony immunodiffusion technique, and autoradiographic visualisation of radioactive precipitates were used as described before[10–12].

Disc electrophoresis

Disc electrophoresis was run at pH 8.9 as described by Davis[13].

Analytical ultracentrifugation

Analytical ultracentrifugation was carried out in the Beckman Model E ultracentrifuge equipped with a photoelectric scanner. The samples contained 0.2–0.8 mg protein per ml in 0.1 M NaCl buffered to pH 7 with 0.01 M sodium phosphate. Sedimentation velocity measurements were made as described by Schachman[14] and equilibrium centrifugations in water and $^2H_2{}^{18}O$ as described by Edelstein and Schachman[15].

Density gradient ultracentrifugation

The sedimentation behaviour of the salivary vitamin B_{12} complex was investigated essentially as described by Martin and Ames[16], using a Beckman-Spinco L2 preparative ultracentrifuge. The runs were performed at 3 °C and 40 000 rev./min for 20 h in the SW 50 L rotor. The sample was applied in a 0.1-ml volume on a 4.7-ml sucrose gradient (10–30%) in 0.1 M sodium phosphate buffer (pH 7.4).

Electrofocusing

Electrofocusing was performed in the standard LKB 110-ml column using either Ampholines (0.5–1.0%) or an acid gradient developed by us[17]. The density gradient was produced with glycerol. The temperature was 4 °C, the potential 450 V and the current was applied for 4–5 days. Fractions of 1 ml were collected and the pH measured at room temperature.

Molecular parameters by gel filtration

Elution volumes (V_e) were measured by filtration through a column of Sephadex G-150 (2.5 cm × 93 cm) at a flow rate of 15 ml/h in 0.01 M sodium phosphate buffer (pH 7.0) containing NaCl in a concentration of 0.1 M. Blue Dextran 2000 was mixed with the sample to determine the void volume (V_0) and molecular weights were estimated with bovine albumin (Armour) and ovalbumin (Koch-Light) as reference proteins. The K_D values were derived as described by Gelotte[18]. Stokes radii were calculated according to the method of Ackers[19].

The vitamin B_{12}-binding power

The vitamin B_{12}-binding power of saliva was determined by the coated charcoal method[20].

FRACTIONATION AND RESULTS

Fractionation procedure

Initial steps

Two batches of saliva were processed, 30 and 4 l in volume, respectively. The processing of the first one has been described in a brief note[4]. The second batch, described here in detail, was processed to obtain more material for characterization of the purified product; simultaneously the fractionation process was improved.

The saliva samples forming the second batch were saturated with ^{57}Co-labelled vitamin B_{12} (spec. act. 27 mCi/g) immediately after collection and stored frozen in saturated form. After thawing 700 g $(NH_4)_2SO_4$ per l were added, and the precipitate containing the radioactivity was collected by centrifugation at 10 000 × g for 20 min at room temperature (in Table I the properties of this concentrate are mentioned on the line "concentration"). The precipitate was extracted 5 times with an equal volume of distilled water. The combined extracts contained 93.5% of the radioactivity of the saliva batch in 1/8 of its original volume. To the extract $(NH_4)_2SO_4$ was added to give a final concentration of 400 g/l followed by mixing for 1 h at room temperature and centrifugation as described. The precipitate was discarded and 300 g of $(NH_4)_2SO_4$ were added per l of supernatant. The precipitate was collected by centrifugation and processed by DEAE-cellulose chromatography.

DEAE-cellulose chromatography

Whatman DE-52 microgranular DEAE-cellulose was packed into a column (1.5 cm × 12 cm) and equilibrated with the buffer against which the sample had been dialysed, *i.e.* 5 mM ammonium phosphate (pH 7.9). The sample was introduced and the column washed with 100 ml of equilibration buffer. The linear gradient used for elution started with the equilibration buffer and ended with 0.25 M ammonium phosphate (pH 6.0), the gradient mixer containing 450 ml of each buffer. The flow rate was about 30 ml/h and the temperature 4 °C. The radioactivity was eluted as one peak at a buffer concentration of 10–70 mM. The radioactive fractions were combined and 700 g of $(NH_4)_2SO_4$ per l added. The precipitated material was collected by centrifugation and the precipitate dialysed against the equilibration buffer used in the next step.

CM-cellulose chromatography

Whatman CM-52 cellulose was packed as a column (1.5 cm × 6 cm) and equilibrated with 10 mM ammonium acetate (pH 4.5). The sample was introduced and

the column washed with 50 ml of equilibration buffer. A linear buffer gradient was produced with a gradient mixer containing 200 ml of equilibration buffer and 200 ml of 0.3 M ammonium acetate (pH 4.5). The flow was about 15 ml/h and the temperature 4 °C. The radioactive protein complex was eluted as two partially resolved peaks at a buffer concentration of 30–80 mM. The radioactive eluates were combined and concentrated by precipitation with $(NH_4)_2SO_4$, as described in the preceding paragraph.

Gel filtration

The precipitate was dissolved in 4 ml of the buffer and filtered through the Sephadex G-150 column as described above under Analytical procedures. The radioactivity was eluted as one symmetrical component, which was precipitated and concentrated by dialysis against concentrated $(NH_4)_2SO_4$ solution. The preparation was stored at 4 °C in concentrated $(NH_4)_2SO_4$, which in our experience is the safest way of storing pure vitamin B_{12}–protein complexes.

Processing of the 30-l saliva batch[4]

The main differences in the processing of this batch compared with the procedure described above was the use of ultrafiltration to concentrate the sample before and between the fractionation steps and the stepwise increase of buffer concentration during the ion-exchange chromatographies. The saliva was not saturated with vitamin B_{12} until after the first concentration step.

Yield

Table I summarizes the purification. The recovery of bound vitamin B_{12} was much better in the 4-l run and the two runs resulted in equal amounts of the isolated product. Much vitamin B_{12}-binding power was lost in the large run during the concentration step before vitamin B_{12} was added.

TABLE I

PURIFICATION STEPS AND THEIR YIELDS

30-l, 30-l batch; 4-l, 4-l batch. Volume × $A_{280\ nm}$ is roughly equivalent to amounts of protein in mg.

Fractionation step	*Volume at end of step (ml)*		*Volume × $A_{280\ nm}$ ("mg")*		*Radioactive vitamin B_{12} content (μg)*	
	30-l	*4-l*	*30-l*	*4-l*	*30-l*	*4-l*
Saliva	30 500	3 940	88 500	12 300	1 050	111.0
Concentration	2 332	505	18 830	5 720	612	103.6
$(NH_4)_2SO_4$ fractionation	—	77	—	1 738	—	89.8
DEAE-cellulose	1 660	170	986	245	209	78.2
CM-cellulose	450	74	51	7.7	87	38.2
Gel filtration	32	36	2.2	2.2	33	33.0

Studies on the purified products

Dry weight and absorption spectrum

Samples were dialysed against distilled water and dried to constant weight at 105 °C and weighed with a Cahn Gram electrobalance. The dry weights of the two preparations were both calculated to be 1.8 mg. Absorption spectra were measured

from samples dialysed against 0.05 M sodium phosphate (pH 7). The spectra of the two preparations were identical (Fig. 1). An absorption maximum was found at 363 nm instead of at 361.5 nm as in cyanocobalamin. The ratios $A_{278\ nm}/A_{363\ nm}$ were found to be 3.23 for the product of the 30-l and 3.22 for the 4-l batch. At 278 nm a solution containing 1 mg of the complex per ml gives $A = 1.21$. From the spectrophotometric vitamin B_{12} content of 18.5 μg per mg and assuming a 1:1 molar ratio of vitamin B_{12}/protein, a molecular weight (M_r) of 73 000 was calculated for the complex.

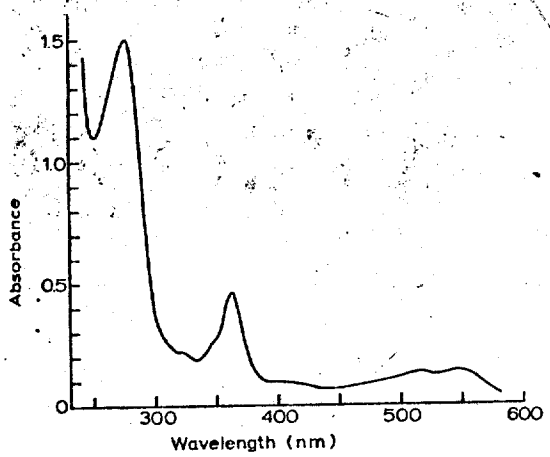


Fig. 1. Absorption spectrum of the vitamin B_{12} complex isolated from the 4-l saliva batch. 1.24 mg of complex per ml.

Ultracentrifugation

In analytical ultracentrifugation light of 278 nm was used. The two preparations sedimented as homogeneous proteins. The $s_{20,w}$ of the 30-l preparation was 3.95 S* and that of the 4-l preparation 4.45 S. The sedimentation equilibrium plot of the 4-l preparation gave a straight line. Equilibrium runs in water and $^2H_2{}^{18}O$ gave $\bar{v} = 0.679$. The data from the equilibrium runs gave $M_r = 66\ 000$. The f/f_0 of the complex from the 4-l batch was calculated to be 1.59 and its Stokes radius 4.17 nm.

Disc electrophoresis

In disc electrophoresis both preparations migrated as single but relatively wide bands. An example is shown in Fig. 2.

Immunodiffusion

When diffused against anti-saliva and anti-leucocyte sera amounts up to 7 μg of the purified binder from the 30-l batch were found to give one precipitation line, visible without staining and after staining with amido black. One line was visualised by radioautography; it could be superimposed on the visible line. As had previously been observed in impure preparations[2,11], the precipitation line of the isolated

* It was previously erroneously calculated and reported[4] to be 3.5. The value 3.9 S was reported in Table II of ref. 21.

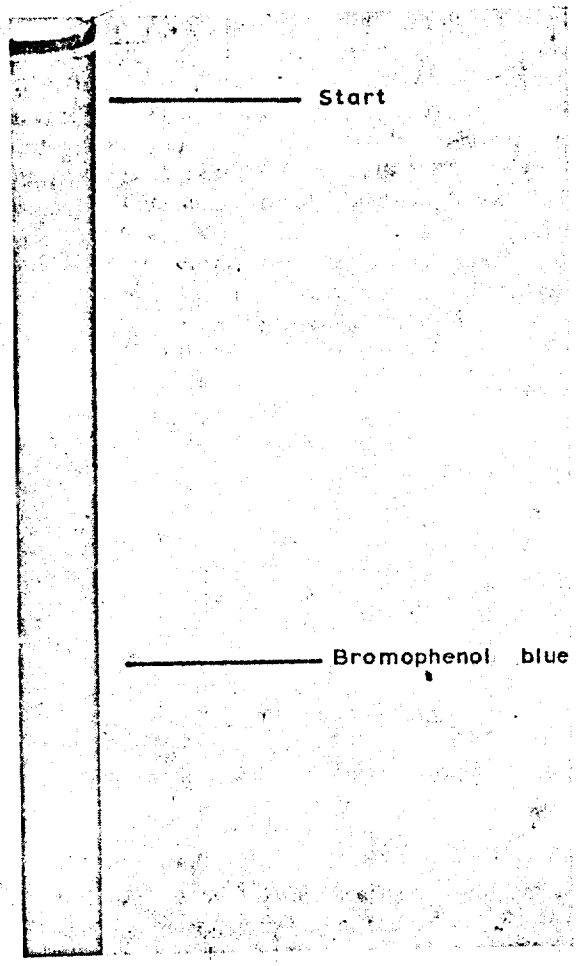


Fig. 2. Disc electrophoresis of the vitamin B_{12} complex isolated from the 4-l batch. 50 μg complex was applied.

salivary vitamin B_{12} complex fused with the line of the vitamin B_{12}–protein extracted from leucocytes (Fig. 3).

Electrofocusing

Both preparations gave several peaks. The main peaks of the 4-l preparation were between pH 4.55 and 5.03 (Fig. 4), and those of the 30-l preparation between pH 4.9 and 5.28.

Amino acid composition

Since sufficient quantities of the components with different isoelectric points were not available, the amino acid composition, presented in Table II, was determined on the unfractionated preparation obtained from the 4-l run.

Carbohydrate composition

In the 4-l batch the content of total hexoses was 15 ± 0.5%. The sialic acid

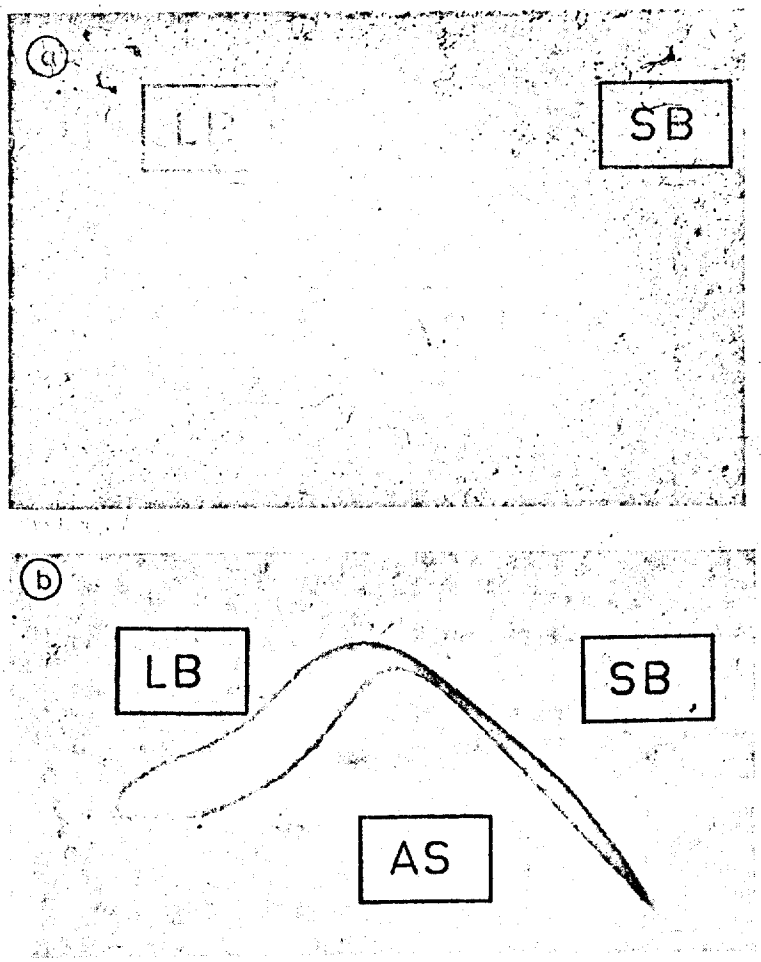


Fig. 3. Immunodiffusion of the isolated salivary vitamin B_{12} complex (SB) and the vitamin B_{12}-protein complex (LB) partially purified from leucocytes as described in ref. 11. AS = anti-saliva immune serum. (a) Amido black stain, (b) radioautography.

content had to be determined on as little as 17.4 μg of this preparation. The result was approx. 0.5 mole of sialic acid per mole of complex, which was at the limit of detection of the method.

Comparison of the isolated material with the complex in unfractionated saliva

In our experience native vitamin B_{12}-binding proteins of R-type are relatively acid[22,23]. Having observed that the isolated complexes consisted of components with pI values around 5, we assumed that the binding protein had lost acid components, most likely sialic acid, during purification. Experiments were performed to elucidate how the isolated complex differed from the original complex.

Electrofocusing

Fresh saliva contains mainly very acid vitamin B_{12}-binding components. The

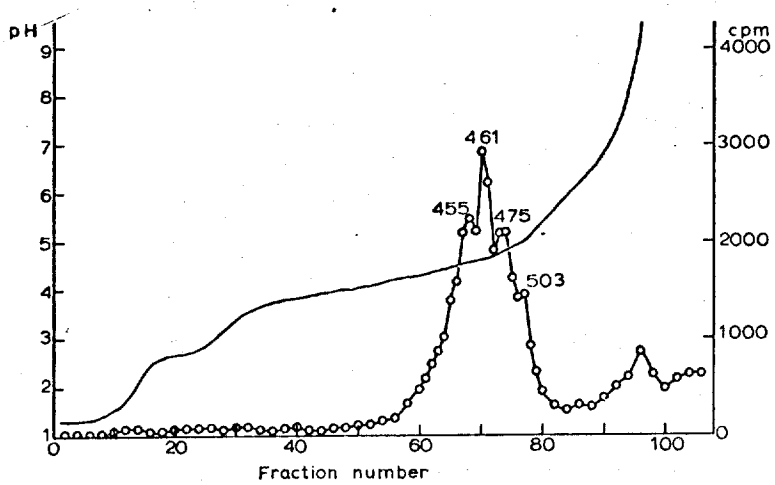


Fig. 4. Electrofocusing of the complex isolated from the 4-l batch. ○——○, radioactivity; ———, pH in 1-ml fractions.

TABLE II

RESULTS OF AMINO ACID ANALYSIS OF THE VITAMIN B_{12} COMPLEX ISOLATED FROM THE 4-l BATCH OF SALIVA

Mean of two samples of 245 μg dry weight. The column marked moles/50 000 gives a rough estimate of the number of amino acids in the peptide portion of the isolated glycoprotein.

Amino acid	*μmoles*	*moles/50 000*
Lys	25.3	19.5
His	4.7	3.6
Arg	9.7	7.5
Asp	65.8	50.7
Thr	29.8	22.9
Ser	35.8	27.6
Glu	66.0	50.8
Pro	27.6	21.3
Gly	29.6	22.8
Ala	28.0	21.6
Cys	9.1	7.0
Val	32.5	25.0
Met	9.5	7.3
Ile	29.0	22.3
Leu	43.1	33.2
Tyr	22.6	17.4
Phe	16.8	12.9

main component in the run depicted in Fig. 5a, isoelectric at pH 3.70, was digested with neuraminidase (Behringwerke, EC. 3.2.1.18). After incubation the pI of the sample had risen to approx. 5 (Fig. 5b), whereas the pI of the control remained essentially unchanged (Fig. 5c).

Gel filtration

Gel filtration of the purified ^{57}Co-labelled complex together with fresh saliva saturated with ^{58}Co-labelled vitamin B_{12} revealed differences in the molecular sizes

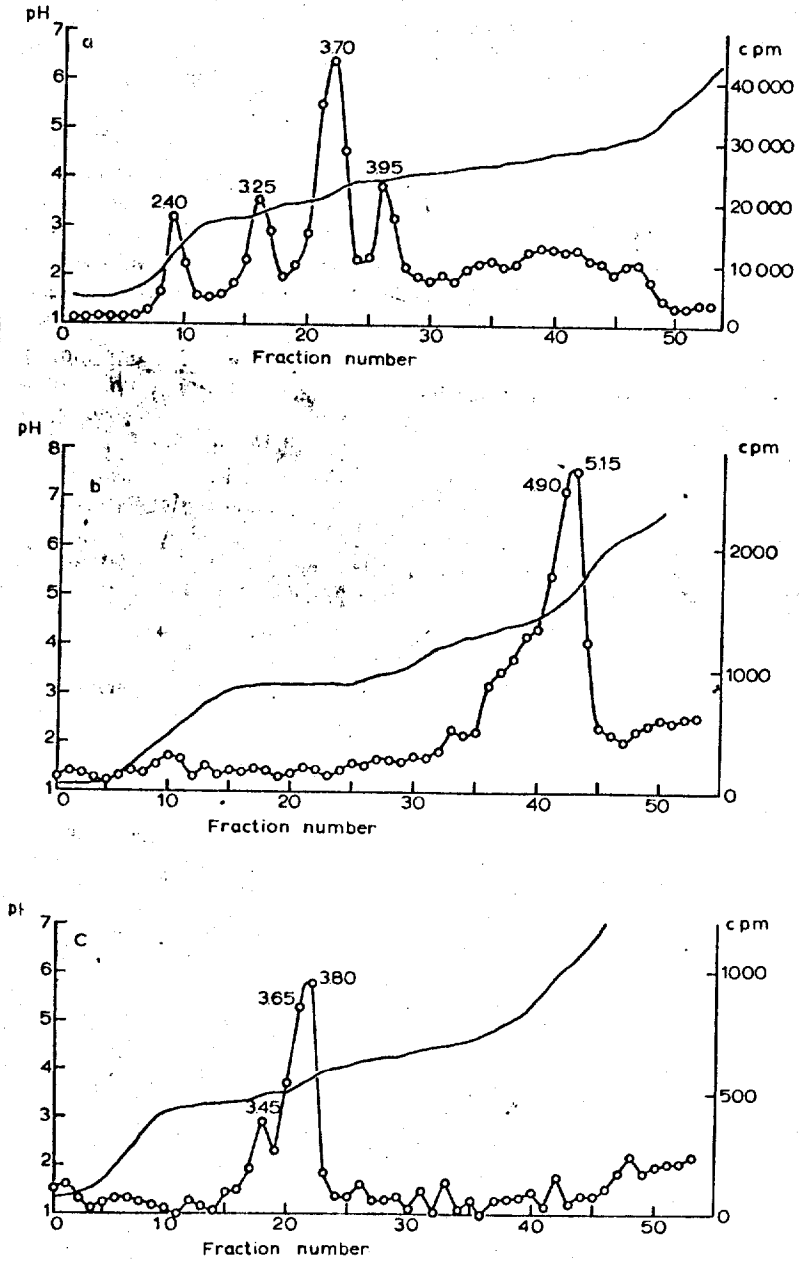


Fig. 5. Effect of neuraminidase on the electrofocusing pattern. (a) 0.2 ml saliva (from a single individual) saturated with ^{57}Co-labelled vitamin B_{12}. The component isoelectric at pH 3.70 was pooled and divided into two halves, one of which (b) was incubated with 1000 units of neuraminidase, the other (c) without neuraminidase followed by electrofocusing. Conditions of incubation: 0.05 M sodium acetate (pH 5.9), 1 g NaCl and 0.5 mg vitamin B_{12} per l, 37 °C for 20 h. Symbols as in Fig. 4. Acid gradient described in ref. 17.

467

1037

of the proteins. The purified complexes had larger elution volumes. When fresh saliva was incubated with neuraminidase, the elution volume of the complex was increased (Table III).

TABLE III

MOLECULAR CHARACTERISTICS OF THE SALIVARY VITAMIN B_{12} COMPLEX

The calculations are based on data from analytical ultracentrifugation (a), density gradient centrifugation (b) and gel filtration (c).

Characteristic	*Vitamin B_{12} complex preparation*		
	Unfractionated material	*4-l preparation*	*30-l preparation*
Sedimentation coefficient $s_{20,w}$ (S)	4.65 (b)	4.45 (a,b)	3.95 (a)
Molecular weight M_r	73 000 (b,c)	66 000 (a) 65 000 (b,c)	51 000 (a,c)
Stokes radius a (nm)	4.44 (c)	4.17 (a) 4.13 (c)	3.65 (c)
Frictional ratio f/f_0	1.63 (b,c)	1.59 (a) 1.58 (b,c)	1.51 (b,c)
Diffusion coefficient D ($\times 10^{-7}$ cm²/sec)	4.83 (c)	5.14 (a) 5.18 (c)	5.87 (c)
Partial specific volume $\bar{v}$ (cm³/g)	—	0.679 (a)	—
Elution volume/void volume ratios in Sephadex G-150 (V_e/V_0)	1.54 ± 0.02*	1.61 ± 0.04	1.8

* Unfractionated, vitamin B_{12}-saturated salivary complex digested with neuraminidase gave 1.8, the control 1.6. In the gel filtration experiments ovalbumin gave 2.07 and bovine serum albumin 1.81 ± 0.01.

Density gradient ultracentrifugation

The complex of fresh saliva was observed to have a sedimentation coefficient of 4.65 S when centrifuged with bovine albumin as the reference protein. Fresh unfractionated salivary binder saturated with ^{58}Co-labelled vitamin B_{12} was mixed with the purified complex and ultracentrifuged. The unpurified complex had a sedimentation coefficient of 4.65 S relative to the assumed value 4.45 S of the purified complex.

Table III summarizes the molecular characteristics of the salivary vitamin B_{12} complex.

DISCUSSION

The two fractionation procedures gave end products that satisfy several criteria of homogeneity. An exception was electrofocusing, which showed the presence of components with different pI values. However, that "pure proteins" are heterogeneous in electrofocusing is the rule rather than the exception (*e.g.* refs 24, 25). It is fairly common for glycoproteins to be heterogeneous[26].

The two preparations gave very similar products, but the complex isolated [illegible] However, both purified products

1037

would be progressive loss of loosely bound carbohydrate from the complex during its purification. The product of the 4-l run had a low content of sialic acid, as evidenced by chemical analysis and the relatively high isoelectric points. Unfractionated saliva gave peaks with low pI values, about the same as those observed with other R-type vitamin B_{12}–protein complexes[23]. Digestion of an acid component with neuraminidase shifted the pI from 3.7 to 5 and the same treatment reduced the molecular size of the complex in unfractionated saliva. It seems likely that much of the material lost during purification is sialic acid.

Evidence has been presented that variations in sialic acid content are the cause of the heterogeneity of the human vitamin B_{12}–intrinsic factor complex in electrofocusing[24]. Most of the differences between the human leucocyte R-type binder and normal and leukemic transcobalamin I in plasma seem to be due to differences in sialic acid content, and chromatographic purification decreased their electrophoretic mobilities; this was apparently related to loss of sialic acid[11]. Other authors have also found variations in sialic acid content among R-type binders[27]. The present view in this laboratory is that the R-proteins are mixtures of "isoproteins" differing in pI, some of which are common to many R-proteins[23]. According to the immunological evidence, the peptide parts of these proteins are at least partially identical. They are apparently coded by one gene, the genetic evidence being the report of two brothers lacking R-proteins[3]. The variability among the R-proteins seems to be an instance of differences in gene expression in different cells. This may be due to modifying genes governing the addition of carbohydrate to the peptide.

In tissue culture Hurlimann and Zuber[28] observed synthesis of R-type binders in serous glandular cells from both submandibular and parotid glands and in gastric mucosa, and in this laboratory leucocytes were found to synthesize an R-protein[29]. It is possible that the different isoelectric components of the salivary binder emanate from different salivary glands or from cells of different types, one of these being leucocytes.

Since the saliva was pooled, genetic variants might be responsible for the different components. However, heterogeneity was also observed in samples from single individuals, and components with similarly heterogeneous pI values have been observed in other R-type protein preparations from single individuals[23]. The heterogeneity is present in unfractionated material and there is no evidence that it increases during purification. On the contrary, desialylation diminished the heterogeneity of intrinsic factor[24]. Close inspection of the elution profiles observed in simultaneous gel filtration revealed that the width of the peak corresponding to the ^{58}Co-labelled vitamin B_{12} complex in unfractionated saliva was greater than that of the isolated ^{58}Co-labelled vitamin B_{12} complex. The variability in molecular size may therefore diminish during purification.

The isolation of other R-type proteins may have taken place. In 1954–1955 Gregory and Holdsworth[30,31] isolated a cyanocobalamin–protein complex from sow's milk. It contained about 23 μg vitamin B_{12} per mg, 7% carbohydrate and 9% hexosamine. An absorption maximum was observed at 362 nm. Assuming a 1:1 molar ratio for vitamin B_{12}/protein, a molecular weight of 55 000 was calculated. Along with their hog intrinsic factors, Holdsworth[32] and Ellenbogen and Highley[33,34] isolated pure proteins that bound vitamin B_{12} but did not promote vitamin B_{12} absorption. Holdsworth's protein had an unsaturated vitamin B_{12}-binding capacity

of 18 μg/mg, a total reducing sugar content of 29% and a sedimentation constant of 4.17 S. The latter authors found the corresponding values to be 14.8 μg/mg, 32% and 3.7 S. The same protein isolated after saturation with vitamin B_{12} had a vitamin B_{12} content of 25 μg/mg, 14% sugars and 4.6 S. None of these proteins were subjected to immunological analysis, and it is not known whether they were R-proteins. However, R-proteins have been definitely identified in milk[27] and hog pylorus[35]. We note that the molecular characteristics reported are fairly close to those of the isolated salivary vitamin B_{12} complex.

The molecular parameters reported here are supported by recent data of Hippe[36], who studied unfractionated saliva by gel filtration and observed a Stokes radius of 4.36 ± 0.18 nm. Slight reduction in molecular size may have occurred in his experiments, since the saliva samples were stored frozen in unsaturated form until analysed.

ACKNOWLEDGEMENTS

This work was supported by grants from the Sigrid Jusélius Foundation, the Finnish Research Council for Medical Sciences and Societas Scientarum Fennica. Drs H. Aro, L. Puutula and K. Simons generously assisted in performing some of the assays.

REFERENCES

1 E. Beerstecher and S. Altgelt, *J. Biol. Chem.*, 189 (1951) 31.
2 K. Simons, *Soc. Sci. Fenn. Commentat. Biol.*, 27 (1964) Fasc. 5.
3 R. Carmel and V. Herbert, *Blood*, 33 (1969) 1.
4 R. Gräsbeck and K. Visuri, *Scand. J. Clin. Lab. Invest. Suppl.*, 101 (1968) 13.
5 R. Gräsbeck, K. Simons and I. Sinkkonen, *Ann. Med. Exp. Biol. Fenn. Helsinki*, 40 (1962) Suppl. 6.
6 R. Gräsbeck, K. Simons and I. Sinkkonen, *Biochim. Biophys. Acta*, 127 (1966) 47.
7 A. M. Crestfield, S. Moore and W. H. Stein, *J. Biol. Chem.*, 238 (1963) 622.
8 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, 28 (1956) 350.
9 L. Warren, *J. Biol. Chem.*, 234 (1959) 1971.
10 K. Simons, T. Weber, M. Stiel and R. Gräsbeck, *Acta Med. Scand. Suppl.*, 412 (1964) 257.
11 U.-H. Stenman, K. Simons and R. Gräsbeck, *Scand. J. Clin. Lab. Invest.*, 21 (1968) 202.
12 S. E. Holm, *Int. Arch. Allergy*, 26 (1965) 34.
13 B. J. Davis, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
14 H. K. Schachman, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 55.
15 S. J. Edelstein and H. K. Schachman, *J. Biol. Chem.*, 242 (1967) 306.
16 R. G. Martin and B. N. Ames, *J. Biol. Chem.*, 236 (1961) 1372.
17 U.-H. Stenman and R. Gräsbeck, *Scand. J. Clin. Lab. Invest. Suppl.*, 116 (1971) 26.
18 B. J. Gelotte, *J. Chromatogr.*, 3 (1960) 330.
19 G. K. Ackers, *Biochemistry*, 3 (1964) 723.
20 C. Gottlieb, K. S. Lau, L. R. Wassermann and V. Herbert, *Blood*, 25 (1965) 875.
21 R. Gräsbeck, *Prog. Hematol.*, 6 (1969) 233.
22 R. Gräsbeck, in H. Peeters, *Protides of the Biological Fluids*, Proc. 16th Colloq., Pergamon Press, London, 1969, p. 401.
23 U.-H. Stenman, *Scand. J. Clin. Lab. Invest. Suppl.*, 116 (1971) 26.
24 R. Gräsbeck, *Acta Chem. Scand.*, 22 (1968) 1041.
25 W. A. Susor, M. Kochman and W. J. Rutter, *Science*, 165 (1969) 1260.
26 A. Gottschalk, *Glycoproteins*, Elsevier, Amsterdam, London, New York, 1966.
27 J. Hurlimann and C. Zuber, *Clin. Exp. Immunol.*, 4 (1969) 123.
28 J. Hurlimann and C. Zuber, *Clin. Exp. Immunol.*, 4 (1969) 141.
29 K. Simons and T. Weber, *Biochim. Biophys. Acta*, 117 (1966) 201.
30 M. E. Gregory and E. S. Holdsworth, *Nature*, 173 (1954) 830.

1057

31 M. E. Gregory and E. S. Holdsworth, *Biochem. J.*, 59 (1955) 335.
32 E. S. Holdsworth, *Biochim. Biophys. Acta*, 51 (1961) 295.
33 L. Ellenbogen and D. R. Highley, *J. Biol. Chem.*, 242 (1967) 1004.
34 D. R. Highley, M. C. Davies and L. Ellenbogen, *J. Biol. Chem.*, 242 (1967) 1010.
35 R. Gräsbeck and H. Aro, *Biochim. Biophys. Acta*, 252 (1971) 217.
36 E. Hippe, *Scand. J. Clin. Lab. Invest.*, 29 (1972) 59.

471

Med. Pharmacol. exp. 14: 379–383 (1966)

Department of Internal Medicine Nr. II, Tîrgu-Mureş (Roumania)

Effect of Vitamin B_{12} on the Intestinal Absorption of Glucose

Cs. HADNAGY and E. HORVÁTH

In cases of Addison's disease, addisonism, myxedema and Simmond's disease the peroral glucose tolerance test constitutes one of the routine clinical diagnostic procedures. In the above diseases the rate of absorption of glucose is diminished. In the thyroidectomized rats this is similarly decreased, while in the case of experimentally induced hyperthyroidism the absorption of glucose is considerably increased. The thyroxin increases the activity of the hexokinase as well as that of the alkaline phosphatase in the intestine (lit. cit. *Cordier,* 1). In the case of adrenal cortex insufficiency the mechanism of the decreased absorption has not been conclusively elucidated. *Verzár* and co-workers (5) explain the selective absorption of glucose with phosphorylation and according to their experiments, the hormones of the adrenal cortex have an important role in the regulation of the above enzymatic processes. Other authors, however, explain the decreased intestinal absorption of the glucose of adrenalectomized rats with other mechanisms (6 and lit. cit. 1). — Ever since *Verne and Herbert's* (4) experiments it is known that, following adrenalectomy, the alkaline phosphatase activity can no longer be demonstrated from the intestinal tract.

Considering that in all probability the alkaline phosphatase activity has a certain role in the intestinal absorption of the glucose, and further that, according to our own findings (2), the vitamin B_{12} has a stimulating effect on the activity of intestinal phosphatase, we examined the effect of vitamin B_{12} on the in-

Received: 29. 7. 1965.

TABLE I

Effect of Vitamin B_{12} upon the absorption of glucose from the intestine

No. of cases	Name of patients	Administration of	Blood sugar level before and after ingestion of glucose mg% before	after 30′	after 60′	after 120′	Blood sugar modification % after 30′	after 60′	after 120′
1.	O. N.	Glucose	70	89	80	56	+27	+14	−20
		Glucose+B_{12}	70	109	121	77	+55	+72	+10
2.	G. I.	Glucose	83	107	110	105	+28	+32	+26
		Glucose+B_{12}	77	119	106	91	+54	+37	+18
3.	K. J.	Glucose	81	89	104	95	+ 9	+28	+17
		Glucose+B_{12}	67	94	115	98	+40	+71	+46
4.	S. E.	Glucose	85	105	125	138	+23	+47	+62
		Glucose+B_{12}	95	122	172	100	+28	+81	+ 5
		Glucose	90	99	116	92	+10	+29	+ 2
		Glucose+B_{12}	89	120	130	111	+35	+46	+24
5.	T. K.	Glucose	85	97	105	123	+14	+23	+45
		Glucose+B_{12}	75	95	108	86	+26	+44	+14
6.	D. E.	Glucose	70	95	88	79	+35	+25	+12
		Glucose+B_{12}	84	130	109	105	+54	+29	+21
7.	P. L.	Glucose	92	103	106	93	+11	+15	+ 1
		Glucose+B_{12}	107	149	142	121	+39	+32	+13
8.	B. J.	Glucose	99	123	114	102	+24	+15	+ 3
		Glucose+B_{12}	93	135	116	95	+45	+24	+ 2
9.	L. M.	Glucose	88	115	104	97	+30	+18	+10
		Glucose+B_{12}	90	136	119	108	+51	+32	+20
10.	T. F.	Glucose	88	106	118	70	+20	+34	− 2
		Glucose+B_{12}	97	139	79	63	+43	−19	−35
11.	A. J.	Glucose	89	98	116	70	+10	+30	−21
		Glucose+B_{12}	82	84	133	109	+ 2	+62	+33
12.	S. M.	Glucose	84	91	110	107	+ 8	+30	+27
		Glucose+B_{12}	82	147	129	75	+79	+57	− 8
13.	V. Z.	Glucose	90	113	106	91	+25	+17	+ 1
		Glucose+B_{12}	97	150	122	81	+54	+25	−16
14.	K. K.	Glucose	75	95	86	70	+27	+15	− 7
		Glucose+B_{12}	70	120	88	75	+67	+24	7
15.	A. A.	Glucose	98	96	85	75	− 2	−13	−23
		Glucose+B_{12}	88	108	113	79	+22	+28	−11
16.	T. S.	Glucose	79	102	114	96	+29	+44	+21
		Glucose+B_{12}	72	116	107	95	+61	+48	+31
17.	E. R.	Glucose	76	110	87	82	+45	+14	+ 8
		Glucose+B_{12}	80	112	131	77	+40	+64	− 4
18.	U. E.	Glucose	99	113	101	92	+14	+ 2	− 7
		Glucose+B_{12}	82	90	147	91	+10	+79	+11
19.	P. S.	Glucose	97	115	118	122	+18	+22	+26
		Glucose+B_{12}	104	143	144	144	+37	+38	+38

TABLE 1

(continued)

No. of cases	Name of patients	Administration of	Blood sugar level before and after ingestion of glucose mg%: before	after 30′	after 60′	after 120′	Blood sugar modification %: 30′	60′	120′
20.	H. S.	Glucose	105	120	130	114	+14	+24	+ 8
		Glucose+B_{12}	95	138	166	106	+35	+64	+12
21.	M. E.	Glucose	90	113	131	132	+25	+45	+46
		Glucose+B_{12}	90	154	168	106	+71	+86	+17
22.	A. P.	Glucose	90	107	125	85	+18	+39	− 5
		Glucose+B_{12}	90	165	104	78	+83	+15	−13
23.	L. K.	Glucose	100	119	148	112	+19	+48	+12
		Glucose+B_{12}	94	142	126	105	+51	+34	+12
24.	N. B.	Glucose	193	213	231	231	+10	+20	+20
		Glucose+B_{12}	200	301	380	193	+50	+90	− 4
25.	H. S.	Glucose	221	228	279	182	+ 3	+26	−17
		Glucose+B_{12}	169	268	275	195	+58	+62	+15
26.	M. N.	Glucose	258	300	308	300	+16	+19	+16
		Glucose+B_{12}	194	248	302	202	+27	+55	+ 4
27.	A. E.	Glucose	90	91	86	60	+ 1	− 4	−33
		Glucose+B_{12}	81	134	118	90	+65	+45	+11
28.	F. F.	Glucose	81	90	85	83	+11	+ 5	+ 2
		Glucose+B_{12}	82	118	116	82	+56	+41	0
29.	P. J.	Glucose	89	91	98	89	+ 2	+10	0
		Glucose+B_{12}	97	101	110	115	+ 4	+13	+18
30.	K. N.	Glucose	81	81	81	82	0	0	+ 1
		Glucose+B_{12}	73	100	85	72	+36	+16	− 1
31.	R. T.	Glucose	89	115	100	84	+29	+12	− 5
		Glucose+B_{12}	90	200	157	97	+122	+74	+ 7
32.	Glucose	Glucose	82	99	119	82	+20	+45	0
		Glucose+B_{12}	82	100	98	91	+22	+20	+11

Diagnosis: No. of cases 1–6 = M. Addisoni
7–11 = Addisonism
12 = M. Simmonds
13–15 = Simmondsoid leannes
16–18 = Sheehan syndrome
19 = Hypophysis adenoma
20–23 = Myxedema
24–26 = Diabetes mellitus and malabsorption of glucose
27–32 = Malabsorption of glucose with unknown origin

testinal absorption of glucose. Our observations were made on patients suffering from Addison's disease, addisonism, myxedema, Simmond's disease and Sheehan's syndrome. The testing of glucose tolerance is an examination frequently performed at our clinic, usually several of these being made daily. In the course of the last few years several cases were observed in which the decreased absorption of glucose remained unexplained; in these cases we have also examined the effect of vitamin B_{12}.

Methods

The examinations were made in the early morning hours with administration of 1 g glucose/kg dissolved in 300 ml water. Determination of the blood sugar level was made according to the Hagedorn-Jensen method before the ingestion of glucose and 30-60 and 120 min later. Each value was determined on the basis of 3 parallels. The glucose absorption tests were repeated 2-3 days after this examination. Then, having performed the blood collection for the determination of the fasting value, the vitamin B_{12} (in the majority of cases 1000 gamma) was injected subcutaneously. The solution of glucose was ingested by the patient 20-30 min after injection.

Results

As can be seen from the attached Table, in the case of all patients submitted to examination, with the exception of case 32 of inexplainable etiology, the administration of vitamin B_{12} increased the absorption of glucose. In all probability this effect can be explained with the activation of phosphorylation. According to our own experiments, the vitamin B_{12} does not influence the peristaltic movements of the isolated rabbit's intestine (3).

Based on the observations made on our own patients, we believe that certain conclusions may be drawn regarding the mechanism of the glucose's intestinal absorption.

Summary

In connection with 32 patients (Addison's disease, addisonism, myxedema, Simmond's disease, Sheehan's syndrome, malabsorption of glucose with unknown origin) the authors established that the administration of vitamin B_{12} has a stimulating effect on the intestinal absorption of glucose. The authors believe that this is due to the activation of phosphorylation in the intestinal tissue.

Zusammenfassung

An 32 Patienten, die an folgenden Erkrankungen litten: Morbus Addison, Addisonismus, Myxoedem, Morbus Simmond, Morbus Sheehan, Malabsorption von Glukose unbekannter Genese, konnte festgestellt werden, daß die Verabreichung von Vitamin B_{12} die intestinale Resorption von Glukose fördert. Die Autoren nehmen an, daß dies auf eine Aktivierung der Phosphorylierungsprozesse im intestinalen Gewebe zurückzuführen ist.

Résumé

Après traitement de 32 malades (maladie d'Addison, myxœdème addisonien, maladie de Simmonds, syndrome de Sheehan, mauvaise absorption de glucose d'origines inconnues) les auteurs ont établi que l'administration de vitamine B_{12} a un effet stimulant sur l'absorption intestinale de glucose. Les auteurs croient que ce phénomène est dû à l'activation de la phosphorylation dans le tissu intestinal.

References

1. *Cordier, D.:* L'absorption intestinale des glucides. Sem. Hôp., Paris *32:* 1-15 (1956).
2. *Hadnagy, Cs.; Feszt, T.; Horváth, E.; Gündisch, M.* und *Kemény, G.:* Wirkung von Vitamin B_{12} auf die Tätigkeit der alkalischen und sauren Gewebsphosphatase. Acta histochem. *5:* 233-235 (1958).
3. *Hadnagy, Cs.; Szabó, I.; Szilágyi, D.; Metz, O.; Szentkirályi, É.* und *Dézsi, Z.:* Die Wirkung von Vitamin B_{12} auf die Histamin- und Acetylcholin-Empfindlichkeit des Meerschweinchendarmes, auf die Bewegungen des Kaninchendarmes, auf den anaphylaktischen Schock und die Antikörperbildung. Int. Z. Vitaminforsch. *32:* 392-398 (1962).
4. *Hébert, S.:* Les phosphatases alcalines de l'intestin. Étude histochimique expérimentale. Arch. Biol. *61:* 235 (1950).
5. *Verzár, F.:* Lehrbuch der Inneren Sekretion (Ars Medici, Liestal 1948).
6. *Vidal-Sivilla, S.:* Sobre los resultados de absorción intestinal en los animales adrenalectomizados. Rev. esp. Fisiol. *10:* 203-223 (1954).

Authors' address: Dr. Cs. Hadnagy and Dr. E. Horváth, Departement of Internal Medicine Nr. II., Str. Kossuth 4, *Tirgu-Mures* (Rumänien).

Long-Term Excretion of Co^{57}-Vitamin B_{12} and Turnover Within the Plasma

CHARLES A. HALL, M.D.,* WITH THE TECHNICAL ASSISTANCE OF JANE G. HEGEMAN

CLARIFICATION of two aspects of the frequently studied problem of vitamin B_{12} metabolism in man—excretion and plasma turnover—is needed. Vitamin B_{12} can be found in urine,[1] but recent work[2] suggests that bile and feces may be a more important route of excretion. Although microbiologic assay of vitamin B_{12} in urine is possible, it can be destroyed or produced in the gut, and assay of vitamin B_{12} in the feces is meaningless. Moreover, such measurements determine only that the vitamin is sufficiently intact to promote growth of the organism used in the assay, and possibly much of the excreted vitamin B_{12} enters the urine or gut in an inactive form. Tracer studies circumvent some of these problems, but in the past neither specific activity of the radioactive vitamin B_{12} nor the technic for measurement have been adequate for long-term studies. Consequently, the route and magnitude of vitamin B_{12} excretion have remained unknown.

Initially, loss of injected vitamin B_{12} from the circulation is very rapid even when the vitamin is bound to plasma protein prior to injection.[3] A rapid but changing rate of loss persists for one or two months after the vitamin is either ingested or injected.[4] However, since the total body turnover of vitamin B_{12} is slow,[5,6] the question arose as to when this early, rapid plasma loss changed to a rate equivalent to total body turnover. The solution of this problem, too, has been beset by technical difficulties.

A fresh approach became possible because of an improvement in the technics of counting,[3,7] the greater counting efficiency of Co^{57} as compared to previous labels,[7] and the availability of high specific activity of Co^{57}-vitamin B_{12}. The experimental plan was simple: to give control subjects small amounts of Co^{57}-vitamin B_{12} by various routes and to follow the radioactivity of the blood, urine and feces as long as physically possible.

METHODS

Eight subjects were given Co^{57}-vitamin B_{12}: three orally, four intravenously and one intramuscularly. The subjects, all men, were from the chronic service of the hospital. Six were between sixty-five and sixty-seven years of age, two were ninety years old. Five had peripheral vascular disease, venous or arterial; one had mild generalized arteriosclerosis; and two had recovered from cerebral thrombosis. These subjects were carefully selected from a large group of patients for their ability to cooperate and for the absence of congestive failure, renal disease, diabetes or other endocrine disorders, abnormalities of gastrointestinal system, liver disease, blood disorders and neoplasia (one was found to have operable carcinoma of the lung after nine months of the present study). None had a vitamin B_{12} deficiency as measured by assay of plasma vitamin B_{12}.

The Co^{57}-vitamin B_{12} was taken from two lots purchased from the same source and at the time of use ranged from 12 to 30 μc. per μg. From 0.6 to 1.4 μg. (average 1.0 μg.) was given. Plasma samples were taken frequently during the first three days, then weekly for 300 to 400 days and then monthly. These samples were counted in a well crystal and scintillation spectrometer by previously described technics.[3,7] Erythrocytes from some patients were counted weekly but not beyond 250 days. They were washed twice in saline solution prior to counting. Feces and urine were collected for periods of five to seven days as individual stools and twelve hour urines. The patients and the ward personnel were carefully trained in the collection procedure.

From the Radioisotope and Medical Services, Albany Veterans Administration Hospital, Albany, New York.
* Hematologist.

If there was any doubt as to the completeness of collection, the collection period was abandoned and a new week of collections was started. Midway in the study, a formal metabolic unit was organized and, after day 215, all patients were transferred to this unit for collection. All collections from some short-term patients were performed in this unit. A total of twenty-one, five to seven day periods of collection were included in the study. The number and timing of collection varied according to the route of administration of the Co^{57}-vitamin B_{12} and with the purpose of the study. We examined fecal and urinary excretion shortly after the administration of Co^{57}-vitamin B_{12} in some cases, and much later in others. The stools were counted individually, and the urines were counted in 600 ml. aliquots of a twenty-four hour collection using a system described previously.[7] Usually, the counting error for blood and excreta was under 5 per cent (it was always under 10 per cent). All radioactivity was corrected for decay to the date of administration of the Co^{57}-vitamin B_{12}.

RESULTS

Plasma Disappearance. The long-term results are based on four subjects still being followed 539 to 686 days after the administration of Co^{57}-vitamin B_{12}. The short-term data are from the same subjects plus four others followed for 70 to 181 days. During the first twenty-four hours the disappearance of an intravenous injection followed the pattern described previously.[3] The plasma peak after intramuscular injection occurred at sixty to ninety minutes, and all three subjects receiving vitamin B_{12} orally showed a plasma peak at eight hours. The data presented here will be concerned with events after twenty-four hours. There were no definite, consistent differences among the subjects receiving vitamin B_{12} by different routes. The disappearance curves from day 1 to day 70 in two subjects are illustrated in Figures 1 and 2. Analysis of all curves showed a rapid, complex phase of plasma loss of vitamin B_{12} during the first week. During the period between day 7 and 45 the loss followed a simple exponential function with a half-time of seven to ten days. In one case (Fig. 1), the analysis was carried back to day 1, and there appeared to be a two component function in operation during the first two months. At about days 60 to 70

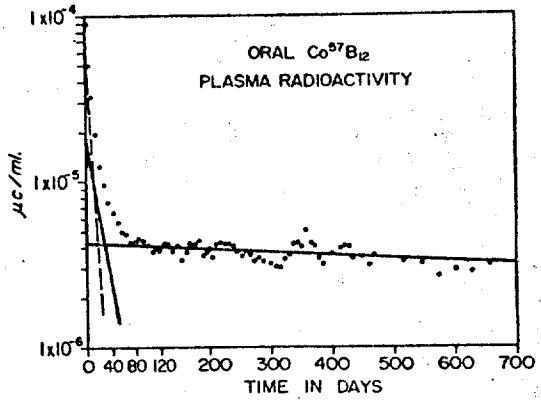


FIG. 1. Plasma disappearance of subject receiving Co^{57}-vitamin B_{12} orally. The function of the slow phase of loss was calculated from the data of days 71 to 574 by the method of least squares. The other functions were obtained by subtraction.

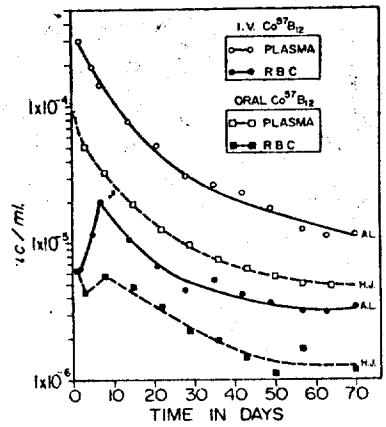


FIG. 2. Plasma and erythrocyte Co^{57}-vitamin B_{12} levels after two routes of administration during the period of one to seventy days.

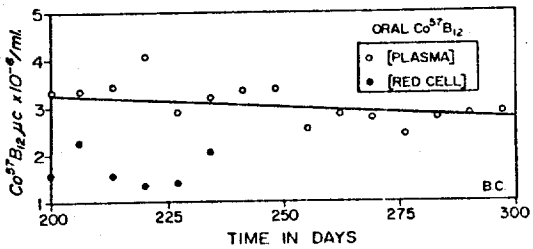


FIG. 3. Slow phase of loss of Co^{57}-vitamin B_{12} from plasma and erythrocytes after oral administration.

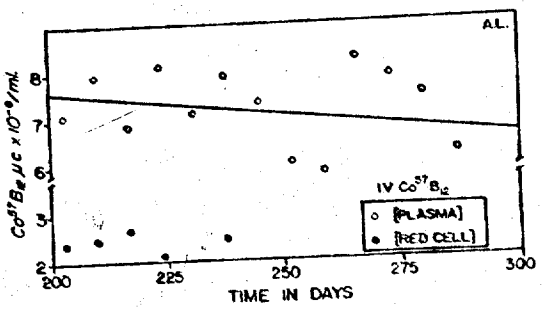


FIG. 4. Slow phase of loss of Co^{57}-vitamin B_{12} from plasma and erythrocytes after intravenous administration.

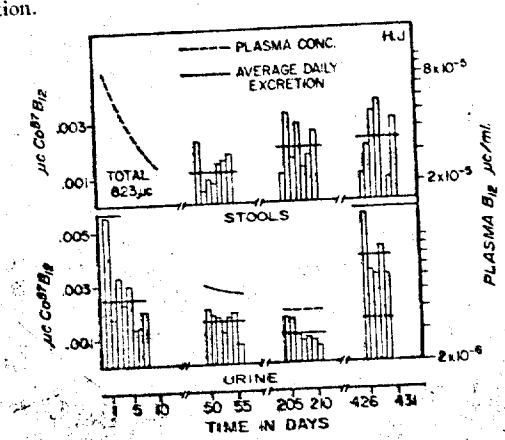


FIG. 5. Fecal and urinary Co^{57} during four collection periods after the oral administration of 1.0 μg. (0.86 μg. absorbed) of Co^{57}-vitamin B_{12}. Stools and urine are plotted as daily totals. Superimposed is the plasma Co^{57}-vitamin B_{12} disappearance curve during each collection period with the ordinate of this curve on the right.

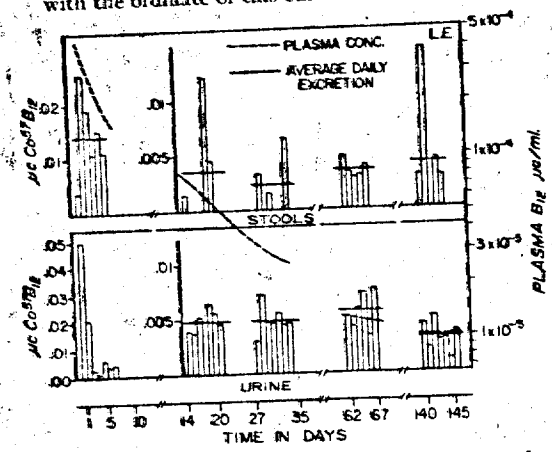


FIG. 6. Excretion after the intravenous administration of 1.2 μg. Co^{57}-vitamin B_{12} plotted in the same manner as Figure 5. Note the change in ordinates between the first and second collection periods.

there was always a sharp break in the curve. Subsequently (Fig. 1, 3 and 4), loss took place at a very slow rate. It was impossible to determine whether this loss represented a phase of an exponential function which only appeared linear (as Figure 1 suggests), or whether this disappearance phase of vitamin B_{12} from plasma was separate from previous events and was truly linear. For mathematical considerations, it was preferable to assume that the function was linear and in four subjects the rate of loss was calculated from the data of days 70 to 90 to days 456 to 574 by the method of least squares. The half time ranged from 480 to 1,284 days and did not appear to be related to the method of administration. During the slow phase, there was a greater fluctuation than could be accounted for by errors of counting. As can be seen in Figures 3 and 4, there was a cyclic pattern to this fluctuation.

While this study was concerned chiefly with subjects with normal vitamin B_{12} metabolism, short-term plasma studies only were conducted in two subjects with chronic granulocytic leukemia and in two with pernicious anemia. The former, were followed thirty-four and thirty-six days after the intravenous administration of Co^{57}-vitamin B_{12}, and the latter, twenty and thirty days after. These patients were untreated at the time of the injection, but treatment was started the following day. During the limited period of study, plasma disappearance in these patients after twenty-four hours did not differ from that of the control subjects.

Erythrocyte Concentration. Incorporation of Co^{57}-vitamin B_{12} in the red cells is illustrated in Figures 2, 3 and 4. There was always an erythrocyte peak at the time of the plasma peak followed by a decline and a second peak at about seven days. Thereafter, the decline followed a pattern similar to that of plasma. The disappearance curves in two subjects receiving vitamin B_{12} orally were slightly different from those of subjects receiving vitamin B_{12} intravenously.

Fecal and Urinary Excretion. The results of this part of the study are based on eight subjects, each of whom was subjected to one to five collection periods. The timing of collec-

tion periods was varied in order to study particular phases of excretion. In two subjects, several periods were examined and in the others only early, middle or late phases. The findings were quite consistent for all; two illustrative cases are given in Figures 5 and 6.

Fecal loss of the vitamin during the first five to seven days after the oral dose in three subjects was 17, 56 and 59 per cent of the dose. In one case, excretion had fallen to the level of later periods by day 6, but the others were not at this point by day 7. After an intravenous dose, fecal loss was high from days 1 to 7 in one case, but had reached a consistent rate by days 14 to 21 (Fig. 6). However, another subject excreted three times as much during days 20 to 25 as during days 62 to 67. After these early periods of higher excretion, all subjects continued to excrete surprisingly uniform amounts of Co^{57}. The daily fecal excretion of seven subjects after the first month ranged from 0.013 to 0.061 per cent of the retained dose, with an average of 0.031 per cent.

Although urinary loss tended to be high during the first day or two regardless of route of administration (Fig. 5 and 6), the excretion promptly reached a uniform rate. The daily excretion of eight subjects during the period of uniform excretion ranged from 0.017 to 0.037 per cent of the retained dose, with an average of 0.028 per cent. Single twenty four hour urines were collected from four subjects on some day between days 87 and 667. The average amount of radioactivity was 0.027 per cent of the retained dose. Assuming an average body content of total vitamin B_{12} of 2.2 mg., excretion of the radioactive tracer reflected a mean total urinary excretion of 0.6 μg. vitamin B_{12} per day. Assay of the urine for total vitamin B_{12} by Euglena gracilis showed only an average excretion of 0.06 μg. per day; the most excreted was 0.09 μg.

Three findings in regards to excretion stand out: (1) fecal and urinary excretion of tracer doses of vitamin B_{12} was about equal; (2) both continued at rather uniform rates for many months; and (3) the rate of excretion by either route during the first two months seemed to bear little relation to the rate of plasma loss (Fig. 5 and 6).

COMMENTS

There is no obvious explanation of the peculiar behavior of radioactive vitamin B_{12} in plasma during the first two months after intake into the body. Some of the complexity of the pattern of plasma loss during the first day or two is the result of binding to more than one plasma protein.[8] However, the break in the disappearance curve at days 60 to 70 cannot be explained on this basis since binding by a single protein is reached long before that time. The early rapid plasma loss could not be explained on the basis of higher excretion. The half-times of 480 to 1,284 days of the slow phase of plasma loss are comparable to the slow phase of total body loss to the half-time of 1,300 days found by Heinrich and Pfau,[5] and of 407 to 770 days by Bozian et al.[6] After sixty to seventy days, the plasma vitamin B_{12} appeared to be at least close to equilibrium with that of the total body. The plasma vitamin B_{12} can never be in complete equilibrium with total body vitamin B_{12}. If newly absorbed vitamin B_{12} goes through the phase of intake and rapid loss from the plasma as illustrated in Figures 1 and 2 each time the vitamin is absorbed from the intestine, plasma vitamin B_{12} will always be somewhat high in proportion to total body vitamin B_{12}. The daily intake of vitamin B_{12} is probably small in relation to total body vitamin B_{12} and for this reason the rate of plasma turnover may appear to be the same as the whole body turnover. The exact nature of the proteins which transport vitamin B_{12} in the blood and their rates of turnover are unknown, but plasma proteins usually turn over rapidly. Certainly, during the slow phase of plasma loss described in this study the Co^{57}-vitamin B_{12} could not have been bound to the same molecules of plasma protein as at the time of entry into the plasma, and the Co^{57}-vitamin B_{12} measured in the plasma at that time must have been in free exchange with at least total vitamin B_{12} in the plasma and possibly with that of the rest of the body.

The early Co^{57}-vitamin B_{12} of the erythrocytes may have been on the surface of the cell, although it was not removed by washing. The second peak at seven days probably

represented incorporation of Co^{57}-vitamin B_{12} into the cells as they were made rather than direct entry from the plasma. Doscherholmen and Hagen[9] observed an erythrocyte peak at the same time. The loss of Co^{57}-vitamin B_{12} from the cell during the month after this peak was too rapid to be accounted for solely on the basis of release with the death of the cell. The Co^{57}-vitamin B_{12} in or on the erythrocytes after about day 120 obviously did not get there at the time the dose was administered since the life span of erythrocytes made at that time had been exceeded. The ultimately parallel curves of loss from plasma and erythrocytes could be explained by either a free exchange of vitamin B_{12} between the two, or by receipt of the vitamin B_{12} from a common pool.

Both feces and urine were shown to be sources of excretion of vitamin B_{12} or breakdown products of vitamin B_{12}. Calculation of the expected amount of vitamin in the urine, based on measured excretion of the tracer and estimation of the total body vitamin B_{12}, indicate a total urinary excretion of 0.6 μg. per day. Only 10 per cent of this was recovered by microbiological assay in the present study suggesting either that much of the tracer was excreted in a form inactive to Euglena gracilis or that the assay did not measure all the vitamin B_{12} present. The latter possibility is discounted by studies which showed good recovery of vitamin B_{12} added to various dilutions of urine. The form in which Co^{57}-vitamin B_{12} reached the feces is unknown. However, in studies of the microbiologically measured vitamin B_{12} in bile Reizenstein[10] found that about 4 μg. entered the bile daily and 1.3 μg. of this left the body in the feces. The latter figure is of the same order of magnitude as calculated fecal loss in the present study. Apparently, the vitamin B_{12} which is excreted reaches the bile in an active form, is partly reabsorbed and the remainder is then undoubtedly altered as it passes through the small and large intestines.

Early studies based on tracer technics suggested that body excretion was primarily fecal and rather high.[2] The labeled vitamin B_{12} was given intravenously in those studies, and excretion was measured for a short time only. As shown in the present study and previously,[2,11] fecal loss was high shortly after the intravenous administration of vitamin B_{12}; from present data, fecal excretion of labeled vitamin B_{12} does not appear to reach a baseline level until about one month after injection. Measurements of fecal excretion in the earlier studies were not carried on for a long enough period of time to permit the excretion of Co^{57}-vitamin B_{12} to act as a true tracer.

Invariably, those who have investigated some aspect of the dynamics of body vitamin B_{12} have tried to estimate total body turnover from their data. One or more vital assumptions have always been necessary. The measurement of excretion of the tracer dose of vitamin B_{12} in the present study appears reliable, and all the data support the assumption that the Co^{57} which was excreted truly reflected the excretion of endogenous vitamin B_{12}. The major weakness in an attempt to calculate total daily loss and daily needs of vitamin B_{12} from measured excretion of a tracer is in the estimation of the total body content of vitamin B_{12}. Adams,[12] by an isotope dilution technic, measured the total body vitamin B_{12} of patients dying from various diseases. The mean body content of the group was about 2 mg. and if only those subjects are included who received the radioactive vitamin B_{12} at least two weeks prior to death, the average was 2.2 mg. The exact time of tissue equilibrium of labeled vitamin B_{12} in man is unknown. Tracer doses of vitamin B_{12} given intravenously to the rat were in equilibrium with tissue vitamin B_{12} by day 15[13] (by day 14 in the dog[14]). Therefore, the figure of 2.2 mg. was used as an average figure for body vitamin B_{12} in the present calculation. This figure is in agreement with an estimate of body vitamin B_{12} derived from microbiological assay in our laboratory of ten to twelve tissue specimens from ten subjects dying from various causes. The calculated total daily loss of vitamin B_{12} or its breakdown products in the feces was 0.29 to 1.3 μg., mean 0.68 μg. A similar calculation of urinary excretion was 0.37 to 0.82 μg. per day, mean 0.62 μg. The average daily excretion of the vitamin by both routes was

0.66 to 2.1 μg., mean 1.3 μg. These calculations are based on excretion after a uniform rate had been reached, and all subjects regardless of route of intake were included. There was no difference in excretion between the two groups when the subjects who received the Co^{57}-vitamin B_{12} orally, the more physiological route, were separated from those who received it intravenously. Separation of data collected after 300 days, when equilibrium of the tracer was most likely, did not change the estimate of excretion. Others[2,5] assume a total body vitamin B_{12} level of 4 to 5 mg. If these estimates are closer to fact than the 2.2 mg. assumed here, this calculation of total excretion should be doubled. It is interesting that Heinrich and Pfau[5] calculated the excretion of the vitamin to be 2.6 to 3.0 μg., based on total body counting in three subjects (assumed 5 mg. total body content). Similar calculations from the body counting data of the three normal subjects of Bozian et al.[6] give an excretion value of 4.8 μg. per day. Estimation of total excretion implies estimation of total need. However, we are discussing calculations based on measurements which were made in subjects who were presumed to have a normal vitamin B_{12} metabolism and who were subsisting on a normal diet. Possibly health can be maintained on a much smaller intake than implied by our data, and a greater intake may be optimal.

SUMMARY

Eight control subjects were given an average of 1.0 μg. of Co^{57}-vitamin B_{12} by different routes. The Co^{57} in plasma, urine and feces was measured for at least seventy days in all subjects and for more than 500 days in half of them with the following results:

(1) The Co^{57}-vitamin B_{12} disappeared rapidly from the plasma in a complex manner until about day 70. Subsequently, the loss could be described by a linear function with an average half-time of 690 days.

(2) Urinary excretion was higher the first few days than subsequently regardless of route of intake. Fecal excretion did not become uniform until about one month after intravenous administration.

(3) Urinary excretion during the period of uniform loss was 0.028 per cent of the retained dose per day. Simultaneous fecal excretion was 0.031 per cent per day. Comparison of urinary excretion of Co^{57} and total vitamin B_{12} as measured by microbiological assay suggested that only 10 per cent of the vitamin B_{12} excreted in the urine was in an active form.

(4) The total daily excretion of vitamin B_{12} was calculated to be 0.66 to 2.1 μg., mean 1.3 μg.

ACKNOWLEDGMENT

I wish to express my appreciation to the staff of the Chronic Medicine Section, Albany Veterans Administration Hospital, for their assistance.

REFERENCES

1. Heinrich, H. C. Die Biochemischen Grundlagen der Diagnostic und Therpie der Vitamin B_{12} Mangelzustand de (B_{12} hypo und Avitaminosen) des Menschen und der Haustiere II. Untersuchungen-Zum Vitamin B_{12} Stoffwechsel des Menschen Wahrend der Graviditat und Lactation. *Klin. Wshnschr.*, 32: 205, 1954.
2. Gräsbeck, R., Nyberg, W. and Reizenstein, P. Biliary and fecal vitamin B_{12} excretion in man. An isotope study. *Proc. Soc. Exper. Biol. & Med.*, 97: 780, 1958.
3. Hall, C. A. The plasma disappearance of intravenously administered cobalt^{58}-vitamin B_{12}. *J. Clin. Invest.*, 39: 1312, 1960.
4. Reizenstein, P., Cronkite, E. P. and Cohn, S. A. Plasma clearance and whole body turnover of injected and absorbed radioactive vitamin B_{12} in man. *J. Lab. & Clin. Med.*, 62: 255, 1963.
5. Heinrich, H. C. and Pfau, A. A. Mit Hilfe Eines Gesamtkorper-Radioaktivitats-Detektors Durchgefuhrte Untersuchungen zur Resorption, Biologischen Halbwertzeit und Umsatzrate des Vitamin B_{12} im Menschlichen Organismus. In: Vitamin B_{12} und Intrinsic Faktor, p. 351. Edited by Heinrich, H. C. Stuttgart, 1962. Ferdinand Enke.
6. Bozian, R. C., Ferguson, J. L., Heyssel, R. B., Meneely, G. R. and Darby, W. J. Evidence concerning the human requirement for vitamin B_{12}. *Am. J. Clin. Nutrition*, 12: 117, 1963.
7. Allen, R. A. and Moore, B. T. Comparative counting efficiencies of Co^{57} B_{12} and Co^{58} B_{12} for three counting systems using two detectors. *J. Nuclear Med.*, 3: 300, 1962.
8. Hall, C. A. and Finkler, A. E. A second vitamin B_{12} binding substance in human plasma. *Biochim. et biophys. acta*, 78: 234, 1963.
9. Doscherholmen, A. and Hagen, P. S. Kinetics of ingested radiocyanocobalamin in man. In: Vitamin B_{12} und Intrinsic Faktor, p. 381. Edited

by Heinrich, H. C. Stuttgart, 1962. Ferdinand Enke.

10. Reizenstein, P. G. Excretion of non-labeled vitamin B_{12} in man. *Acta med. scandinav.*, 165: 313, 1959.

11. Cronkite, E. P., Henley, E., Driscoll, D. H., Meyer, L. M., Dohan, F. C., Rubini, J. R. and Wolins, W. Studies on the kinetics of intravenously injected ^{60}cobalt labeled vitamin B_{12} in man. *Haematologica Latina*, 2: 265, 1959.

12. Adams, J. F. The measurement of the total assayable vitamin B_{12} in the body. In: Vitamin B_{12} und Intrinsic Faktor, p. 397. Edited by Heinrich, H. C. Stuttgart, 1962. Ferdinand Enke.

13. Newman, G. E., O'Brien, J. R. P., Spray, G. H. and Witts, L. J. Distribution of vitamin B_{12} in rat liver cell fraction. In: Vitamin B_{12} und Intrinsic Faktor, p. 424. Edited by Heinrich, H. C. Stuttgart, 1962. Ferdinand Enke.

14. Cooperman, J. M., Luhby, A. L., Teller, D. N. and Marley, J. F. Distribution of radioactive and nonradioactive vitamin B_{12} in the dog. *J. Biol. Chem.*, 235: 191, 1960.

1108

REVIEW

Vitamin B_{12}-Binding Proteins of Man

CHARLES A. HALL, M.D., Albany, New York

Body proteins that bind vitamin B_{12} are now finding multiple uses in clinical medicine. Intrinsic factor in the intestinal tract and transcobalamins I and II in the plasma participate in normal vitamin B_{12} transport. Several other binding proteins have been identified in various cells and body fluids, but the reasons for their existence are not known. The absence of intrinsic factor, the basis for the vitamin B_{12} deficiency in pernicious anemia, is obviously useful in differential diagnosis. The different degrees of increase in transcobalamin I in myeloid metaplasia, chronic myeloid leukemia, and polycythemia vera are useful in the differential diagnosis of these diseases. In addition, there is a large amount of a binder of unknown function in the plasma of polycythemia vera. Some of the natural substances that bind vitamin B_{12} have been used as a reagent in isotope-dilution assays of the vitamin.

THIS BRIEF REVIEW was prompted by a growing awareness that those of us who write about substances that bind vitamin B_{12} (B-12) have created a confusing picture. Since these substances are now becoming important clinically (1) as well as scientifically, it is our obligation to present the basic and important facts clearly.

Substances that carry vitamin B_{12} from one part of the body to another must bind it, but many bind B-12 without having any apparent physiologic function in B-12 metabolism. Probably all B-12 binding substances are proteins, and all those that are well described appear to be glycoproteins. They can be put into five, sometimes overlapping, groups: transport proteins, binders that exist in vivo with unknown functions, binders useful in diagnosis, binders useful in performance of isotope-dilution assays of B-12, and artifacts of methods of analysis.

From the Hematology Research Laboratory, Veterans Administration Hospital; and the Department of Medicine, Albany Medical College of Union University; Albany, N.Y.

Annals of Internal Medicine 75:297-301, 1971

Transport Proteins

Perhaps one of the most confusing aspects of the B-12 binders is nomenclature. All were not discovered simultaneously, and new observations have changed old names. Improvements in methods of evaluation have changed concepts and terminology. Different methods of isolation used in different laboratories are also responsible for confusion in nomenclature.

Intrinsic factor, the best established of this group, is discussed well in a recent review (2). Little more will be said here except to note that in man intrinsic factor is required to transport the B-12 of food to the ileum.

Transcobalamin II, alternately referred to as the beta B-12 binder of plasma, is one of two transport proteins in normal plasma. There have not been many studies of its function, but those available all support an active role for this protein. Transcobalamin II takes up B-12 as the B-12 enters the blood after oral administration either as the vitamin (3) or in food (4). Vitamin B_{12} bound to transcobalamin II leaves the plasma rapidly for the tissues (5-7). In vitro it promotes the uptake of B-12 by cells in culture or suspension (8, 9). Correlation of all of these observation suggests that transcobalamin II transports B-12 from the ileum to tissues during the early stages of distribution. There are no published data supporting a later role in plasma transport, but unpublished studies from our own laboratory suggest there may be one. Recently, congenital absence of transcobalamin II was found to result in rapidly developing megaloblastic anemia, confirming its im-

portance in the transport of B-12 (10). Transcobalamin II is found in cerebrospinal fluid and seminal plasma, as well as in blood plasma (11).

Transcobalamin I, also referred to as the alpha B-12 binder of plasma, has a somewhat less well defined role in transport. It does carry B-12 that has been in the body for more than 24 hours. Transcobalamin I does not seem to be an efficient transport protein for moving B-12 into cells of homologous and physiologic systems since it leaves the plasma slowly (5–7) and does not promote cell uptake of B-12 in vitro (8, 9). There is, however, evidence that for months after intake B-12 recirculates via the plasma while bound to transcobalamin I, suggesting some active transport role (12). The B-12 entering HeLa cells bound to transcobalamin II exits bound to transcobalamin I (9), and there is evidence of a similar process in intact man (6). The fate of this transcobalamin I-B-12 is unknown. Transcobalamin I is also discussed below as an "R" binder since it has many properties in common with that group.

Well-Defined Vitamin B_{12} Binders of Unknown Function

There are in the body several substances of various sources that bind B-12 when it is added to them, but their functions are unknown, and, in fact, they may have none. They are most often identified by their sources—for example, saliva, tears, milk, fetal plasma, polycythemia vera plasma, bile, gastric juice, cerebrospinal fluid, seminal plasma, leukocytes, and erythrocytes. Reviews should be consulted for details of the individual substances (2, 11). Almost all of the work with these substances has identified them by radioactive B-12 bound artifically to them, and little is known about their binding of the B-12 that occurs naturally in their sources. The binder peculiar to fetal plasma, for example, binds B-12 added in vitro very readily but does not seem to carry B-12 naturally (13). The B-12 binder in milk does, however, carry native B-12 (14). Some body fluids—gastric juice, for example—contain both functioning transport proteins and binders that probably have no function, adding to the confusion.

Gräsbeck (2) has grouped all of the body B-12 binders other than intrinsic factor and transcobalamin II into a category called "R." Initially the "R" referred to rapid electrophoretic mobility, although as the list of "R" proteins has grown the range of mobilities has widened. Transcobalamin II was originally included in the group, but it is now clear that it is separate. With the exception of saliva "R," which has a molecular size of ± 60,000 mol wt (2), all members of the group are approximately 120,000 in molecular weight, as studied by gel filtration. The "R" binders are immunologically identical and do not react with antitranscobalamin II or anti-intrinsic factor (11). No reactivity has been found between antihuman transcobalamin I and transcobalamin I of other species, although antihuman transcobalamin II reacts with transcobalamin II from monkey and dog (15).

"R" binders are separated from each other mainly by their source of origin. There are differences in isoelectric points, but these differences alone would not always separate them in a mixture. Moreover, every "R" protein that has been well studied by isoelectric focusing has shown heterogeneity (2). Close similarities between the "R" binders has suggested common origin or conversion from one to another. Creation of transcobalamin I from the leukocyte binder by adding molecules of sialic acid has been the most discussed of possible interrelationships (2). The "R" binders do contain sialic acid, and the differences among members of the group are in electrophoretic properties. Therefore, the "R" binders could all come from a common source by manipulating the amount of sialic acid.

In summary, there are in man two distinct and functioning B-12 transport proteins—intrinsic factor and transcobalamin II. There is also a group of binders called "R," one of partially identified function, transcobalamin I, and others of unknown function. The properties distinguishing the three classes, in the form of their complex with B-12, are given in Table 1.

Vitamin B_{12} Binders Useful in Diagnosis

The deficiency of intrinsic factor in pernicious anemia is well known, and direct or indirect measurement of this binder can be used in the differential diagnosis. The intrinsic factor deficiency of pernicious anemia is a fundamental part of the disease. There are abnormalities in binders other than intrinsic factor in disease, but in these cases possible relationships to other variables of the disease remain unknown. The increase in transcobalamin I in the chronic myeloproliferative states has been documented repeatedly and can serve as a basis for a diagnostic test (1, 16). Increased transcobalamin I has been observed in leukemoid reactions (17), and the use of the increase in diagnosis must be modified. The decreased transcobalamin II of myeloproliferative disease (18, 19) was not observed in leukemoid reactions, and this may prove to be a diagnostic feature. The presence of an "R" binder, in addition to transcobalamin I, in the plasma of persons with

polycythemia vera has as yet undeveloped diagnostic potential (19).

The capacity of serum to bind added B-12, the summation of the capacities of the individual binders, is also useful in the differential diagnosis of myeloproliferative disease (1).

Vitamin B_{12} Binders as Reagents in Isotope-Dilution Assay

Vitamin B_{12} can be measured by assays based on the principles of isotope dilution. General principles of this type of assay and some information on its application to measurement of B-12 are given in a recent symposium (20). Such an assay requires a reagent that will bind the substance to be measured. The several assays of B-12 use either intrinsic factor preparations, whole human serum, transcobalamin I, or saliva "R" as a binding reagent. The binding of B-12 in these assays is an in vitro phenomenon necessary to the technique. The protein selected as a reagent may or may not participate in natural B-12 transport, but in any case the use of this protein in the in vitro test in no way relates to any in vivo events in the source of the serum being assayed.

The ideal binding reagent has certain well-identified properties (20). It must bind quickly, reliably, and firmly. It must bind both the radioactive form of the substance to be assayed and the nonradioactive form equally, but it must not bind any other substance encountered. Separation of the free and bound material must be feasible. Although several B-12 binding substances have been used in successful assay systems, there are theoretical disadvantages to some of them. Intrinsic factor preparations, for example, contain more than one B-12 binder. Investigators devising an isotope-dilution assay for B-12 should become thoroughly acquainted with the properties of B-12 binders before making a selection.

Nonspecific Binders and Artifacts

The presence of nonspecific B-12 binders has been suspected for many years. We have evidence that when B-12 is added to whole serum in amounts over 1.0 ng/ml there is binding to proteins that probably have no part in B-12 transport (18). These observations have recently been confirmed by Gizis and associates (21). We have found, in unpublished work, that there is much more binding to these proteins when large amounts of hydroxycobalamin are added to serum than when equal amounts of cyanocobalamin are used. Hom and Ahluwalia (22) found a minor binder of high molecular weight when separation was performed by gel filtration. Its significance

Table 1. Properties of the Three Groups of Human Vitamin B_{12} Binders

	Intrinsic Factor-B_{12}	Transcobalamin II-B_{12}	Group "R"-B_{12}
Molecular size	60,000	38,000	±120,000*
CM†-cellulose elution starts	0.02 M pH 5.4	0.2 M pH 7.8	0.02 M pH 5.4
Uptake by ileal tissue	Yes	No	No
Uptake by HeLa cells	No	Yes	No
Reacts with			
Antitranscobalamin II	No	Yes	No
Antisaliva "R"	No	No	Yes
Anti-intrinsic factor	Yes	No	No

* Saliva "R" is 60,000.
† CM = carboxymethyl.

and relationship to other minor components is not clear.

Several workers in the field have produced artifacts by some phase of their separation technique. We, for example, found that repeated freezing and thawing changed the electrophoretic mobility of plasma binders. Perchloric acid changes transcobalamin II into an "R" protein (11). Hom (23) reported that transcobalamin II formed a larger-sized complex when exposed to buffers of low ionic strength, and we have had the same experience. Heparin will also complex with transcobalamin II (24) and change the interpretation of a separation technique based on molecular size. The anticoagulant ethylene diaminetetracetic acid affects the properties of binders to be separated by gel filtration (24). Extensive purification of transcobalamin II by even comparatively gentle means will change its molecular size and isoelectric point (25).

The above discussion is presented to point out that not all B-12 binders described in the literature are important, except to workers in the field. Clearly anyone claiming to have isolated a new binder should make sure that it is not a creation of his methods. A possible case in point is the reported binder of ± 120,000 mol wt and beta mobility in normal plasma (26). The buffers used in the gel filtration were 0.075 M, and transcobalamin II is known to form larger-sized complexes at low ionic strengths (23).

Choice of Techniques

Methods of detection, quantitation, and evaluation of substances that bind vitamin B_{12} have been presented in detail elsewhere (27). All techniques use the label of radioactive B-12 to detect or measure; none measures the protein directly. Detection requires only enough of a radioactive label to measure; quantitation requires saturation of the binder by B-12.

(Precise quantitation also requires a knowledge of how much B-12 was bound to a protein before the label was added.) Where more than one substance that can bind B-12 is present, a method of separation must be applied if the individual proteins are to be measured. The commonly used methods are based on differences in molecular size or isoelectric point, or both.

Methods of separation are valid only if the distinquishing property of a binder is confined to that one binder. The polycythemia vera binder, for example, has a molecular size of about 120,000 and cannot be separated from transcobalamin I by gel filtration since transcobalamin I has the same molecular size (19). The polycythemia vera binder is difficult to separate from transcobalamin II by electrophoresis or by anion exchange chromatography because the isoelectric points are close (19), but differences in molecular size permit ready separation by gel filtration. Obviously the source of the body fluid is a factor in its analysis. The polycythemia vera and fetal plasma binder cannot be separated (and possibly are identical), but their very different sources eliminate any problem in separation.

A choice of methods of studying B-12 binders depends, then, very much on which binder is to be detected and what other binders are expected to be present. The artifacts of certain methods discussed above must be avoided. Simple, repetitive evaluation in a well-defined situation can often be managed through simple methods. When, however, a new substance is described or the situation is otherwise complex, the evaluation must be rigorous, with multiple crosschecks on the final product (27).

Nomenclature

The old expression "the plasma B-12 binding protein" obviously no longer fits the current scene. One approach to naming the different binders has been according to their source. This is adequate when there is only one binder from that source, as in the case of the saliva binder. Where there are two or more binders, as in plasma, electrophoretic mobility can be used to designate them, for example, the alpha B-12 binder or beta B-12 binder. The classification by mobility is patterned after mobilities of other serum proteins at pH 8.6 in common supporting media. This terminology has limitation where there is more than one binder of similar mobility in a fluid or tissue. Designation by mobility has the advantage of nonspecificity, a desirable feature when describing a system of poorly evaluated components. Mobility is not, however, a critical property in the function of B-12 binders. Moreover, studies by electrofocusing show isoelectric heterogeneity in most instances (2).

We devised the terminology transcobalamin I and II for the two normal and functioning transport proteins in plasma because they are specific transport proteins (5, 28). We have not given as specific a designation to other binding substances because functions have not been identified.

Nomenclature as suggested by Gräsbeck (7) may eventually prove to be the best. The two functionally and immunologically separate and distinct proteins, transcobalamin II and intrinsic factor, are classified as such; all other binders are put in the group of "R" proteins. The "R" binders have enough properties in common to form a group, but too little is known about their function to permit grouping with complete confidence. The only certainty in classification is that the system will be changed.

ACKNOWLEDGMENTS: Supported in part by grant AM02808, National Institutes of Health, Bethesda, Md. 20014

Received 10 March 1971; revision accepted 5 May 1971.

► Requests for reprints should be addressed to Charles A. Hall, M.D., Research Service, Veterans Administration Hospital, Albany, N.Y. 12208

References

1. Gilbert HS, Krauss S, Pasternack B, et al: Serum vitamin B-12 content and unsaturated vitamin B-12-binding capacity in myeloproliferative disease. *Ann Intern Med* 71:719-722, 1969
2. Gräsbeck R: Intrinsic factor and the other vitamin B-12 transport proteins, in *Progress in Hematology*. New York, Grune & Stratton, Inc., 1969, pp. 233-245
3. Hall CA, Finkler AE: A second vitamin B-12-binding substance in human plasma. *Biochim Biophys Acta* 78:233-236, 1963
4. Hall CA, Finkler AE: Function of transcobalamin II: a B-12 binding protein in human plasma. *Proc Soc Exp Biol Med* 123:55-58, 1966
5. Hall CA, Finkler AE: The dynamics of transcobalamin II. A vitamin B-12 binding substance in plasma. *J Lab Clin Med* 65:459-468, 1965
6. Hom BL: Plasma turnover of ^{57}cobalt-vitamin B-12 bound to transcobalamin I & II. *Scand J Haemat* 4:321-332, 1967
7. Gizis EJ, Arkun SN, Miller IF, et al: Plasma clearance of transcobalamin II-bound Co^{57} vitamin B-12. *J Lab Clin Med* 74:574-580, 1969
8. Retief FP, Gottlieb CW, Herbert V: Delivery of Co^{57} B_{12} to erythrocytes from α and β globulin of normal, B_{12} deficient, and chronic myeloid leukemia serum. *Blood* 29:837-851, 1967
9. Finkler AE, Hall CA: Nature of the relationship between vitamin B-12 binding and cell uptake. *Arch Biochem* 120:79-85, 1967
10. Neiman PE, Hakami N, Canelos G, et al: Neonatal megaloblastic anemia due to transcobalamin II deficiency (abstract), in *13th Annual Meeting, American Society of Hematology*, San Juan, P.R., 6 December 1970, p. 72
11. Finkler AE, Green PD, Hall CA: Immunological properties of human vitamin B-12 binders. *Biochem Biophys Acta* 200:151-159, 1970
12. Hall CA: Long-term excretion of Co^{57} vitamin B-12 and turnover within the plasma. *Amer J Clin Nutr* 14:156-162, 1964
13. Kumento A: Studies on the serum binding of vitamin B-12 in the newborn human infant. *Acta Paediat Scand* (suppl 194):9-55, 1969

14. Finkler AE, Rappazzo ME, Hall CA: Cyanocobalamin binding protein in human milk (abstract). *Fed Proc* 26:695, 1967
15. Finkler AE, Hall CA, Green P, et al: Cross reactivity of mammalian B-12 transport proteins (abstract). *Fed Proc* 28: 700, 1969
16. Mikkonen L, Finkler AE, Hall CA: A diagnostic test for chronic myelogenous leukemia based on abnormalities of B-12-binding. *Acta Med Scand* 181:591-596, 1967
17. Carmel R, Coltman CA: Non-leukemic elevation of serum vitamin B-12 and B-12-binding capacity levels resembling that in chronic myelogenous leukemia. *J Lab Clin Med*. In press
18. Hall CA, Finkler AE: Measurement of the amounts of the individual vitamin B-12 binding proteins in plasma. *Blood* 27:611-622, 1966
19. Hall CA, Finkler AE: Vitamin B-12-binding protein in polycythemia vera plasma. *J Lab Clin Med* 73:60-69, 1969
20. Hayes RL, Goswitz FA, Murphy BEP (editors): *Radioisotopes in Medicine: In Vitro Studies*. Oak Ridge, Tenn., U.S. Atomic Energy Commission, 1968, pp. 3-57, 365-412
21. Gizis EJ, Dietrich MF, Ohoi G, et al: A ^{57}Co vitamin B-12 binder in normal serum eluted by DEAE-cellulose chromatography with 0.1M sodium phosphate buffer, pH 5.8. *J Lab Clin Med* 75:673-678, 1970
22. Hom BL, Ahluwalia BK: The vitamin B-12 binding capacity of transcobalamin I & II of normal human serum. *Scand J Haemat* 5:64-74, 1968
23. Hom BL: Demonstration of transcobalamin II complex formation and binding to Sephadex G-200 at low ionic strength. *Clin Chim Acta* 18:315-317, 1967
24. Cooper BA: Complexing of transcobalamin 2 and apparent combination with heparin. *Blood* 35:829-837, 1970
25. Puutula L, Gräsbeck R: Personal communication, 1969
26. Lawrence C: The heterogeneity of the high molecular weight B-12 binder in serum. *Blood* 33:899-908, 1969
27. Hall CA, Finkler AE: Isolation and evaluation of the various B-12 binding proteins in human plasma, in *Methods in Enzymology: Vitamins and Coenzymes*, vol. 18C, edited by McCormick DB, Wright LD. New York, Academic Press, Inc., Publishers, in press, 1971
28. Hall CA: Transport of vitamin B-12 in man. *Brit J Haemat* 16:429-433- 1969

Acta Med Scand. 175: 469-471 1104

Acta Medica Scandinavica. Vol. 175, fasc. 4, 1964

From the Laakso Hospital (Head: K. Kivikanervo, M. D.) and the Second Medical Department (Head: Ilmari Vartiainen, M. D.), University of Helsinki, Helsinki, Finland

Malabsorption of Vitamin B_{12} During Treatment with Para-aminosalicylic Acid

A Preliminary Report

By

O. Heinivaara and I. P. Palva

Para-aminosalicylic acid (PAS) is known to have caused e. g. such haematological complications as haemolytic or aplastic anaemia, leukopenia, agranulocytosis and thrombocytopenia (5). We have not found in the literature, however, any report of PAS-induced megaloblastic anaemia. We recently established two cases of this type of anaemia which had developed during PAS therapy. These were not cases of Addisonian pernicious anaemia as the intrinsic factor (IF) did not correct the absorption deficiency of vitamin B_{12} demonstrated by the Schilling test. On the contrary, the Schilling test values after the discontinuance of PAS, even without IF, were normal. Because of the likelihood that PAS might in these cases have had a rôle in the origin of megaloblastic anaemia, we decided to study the effect of PAS on the absorption of vitamin B_{12}.

Material and methods

Our series consisted of 10 patients taking PAS for pulmonary tuberculosis. The Schilling test was performed on a number of the patients before the inception of the PAS regimen and again after some weeks of the therapy. The tests were done in the reverse order on the rest of the patients: initially during long-term PAS therapy and a second time a few weeks after the termination of therapy. The patients received other antituberculous drugs concurrently, but the only medicine added or withdrawn during the test period was PAS.

The Schilling test was performed according to Schilling (4), using 0.7 μg of vitamin B_{12} ^{60}Co (Abbott lab.). In the Schilling II test, one capsule of purified IF preparation (Abbott lab.) was used as the additive.

Results

Table I shows the Schilling values of all the patients during PAS therapy and without PAS, and the Schilling II values during PAS for 5 patients. The Schilling value without PAS was at least 5 times the value obtained during PAS therapy in 7 cases and the difference was 2—3-fold in the other 3 cases. Although the values were not distinctly pathological (under 5 %) during PAS therapy in all the cases, the trend of the changes was the same in

Submitted for publication September 26, 1963.

488

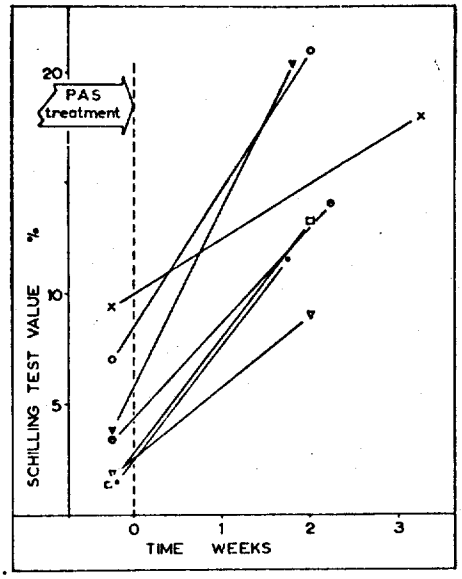


Fig. 1. Change of Schilling test values after the cessation of PAS therapy in 7 cases.

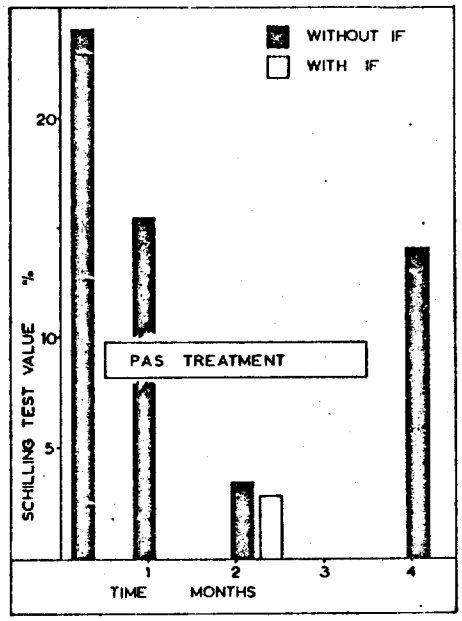


Fig. 2. Schilling test values of the patient tested prior to, during and after PAS therapy.

Table I. Schilling-test values without PAS treatment and during it

Case	Without PAS treatment	During PAS treatment	
		Without IF	With IF
1	19.8	4.8	—
2	9.0	1.8	—
3	21.0	7.0	—
4	18.0	9.4	—
5	11.5	1.5	—
6	20.4	3.8	5.6
7	5.8	2.5	4.8
8	20.0	11.5	8.6
9	24.0	3.4	2.8
10	13.2	1.4	6.6

each case: lowered values during PAS medication. The addition of IF did not normalise the Schilling values.

Fig. 1 shows the cases in which the test was made during therapy and repeated after the termination of PAS. Fig. 2 shows, finally, a case in which it was possible to perform the Schilling test before the institution of PAS therapy, and during and after its termination. The figure shows that administration of PAS for roughly a month and a half sufficed to lower the Schilling value to a pathological level but that the change was reversible and the Schilling value returned to normal about 2 weeks after the termination of PAS.

Discussion

The method used, the Schilling test, naturally does not measure the absorption of vitamin B_{12} from the intestine directly. The decreased urinary excretion of radioactive cyanocobalamin may indicate a change in the absorption, storage or renal excretion of vitamin B_{12}. However, if the reason for the lowered

Schilling values during PAS therapy were a change in storage or in renal excretion, greater quantities of vitamin B_{12} than usual should be retained in the organism. Our 2 cases of megaloblastic anaemia which developed during PAS therapy argue, however, for a vitamin B_{12} deficiency in the organism. We therefore regard it as justifiable to interpret the lowered Schilling values during PAS therapy as due to impairment of absorption caused expressly by PAS.

Although PAS is known to cause gastric distress very frequently, the B_{12} absorption disturbance demonstrated can hardly be due to a possible PAS-induced gastritis and reduced secretion of IF since the addition of IF did not normalise the Schilling values. Some calcium-chelating agents may inhibit the absorption of vitamin B_{12} (1, 2), but since PAS does not as far as is known exert any such calcium-chelating effect, even this cannot explain the lowered absorption of vitamin B_{12}. We would imagine that the question concerns an absorption disturbance in the area of the small intestine. More detailed clarification of its character calls, however, for further studies.

Although the preliminary series reviewed here is small, lowered absorption of vitamin B_{12} during PAS therapy was demonstrated in every patient. However, in spite of the extensive use of PAS, megaloblastic anaemia is by no means a common complication of PAS therapy. This is partly because of the fairly considerable vitamin B_{12} stores of the organism; it takes a long time for the deficient supply of vitamin B_{12} due to PAS to be manifested as megaloblastic anaemia. Secondly, although long-term use of PAS is practised in the treatment of tuberculosis, its side effects, gastric distress and nausea, make its ingestion unpleasant for many and there may consequently be interruptions in the therapy. According to studies made (3), as many as half of the out-patients neglect to take the drug regularly. The third reason for the uncommon occurrence of megaloblastic anaemia may be that the patients are often given various vitamin preparations during PAS therapy which nowadays frequently contain vitamin B_{12} as well. As the daily diet thus contains unusually large quantities of it, it is possible that the organism can, despite impaired absorption, obtain the minimum requirement of vitamin B_{12} and thus evade manifest anaemia.

Summary

After observing two cases of megaloblastic anaemia which developed during PAS therapy, the authors studied the effect of PAS on the Schilling test values. The Schilling value was lowered during the PAS regimen in all the 10 cases examined. The difference was at least 5-fold in 7 cases. As IF failed to normalise the urinary excretion of radiovitamin B_{12}, it was considered possible that malabsorption of vitamin B_{12} in the area of the small intestine was involved. Its detailed clarification requires further studies.

References

1. Gräsbeck, R. & Nyberg, W.: Scand. J. clin. Lab. Invest. *10:* 448, 1958.
2. Herbert, V.: The megaloblastic anemias. Grune & Stratton, New York 1959, p. 35.
3. Riebe, J.: Acta tuberc. scand. *39:* 25, 1960.
4. Schilling, R. F.: J. Lab. clin. Med. *42:* 860, 1953.
5. Stich, W.: In Klinik und Therapie der Nebenwirkungen. Ed. by H. P. Kuemmerle et al. Thieme, Stuttgart 1960.

Am. J. Clin. Nutrition 5 (1957)

Vitamin B_{12} Absorption in Pregnancy and in the Newborn

By ANDRE HELLEGERS, M.D., KUNIO OKUDA, M.D., ROBERT E. L. NESBITT, JR., M.D., DAVID W. SMITH, M.D., AND BACON F. CHOW, PH.D.

INCREASING interest is being shown in the role of vitamin B_{12} in pregnancy and in its transfer from mother to fetus. In a previous communication,[1] it was reported that a considerable quantity of radioactivity is transferred to the fetus when pregnant rats are injected with vitamin B_{12} labeled with Cobalt^{60}. Later it was demonstrated that serum vitamin B_{12} levels in the fetus at the time of delivery, measured from the umbilical cord blood, are invariably higher than those of the mother obtained at the same time.[2] This apparently "parasitic" action of the fetus in relation to its mother's vitamin B_{12} content has led us to study whether the pregnant woman, or rat, possesses a mechanism of compensation against excessive depletion of her vitamin B_{12} stores. The results of such a study, and some additional incidental findings regarding methods of studying this problem, are given in this report.

DETERMINATION OF THE ABSORPTION OF VITAMIN B_{12} BY PREGNANT AND NON-PREGNANT WOMEN

In view of a general, and understandable hesitancy on the part of obstetricians to ask mothers to allow their unborn or newborn infants to be subjected to the effects of radioactivity, however safe this may be, the following method of studying absorption was used:

Two groups of women, one pregnant and one non-pregnant group, were subdivided into three subgroups. The non-pregnant group consisted of women of childbearing age and of comparable background to the pregnant group. All women in subgroup A were given an oral dose of 1,000 μg of vitamin B_{12} while subgroup B and C received 500 μg and 250 μg respectively. Serum vitamin B_{12} levels were obtained on all subjects prior to vitamin B_{12} administration and one and one-half and three hours after the administration.

The resultant serum vitamin B_{12} levels are shown in Table I. An increase in serum vitamin B_{12} level of more than 160 μg is taken as a positive response, since this order of magnitude is beyond the error of our routine assay for this vitamin.

ABSORPTION OF VITAMIN B_{12} IN PREGNANT AND NON-PREGNANT RATS

Determination of Serum Level: In two separate studies, conducted three months apart, 26 adult female rats (three to four months old) were used. In each study the animals were bled by cardiac puncture, and 1.5 ml of blood was obtained for vitamin B_{12} assay according to the procedure described by Okuda[2,3]. The rats were randomly divided into two groups, A and B. Animals in group B were mated with adult male rats, while animals in group A served as controls. In both studies all mated animals became pregnant within four weeks. Shortly (one or two days) prior to delivery, rats in both groups A and B were again bled for B_{12} assay. The results of this study are given in Table II.

Determination of the Absorption of Vitamin B_{12} by Pregnant and Non-Pregnant Rats: The absorption of vitamin B_{12} by pregnant and non-pregnant rats was measured by feeding a measured amount (50 $\mu\mu g$) of radioactive vitamin B_{12} orally with a specific activity of 1,000 microcuries per mg. Two hours after feeding, the animals were injected with 50 $\mu\mu g$ of unlabeled vitamin B_{12}. All animals, housed individually in separate cages, were given a basal casein diet.[4] Urine and feces were collected separately for eight days at

From the Departments of Obstetrics and Pediatrics of the Johns Hopkins Hospital and University, and the Department of Biochemistry of the School of Hygiene of the Johns Hopkins University, Baltimore, Maryland.

two-day intervals. The details for the determination of radioactivity in the excreta are described in our previous communications.[5,6] The results of two studies performed at least three months apart are given in Table III. Half of the animals in each of the two study groups were mated and became pregnant. About ten days before delivery both pregnant and control rats were given 50 mmcg of vitamin B_{12} by mouth. The radioactivity in the urine and feces was determined by scintillation counting. The differences between the radioactivity of orally administered vitamin B_{12} and the fecal radioactivity is taken as a measure of the absorbed vitamin. The radioactivity in the fetus was also determined after digesting the tissues with 30 per cent KOH.

Determination of Radioactivity in Maternal Liver and Kidney and of the Fetus following Subcutaneous Administration of Radioactive Vitamin B_{12}: Twenty-four female adult rats (three to four months old) were used in two separate studies conducted four months apart. Half of the animals in each study were mated with adult males and the other half served as controls. Two or three days before delivery both pregnant and non-pregnant animals were injected subcutaneously with 10 $\mu\mu g$ of

TABLE I

Response of Pregnant and Non-pregnant Women to Oral Tolerance Test for Vitamin B_{12}

Dosage in μg	Pregnant		Non-pregnant	
	Av. change in μg	Response, %	Av. change in μg	Response, %
250	15.5 (10)*	0	47 (10)	0
500	198.3 (13)	41.5	128 (12)	16.6
1,000	262.8 (9)	88.8	157.5 (7)	28.5

* Numbers in parenthesis denote the number of subjects used.

TABLE II

Plasma Vitamin B_{12} Levels of Pregnant and Control Rats

Study	Group	No. of rats	Serum B_{12} content in $\mu\mu g/ml \pm$ S.E.M.*		
			Before mating	Before delivery	6 weeks later
I	A	6 (control)	1.2 ± 0.06	—	1.1 ± 0.1
	B	8 (mated)	1.2 ± 0.12	0.66 ± 0.04	—
II	A	6 (control)	1.15 ± 0.09	—	1.1 ± 0.08
	B	6 (mated)	1.2 ± 0.14	0.6 ± 0.02	—

* S.E.M. Standard error of the mean.

TABLE III

Absorption of Orally Administered Vitamin B_{12} to Pregnant and Non-pregnant Rats

Study	No. of rats	Vitamin B_{12} activity in*			
		Urine	Feces	Absorbed B_{12}	B_{12} in fetus
I	8 (pregnant)	0.6 ± 0.05	10 ± 1.2	40	24 ± 3.6
	8 (control)	0.4 ± 0.12	29 ± 3.6	21	—
II	6 (pregnant)	0.5 ± 0.10	9.6 ± 0.8	40.4	26 ± 2.8
	6 (control)	0.35 ± 0.15	31 ± 2.9	19	—

* Expressed in $\mu\mu g$ of vitamin B_{12}.

radioactive vitamin B_{12} and were sacrificed four days later. The livers and kidneys of the mothers and of the non-pregnant rats were assayed for their radioactivity contents, as were the offspring in their entirety. The results are shown in Table IV.

RESULTS

Pregnant women who were fed a dose of 1,000 μg of vitamin B_{12} showed a statistically significant increase in absorption of the vitamin as compared to non-pregnant women. An increase in serum vitamin B_{12} of 160 $\mu\mu g$ per ml or more was found in eight out of nine pregnant women, as compared with only two out of seven non-pregnant women. When the test dose was reduced to 500 μg the difference was not statistically significant, although it is suggestive of the greater absorption by the pregnant subjects. When the dosage was reduced further to 250 μg no significant increase in the serum level was observed in either group. When the results of the two higher dosages are taken together, the data again show that in pregnant women there is statistically significant greater absorption of vitamin B_{12}.*

This phenomenon was supported by results of experiments with rats. Thus, data tabulated in Table II clearly indicate that the vitamin B_{12} serum level decreased to almost one-half of its original value in the course of pregnancy. This result is clearly shown by comparing the serum vitamin B_{12} level of the rats with either the mean value of the same rats before mating or with the mean value of the non-pregnant controls. The serum vitamin B_{12} level of the control animals would suggest that the relatively lower levels during pregnancy are not the result of the process of bleeding.

Results in Tables III and IV indicate that the absorption of orally administered radioactive vitamin B_{12} is greater in the pregnant than in the non-pregnant rats. The increase in the absorption is approximately twofold (from approximately 20 $\mu\mu g$ to 40 $\mu\mu g$). It is interesting to note also that 60 per cent or more of the absorbed vitamin was concentrated in the fetus. The extraordinary ability of the fetus to draw vitamin B_{12} is again demonstrated in the experiment in which both pregnant and control rats were given 10 $\mu\mu g$ of Co^{60}-labeled B_{12} by the subcutaneous route. About 60 per cent of the injected vitamin B_{12} was concentrated in the fetus. More surprising, however, is the fact that the target organs of the pregnant animals contained much less radioactivity than those of the controls. These results, taken as a whole, indicate clearly that in pregnancy the absorbed vitamin B_{12} is concentrated in the fetus.

TABLE IV

Comparison of the Fate of Vitamin B_{12} Injected into Pregnant and Non-pregnant Rats

Study	No. of rats	Vitamin B_{12} content expressed as $\mu\mu g$ in			
		Liver	Kidneys	Fetus	$\mu\mu g$/fetus
A	6 (pregnant)	0.32 ± 0.06	0.17 ± 0.04	6.2 ± 1.0	0.6 ± 0.2
	6 (control)	1.3 ± 0.12	0.8 ± 0.10	—	—
B	6 (pregnant)	0.75 ± 0.17	0.28 ± 0.09	5.96 ± 0.8	0.46 ± 0.04
	6 (control)	1.50 ± 0.08	0.86 ± 0.10	—	—

Possible Interrelationship between Thyroid Activity and Vitamin B_{12} Metabolism: From what has been stated before, the fetal capacity for obtaining vitamin B_{12} from the available maternal supply is remarkable. It has been previously reported by the authors[2] that in spite of very low maternal serum vitamin B_{12} levels, the fetus could still show adequate or high serum levels. At that time allusion was made to the fact that the fetus of Case V,[2] in which neither the mother nor the fetus showed an appreciable serum level of vitamin B_{12}, had been admitted to the Pediatric Service of the Johns Hopkins Hospital with a classic picture of cretinism. An initial speculation as to a possible relationship between a depleted fetal vitamin B_{12} level and hypothryroid-

* X^2 = 4.33; significant X^2 = 3.8 (X^2 = chi sq)

ism was made at that time. Since then, a second case of athyroid cretinism has been admitted to the Johns Hopkins Hospital, in which the same fetal phenomenon was observed.

As a result of the findings in these cretins, studies were carried out in the Harriet Lane Home for Children which show that the administration of thyroid extract to cretins in infancy will rapidly return the vitamin B_{12} levels to normal.

At birth the second infant had no appreciable amount of vitamin B_{12} in the serum, while none was detected in the mother's blood at delivery. Six weeks postpartum, the mother's serum vitamin B_{12} level had returned to normal without thyroid or vitamin B_{12} therapy. The infant's serum vitamin B_{12} level did not return to normal levels until after thyroid therapy had been initiated, the rise was rapid thereafter.

At the time of the initial diagnosis of cretinism (seven weeks after birth) the infant had a protein-bound iodine of 0.6 mg per 100 ml, no uptake of radio-iodine over the lower neck region, and a bone age revealing retardation to a seven months' intrauterine fetal level with epiphyseal dysgenesis of the talus of the foot. This latter finding indicates that this infant had been hypothyroid *in utero*. The second infant's radio-iodine uptake at seven weeks was also zero. Again the rapid rise in the serum vitamin B_{12} level was noticed after initiation of thyroid therapy. Unfortunately there was no maternal vitamin B_{12} serum level available in this case.

Since in our previous report[2] the occurrence of very low maternal serum vitamin B_{12} levels is reported in the presence of normal or high fetal serum levels, and since the low fetal serum vitamin B_{12} levels were only obtained in those cases which clinically showed evidence of cretinism, we suggest that the fetal serum vitamin B_{12} level, rather than the maternal, is a significant finding in cretinism. The results of these studies are reported in Table V.

Because the incidence of cretinism is low, and because there is an even smaller likelihood of obtaining maternal and fetal serum vitamin B_{12} levels at the time of delivery of cretins except by the routine assay of these levels on thousands of cases, we have considered it worthwhile to report our findings on the aforementioned two cases in the hope of stimulating similar studies in other medical centers.

TABLE V

Thyroid Activity and Vitamin B_{12} Serum Level in Cretinism

	Date	Age	P.B.I.*	Clinical picture	Thyroid therapy	B_{12} serum level†
Patient D. W.	8/12/55	Birth	—	—	0	0
	10/7/55	7 weeks	0.6	Severe hypothyroid	Started on 1/4 g/day	0
	11/22/55	3 1/2 months	4.7	Euthyroid	3/4 g/day	157
	12/7/55	4 months	4.9	Euthyroid	3/4 g/day	222
	1/6/56	5 months	—	Euthyroid	3/4 g/day	325
Patient T. F.	3/28/56	7 weeks	2.0	Moderately hypothyroid	Started on 1/2 g daily	<50
	4/11/56	9 weeks	3.7	Slightly hypothyroid	3/4 g/day	303
	6/16/56	4 months	6.2	Euthyroid	1 g/day	—

* Protein-Bound Iodine.
† Expressed in $\mu\mu g$.

SUMMARY AND CONCLUSIONS

Absorption of orally administered vitamin B_{12} is significantly increased by pregnancy in the human and in the rat. As yet, pregnancy is the only known method of increasing vitamin B_{12} absorption in the adult.

In spite of increased vitamin B_{12} absorption,

the serum vitamin B_{12} level and the vitamin B_{12} content of the liver and kidney of pregnant rats actually show a decrease, presumably due to the demands of the fetus which seems to be the main beneficiary of the increased vitamin B_{12} absorption rate in pregnancy.

Oral tolerance tests can be used as a method of proving increased vitamin B_{12} absorption, provided the administered dose is large enough. The critical test dose in the experiment reported was 1,000 μg. This method of study obviates the use of radioactive materials in the pregnant state.

There is an, as yet, imperfectly understood relationship between thyroid function and vitamin B_{12} absorption in the earlier stages of life. It is suggested that abnormally low vitamin B_{12} serum levels may be found in the blood of newborns with cretinism. Administration of thyroid to newborn cretins rapidly raises the serum vitamin B_{12} to normal levels.

ACKNOWLEDGMENT

The authors wish to thank Miss Ella Rowe, R.N., Nursing Instructor and Supervisor of the Prenatal Clinic of The Johns Hopkins Hospital for her invaluable aid in this study.

REFERENCES

1. Chow, B. F., Barrows, L., and Ling, C. T.: The distribution of radioactivity in the organs of the fetus or of young rats born by mothers injected with vitamin B_{12} containing Co^{60}. *Arch. Biochem.* 34: 151, 1951.
2. Okuda, K., Hellegers, A., and Chow, B. F.: Vitamin B_{12} in pregnancy. Am. J. Clin. Nutrition (in press 1957).
3. Okuda, K., Wood, R., Lang, C. A., and Chow, B. F.: Serum levels of vitamin B_{12} in man. *Fed. Proc.* 13: 1546, 1954.
4. Ling, C. T., and Chow, B. F.: The influence of vitamin B_{12} on carbohydrate and lipid metabolism. *J. Biol. Chem.* 206: 797, 1954.
5. Chow, B. F., and Barrows, L.: Storage and elimination of vitamin B_{12} in the rat. *J. Nutrition* 49: 669, 1953.
6. Becker, B., Lang, C. A., and Chow, B. F.: Vitamin B_{12} excretion and diabetic retinopathy. J. Clin. Nutrition 1: 417, 1953.

3rd 8-20
Hemat. Gastr. Inves. Radionuclides, Annu. Med.

Chapter 2

B_{12} AND FOLATE METABOLISM

VICTOR HERBERT

Metabolism includes not only the biochemical interrelationships of agents but also all the mechanisms involved in their absorption, transport, distribution, fate, and excretion. These phenomena will all be touched on examining B_{12} and folate metabolism (1).

Practically all that is currently known about metabolism of B_{12} and folate, and for that matter about the metabolism of iron, has been learned using radionuclides. Much research has been devoted to discovering the etiologic mechanisms whereby humans may develop deficiency of B_{12} or folate.

The clinical picture of deranged metabolism of B_{12} or folate includes identical hematologic changes and morphology in the peripheral blood. One sees hypersegmented nuclei in the polymorphonuclear leucocytes, and there are macroovalocytes; when these two phenomena are seen together there is a deranged DNA synthesis. These two phenomena result not only from lack of B_{12} or folate but also from anything else that deranges DNA synthesis. The bone marrow in megaloblastosis shows the classic nuclear-cytoplasmic dissociation, characterized by a "young" nucleus (i.e. finely divided chromatin) and by an "old" cytoplasm (i.e. containing hemoglobin). This is the morphology of deranged DNA synthesis, with the primary lesion being that the cell is trying to double its DNA in order to divide, but its proliferation is arrested in this DNA synthetic phase, usually because of lack of B_{12} or folate (1–3).

B_{12} is absorbed primarily from the ileum. The intrinsic factor dependent mechanism for B_{12} absorption occurs only in the ileum. This mechanism dominates in handling the absorption of the amounts of B_{12} made available from an average meal; it has a maximal capacity to handle a "bolus" of approximately two micrograms of B_{12}, and it is the mechanism we usually study using the Schilling test. An additional mechanism, probably diffusion, allows absorption of about 1 percent of any dose of "free" B_{12} along the entire length of the small intestine (1, 4, 5). With radioactive B_{12} one is able to study both in the patient (6, 5) and in the test tube (7) the mechanism whereby B_{12} gets across the gut. Lyophilized homogenate of gut mucosa can also be used to assay in the test tube whether or not a patient has intrinsic factor in his gastric juice and whether or not the patient has antibody to intrinsic factor in his serum (8), although coated charcoal assay is easier (9).

Figure 2–1, from reference (10), schematically depicts the first step in human absorption of B_{12}. Food B_{12} attaches to intrinsic factor made by the gastric parietal cell, and this complex moves down to the ileum, where it attaches to surface receptors on the ileum in the presence of calcium and pH above 5.5. One can measure the quantitative uptake of radioactive B_{12} by ileum homogenate (5,7,8).

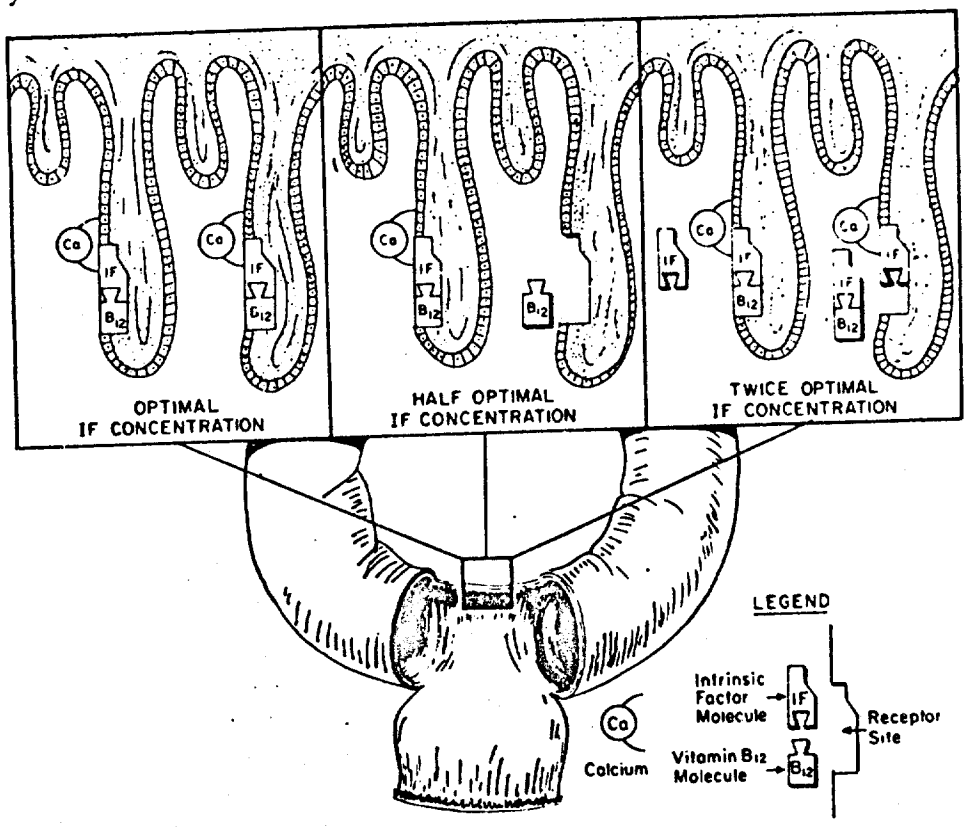


Figure 2–1. Schematic depiction of the first step in human absorption of B_{12}.

Lyophilized homogenate of guinea pig gut mucosa has set on our laboratory shelf for almost a decade without deteriorating. One can take a pinch of it, add a tenth of a cubic centimeter of the patient's gastric juice, add a drop of radioactive B12, shake it up, and spin it down. If the gastric juice contains intrinsic factor, it will attach to the radioactive B_{12} in the solution in the test tube, and the complex will attach itself to the particles of lyophilized gut homogenate. When centrifuged, the particles spin down, taking the radioactivity with them. If the gastric juice is devoid of intrinsic factor, this process will not occur and the radioactivity will stay in the supernate. This method is a rapid, simple test for the presence of intrinsic factor in gastric juice (8).

In Figure 2–2, from reference (11), one sees the mechanism for the uptake of B_{12} by reticulocytes; uptake is even greater into bone marrow cells, by the same mechanism. Note how closely similar it is to the mechanism of uptake of B_{12} by the ileum. Again the vitamin is picked up on a transport glycoprotein. In the case of the gut that glycoprotein is gastric intrinsic factor. In the case of bone marrow cells and reticulocytes that glycoprotein is serum B_{12} binding protein. These glycoproteins are different materials of different molecular weights. The serum B_{12} binding protein delivers its load of B_{12} on the surface of the cell only in the presence of ionic calcium and an appropriate pH (11).

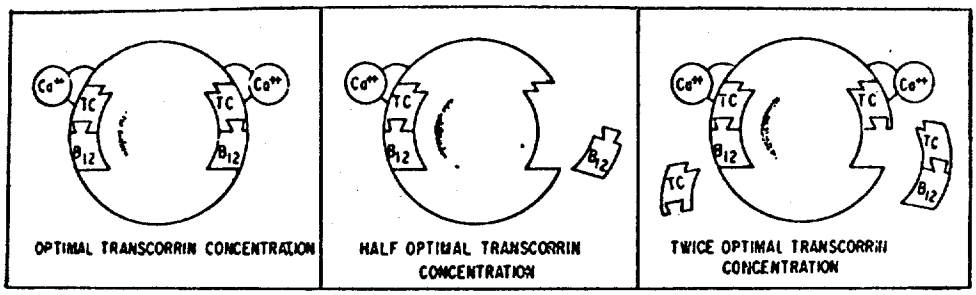


Figure 2–2. Mechanism for uptake of B_{12} by reticulocytes. Schematic depiction of calcium-dependent binding of B_{12}-transcorrin (TC) complexes to reticulocyte receptors.

In studying the mechanism of delivery of B_{12} inevitably one studies th nature of serum B_{12} binding protein. A significant portion of this carri protein for vitamin B_{12} is made in living neutrophils and eosinophils (1 13). One is able to measure reasonably accurately the total blo granulocyte pool simply by determining the serum B_{12} binding prot level. Serum B_{12} binding protein probably measures body granulocyte m in a manner similar to measurement of body myeloma cell mass by m suring myeloma protein (14). Thus, one can use the measurement of B_{12} binding protein level to help in the differential diagnosis of true false malignancy of the blood of various types (1, 15, 16).

Figure 2–3, using data from reference (15), illustrates how mea serum B_{12} binding capacity has been brought to bear on sepa genuine from false polycythemia, including the relative polycy described some years ago by Dr. Lawrence. In normal individu serum B_{12} binding capacity averages about 1800 picograms per r of serum. Thirty-one patients with spurious polycythemia of vario had an average serum B_{12} binding capacity which was normal. I twenty-one patients with true polycythemia, active and untrea an average serum B_{12} binding capacity twice normal. One is able to separate most patients in the category of true polycyth those in the category of spurious polycythemia simply by mea

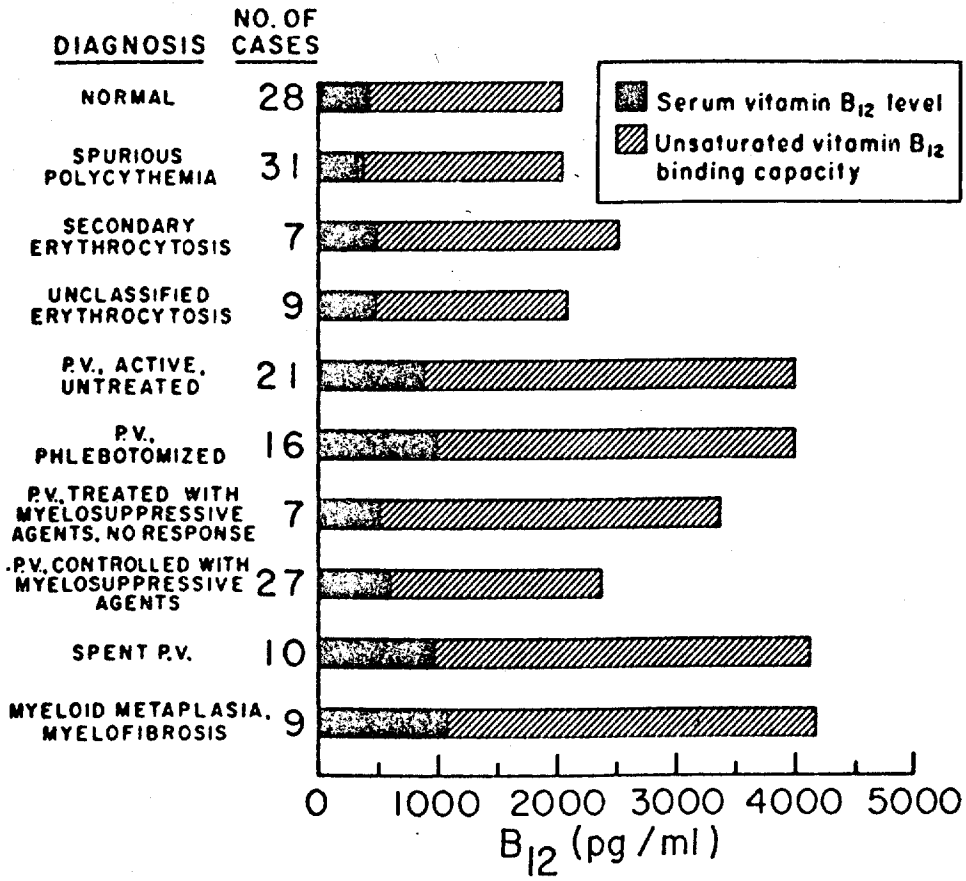


Figure 2–3. Use of serum B_{12} binding capacity in separating genuine from false polycythemia.

serum B_{12} binding capacity. This method probably works because the blood leucocyte pool is increased in polycythemia regardless of whether or not the peripheral white count is increased (15, 17). It has been demonstrated by a number of workers that the peripheral white count may have no relation to the total blood granulocyte pool (17). That is, the patient may have a white cell malignancy or increase in white cell mass for one reason or another, which is not manifested by a high white count in the peripheral blood. Conversely, the patient may have an elevated white count in the peripheral blood and a normal or even a low blood granulocyte pool, as one sees after certain anesthetics which cause release of the marginated white cell pool. (Normally about one half of the white cells are attached ["marginated"] to the blood vessel walls and are not measured when one does a white count) (18). Thus, measurement of serum B_{12} binding capacity distinguishes as normal both false low and false high peripheral white counts, since it allows one to measure the

total blood granulocyte pool by measuring unsaturated B_{12} binding capacity, which is made by circulating living granulocytes and eosinophils (12, 13).

Measurement of serum B_{12} binding capacity has also been of some value in separating leukopenias of different varieties. If the patient has an acute leukopenia, B_{12} binding capacity tends to be normal, because the white cell pool has been normal until very recently. If, on the other hand, the patient has had a chronic leukopenia, the B_{12} binding capacity tends to be low. We use this method in children with leukopenia, to help determine if this is acute leukopenia in association with viral illness, or a serious disease in which chronic leukopenia is a feature. If the B_{12} binding capacity is found to be normal, it is evidence that the leukopenia is probably acute, probably associated with a recent viral infection, and probably will return to normal in short order. It usually does. On the other hand, if the serum B_{12} binding capacity is low, it suggests that the child probably has a serious disorder of white cells with a chronic leukopenia. The test is of ancillary rather than primary value. We have seen patients with normal peripheral white count and differential count, and normal serum B_{12} binding capacity, who actually had aleukemic leukemia diagnosable by a bone marrow full of myeloblasts (which make very little B_{12} binding capacity compared to myelocytes and later stages of granulocyte maturation.

Measurement of serum unsaturated B_{12} binding capacity (UBBC) is simple (9). One takes one-half cubic centimeter of the patient's serum, adds a drop of radioactive B_{12}, shakes it up, lets it sit for a few minutes at room temperature, adds some coated charcoal, shakes it up, spins it down, and counts the radioactivity in the supernate. The radioactivity in the supernate measures the B_{12} binding protein level of the patient. It is an exceptionally simple test of considerable clinical value, and several commercial laboratories now advertise its availability in the *New England Journal of Medicine.*

The active center of the vitamin B_{12} molecule is in the middle of the corrin nucleus, which is almost identical to the porphin nucleus in heme. There are only a few minor differences between the B_{12} corrin nucleus and the porphin nucleus in heme of which the most striking is that the ligand in the center of corrin is cobalt instead of iron (1).

Figure 2–4, from reference (19) summarizes current knowledge of ingestion, absorption, transport, delivery and excretion of B_{12}. Practically all of this information was obtained using radionuclides. After delivery into the cell, B12 is reduced and converted to its two metabolically active forms in man, coenzyme B_{12} and methyl B_{12}. The pharmaceutical preparation, cyanocobalamin, which we inject into our patients to treat their

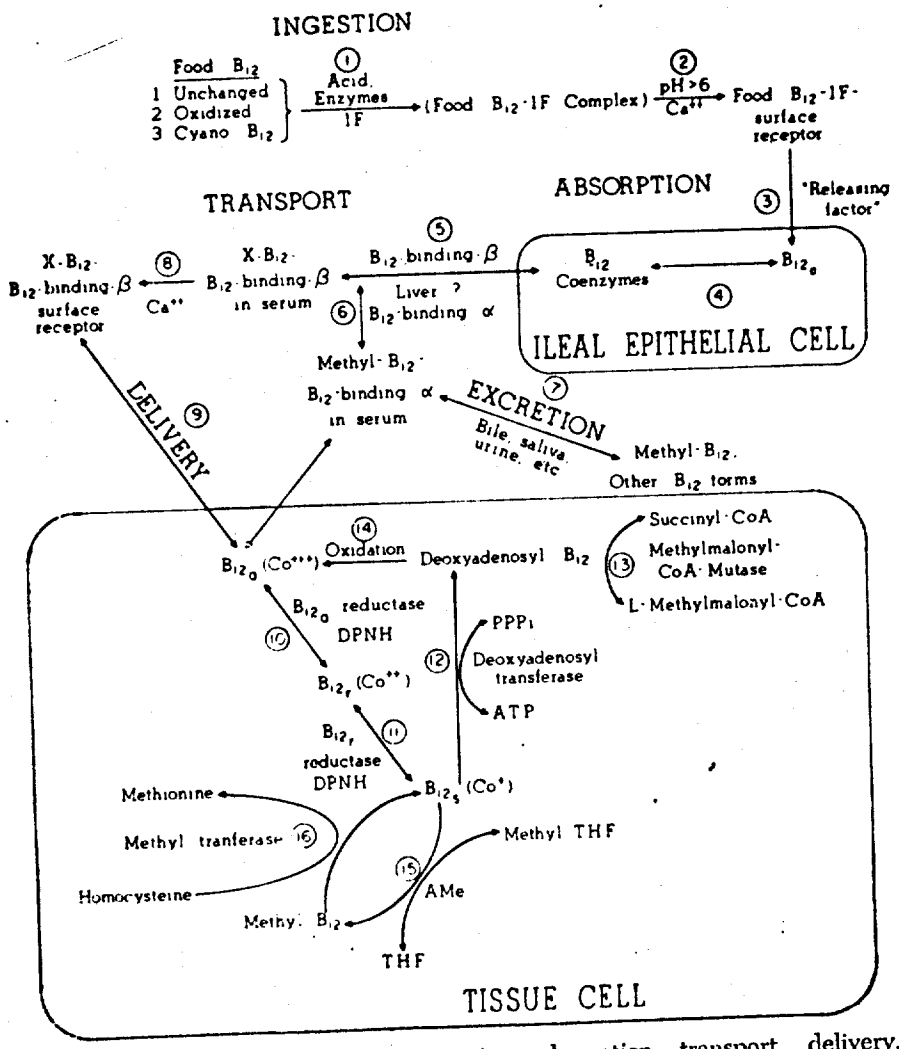


Figure 2–4. Current knowledge of ingestion, absorption, transport, delivery, and excretion of B_{12}.

B_{12} deficiencies is not known to be metabolically active. In the patient's body its cyanide is eliminated and replaced by either a methyl group or a 5′ deoxyadenosyl group, which make the B_{12} metabolically active in human biochemical processes.

B_{12} biochemically has several functions: as a reducing agent; in certain isomerization reactions in which the two end carbons of a long carbon skeleton are picked off and redeposited further along the carbon skeleton; and in folate metabolism (1, 19).

Folate absorption by humans is beginning to be as thoroughly studied as B_{12} absorption (20, 21). Again, research is dependent on the use of radionuclides of folic acid. Most studies have been done with pteroyl-

glutamic acid, which is a stable pharmaceutical preparation, but metabolically dead. In order to be active in human metabolism, it must be reduced. When reduced to tetrahydrofolate, it functions metabolically to pick up one-carbon units from wherever they are not needed and delivers them to wherever they are needed.

Figure 2–5 illustrates a study (22) of folate absorption using tritiated PGA and a triple lumen tube (23). In normal individuals about 36 percent of this particular dose of tritiated folic acid is absorbed when fed through a triple lumen tube into the upper third of the small bowel. If that dose of folic acid is fed through the tube with Dilantin,® its absorption is cut

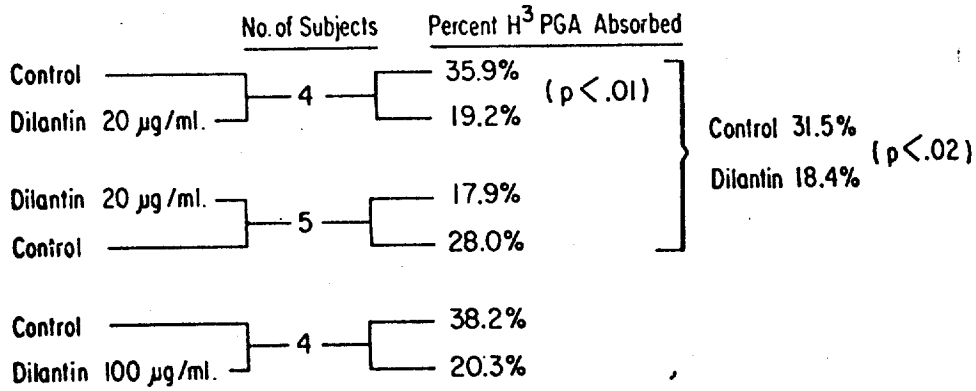


Figure 2–5. Study of folate absorption demonstrating effect of Dilantin.

in half. This demonstrates that the anticonvulsant Dilantin reduces ability to absorb folic acid. This may help explain why patients on the anticonvulsant Dilantin have a higher incidence of folic acid deficiency than people not taking this anticonvulsant. The other two groups of patients in Figure 2–4 illustrate the same phenomenon at different dosage levels.

The same modalities of study applied to B_{12} transport were applied to folate transport, as shown in Figure 2–6 from reference (24). A weak binder for folate has been found (25), with affinity for folate much weaker than the affinity for B_{12} of serum transport protein or the affinity for iron of serum transport protein. Folate is delivered to bone marrow cells and to reticulocytes in a fashion dependent on metabolic activity (24). Delivery of tritiated folic acid to human bone marrow cells in the test tube is practically nil at 4 degrees centigrade but proceeds increasingly with increasing time at 37 degrees centigrade (body temperature).

In the folic acid molecule the active center is between the N5 and N10 position.

The interrelationship of B_{12} and folate in human metabolism can be found in the conversion of homocysteine to methionine (which is methyl homocysteine) (19). This conversion is brought about by B_{12} picking up

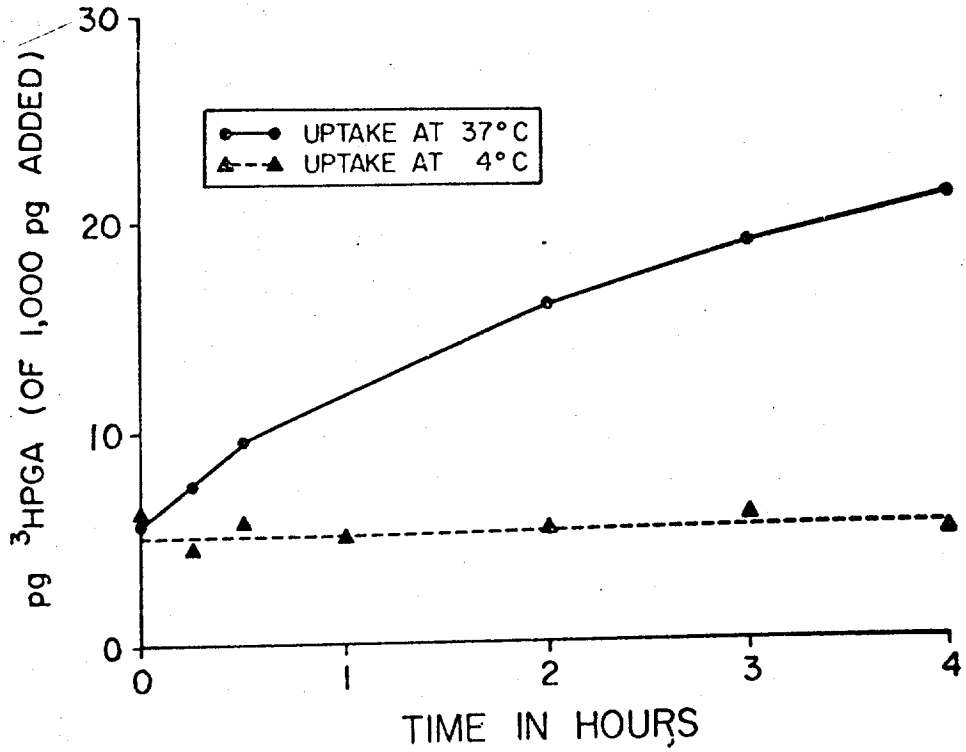


Figure 2–6. Uptake of ³HPGA by bone marrow cells *in vitro*.

a one-carbon unit from methyl tetrahydrofolate and delivering it to homocysteine. Methyl tetrahydrofolate is the dominant folate in the human body. It constitutes two-thirds or more of the circulating folate in the blood stream, and an even greater percentage of the folate in the liver and in other tissues. When a patient is deficient in B_{12}, he is unable to use this dominant form of folate in his serum. Thus, the B_{12}-deficient patient is functionally deficient in folate even if he is eating and absorbing enough folate (1, 19, 26).

The methyl folate, which is the dominant form of folate in the human body, is converted back to tetrahydrofolate (so that it can pick up another one-carbon unit) via the B_{12}-dependent pathway, with B_{12} acting as a coenzyme attached to its apoenzyme, becoming a holoenzyme which picks the methyl unit off methyl tetrahydrofolate and becomes methyl B_{12}. Then this methyl B_{12} delivers its methyl unit to homocysteine, making methyl homocysteine (methionine). Our group has been studying the intertwined relationships of B_6 metabolism with folate and B_{12} metabolism, using radioactive homocysteine, radioactive methionine, radioactive glycine, radioactive serine, and radioactive B_6. We are especially studying patients with congenital homocystinuria in association with Dr. Gaull (3).

Figure 2–7 depicts the crucial interrelationship of B_{12} and folate metabolism focused down at the point of DNA synthesis. When one adds a methyl group to deoxyuridylate, the result is methyl deoxyuridylate (thymidylate), i.e. the product immediately before DNA.

To put this one-carbon unit on deoxyuridylate one needs a form of folate, which is the mediator of most one-carbon transfers in the human body. The form of folate needed is 5,10 methylene. In order to get to that form of folate from the circulating form, B_{12} is required. This explains why

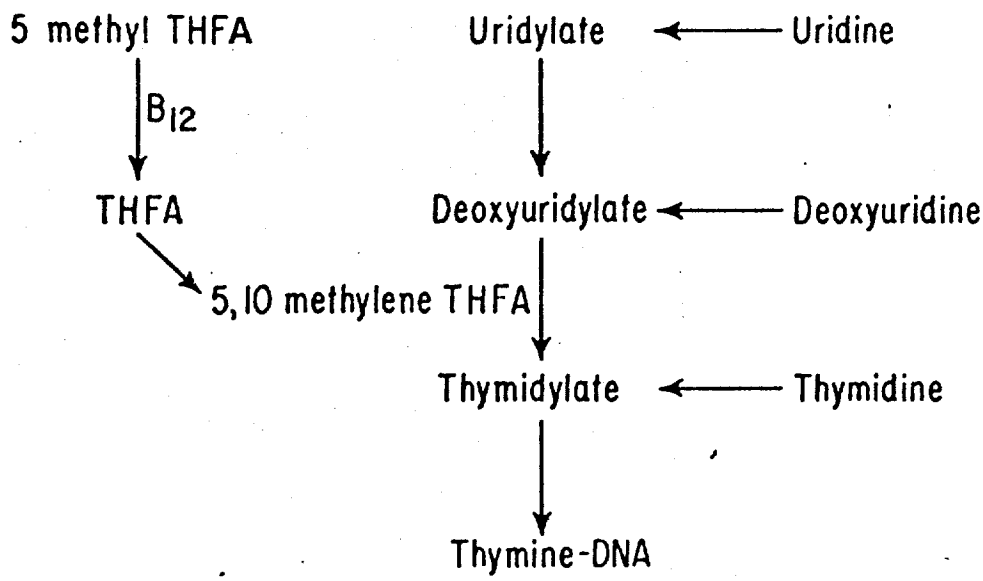


Figure 2–7. Interrelationship of B_{12} and folate metabolism in DNA synthesis.

the patient has exactly the same hematologic and morphologic picture of megaloblastic anemia, regardless of whether he is deficient in B_{12} or folic acid. If he is deficient in folic acid (e.g. if he is not eating any fresh leafy vegetables or fresh fruits), he cannot make DNA normally; therefore he gets megaloblastic anemia. On the other hand, if he lacks B_{12}, even though he may be getting enough folic acid in his diet and absorbing enough folate, it becomes converted to the 5 methyl form which is metabolically dead in the absence of B_{12}. Thus, even if he is eating and absorbing enough folate, he cannot use what he absorbs. The folate piles up as methyl folate, which is metabolically dead when B_{12} is missing; he does not have enough 5, 10 methylene tetrahydrofolate, and he cannot make DNA with normal speed. Therefore, he gets hematologic damage exactly identical to the hematologic damage derived from dietary deficiency of folate.

The making of DNA from uridine is known as the *de novo* pathway, i.e from small building blocks. The reutilization of thymidine from old de-

stroyed DNA, i.e. from old destroyed cells in the body, is called the salvage pathway.

Using this model, and using radioactive tritiated thymidine, it was possible in the test tube to study the relative speed of DNA synthesis by the salvage pathway as compared to the *de novo* pathway. It was also possible in the test tube to predict in a patient with a malignancy which of a battery of chemotherapeutic agents would be effective in suppressing that malignancy. Thus, our studies of B_{12} and folate flowed naturally into studies of malignant cells, where we were able to use the same short-term *in vitro* culture system previously applied to study of bone marrow B_{12} and folate metabolism (27, 28). The percentage of DNA synthesis via the salvage pathway was compared with that via the *de novo* pathway (29). If the malignancy is a solid tumor, with the help of the surgeon we get a piece of tissue and strain it through a tea strainer in order to break up the tumor into single cells and tiny clumps. Then we treat them *in vitro* as if they were bone marrow cells or reticulocytes, to study the effect of chemotherapeutic agents on them.

Figure 2–8 from reference (29) shows such a study. One sees the patterns of DNA synthesis in various human tumor cells of the colon, the breast, the ovary, and of the lung compared to normal. In normal in-

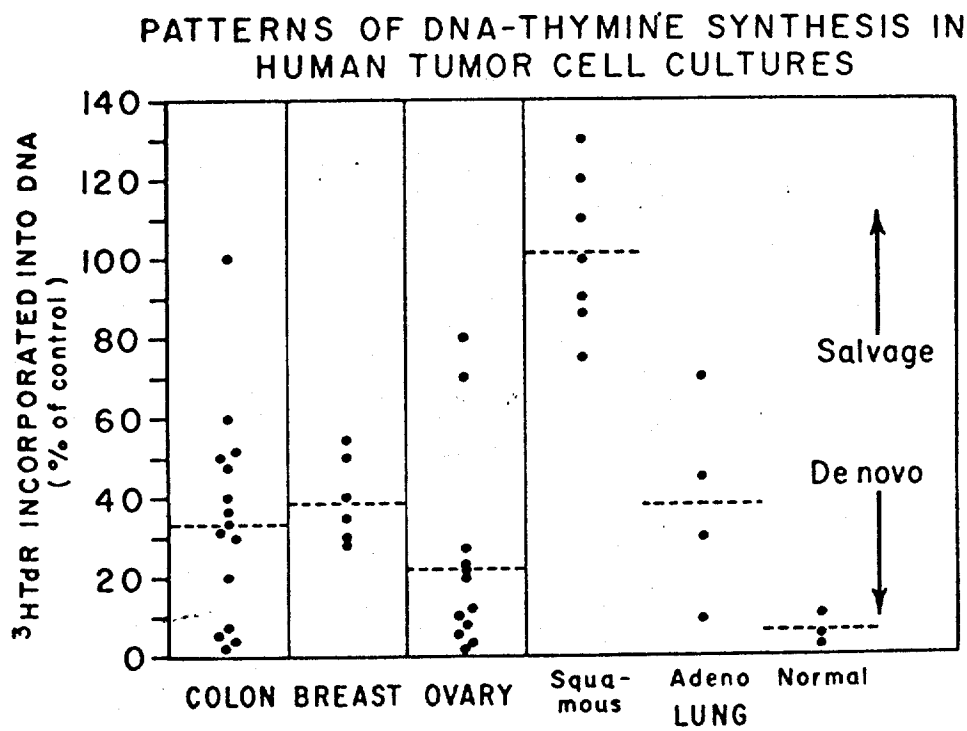


Figure 2–8. Patterns of DNA-thymine synthesis in human tumor cell cultures.

dividuals the *de novo* pathway dominates. Where the *de novo* pathway dominates, one appears able to suppress cell formation by suppressing DNA synthesis using an agent which suppresses the *de novo* pathway. A typical such agent is methotrexate, or one of the other antifols. Methotrexate damages normal cells and produces megaloblastosis in them. In the series of tumors sensitive to methotrexate, i.e., tumor of the colon, breast, ovary and adenocarcinoma of the lung, where the *de novo* pathway dominates, cell growth is suppressed with methotrexate. However, in squamous carcinoma of the lung, we could predict, were it not already known, that methotrexate would not work, because the dominant pathway for DNA synthesis appears to be salvage rather than *de novo*, and the salvage pathway is not suppressed by methotrexate. This is an exciting line of current study whereby it is hoped to predict what chemotherapeutic agent will or will not work in a given tumor and in a given patient, by studying his tumor cells *in vitro* for three hours.

In Figure 2–9 from reference (30), the same bone marrow system is applied to studying pyrimethamine. One sees confirmed that, in fact, the agent pyrimethamine is a strong enough antifol to suppress DNA synthesis in human bone marrow cells in a test tube, and therefore will produce megaloblastosis *in vivo*. Had this tool of short-term bone marrow culture been

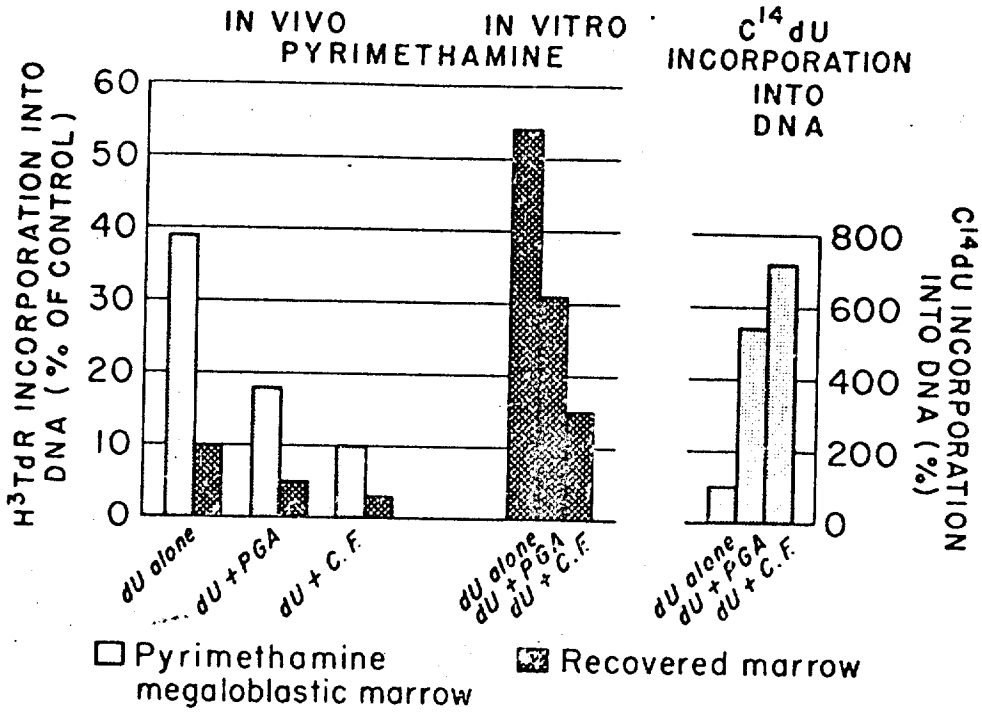


Figure 2–9. Bone marrow system applied to studying pyrimethamine.

available, say fifteen years ago, one could have predicted in the three hour incubation period, that this agent would, as in fact it does, produce megaloblastosis in humans in the doses used to treat toxoplasmosis or malaria.

By this same method one can show *in vitro* that triamterine, the diuretic, is a sufficiently strong antifol that in a patient with marginal folate stores, megaloblastosis will be produced (31).

In summary, much of the knowledge of B_{12} and folate absorption, transport, distribution, fate, and excretion has been worked out using radionuclides of these two vitamins, as well as radionuclides of the various substrates on which these vitamins act in the human body. One can predict on the basis of studies so far that research in this area will have far reaching consequences in other areas, including the area of malignancies.

REFERENCES

1. Herbert, V.: Drugs effective in megaloblastic anemias. *In* Goodman, L.S.: and Gilman, A. (Eds.): *The Pharmacological Basis of Therapeutics,* 4th ed. New York, Macmillan, 1970, p. 1414–1444.
2. Herbert, V.: Megaloblastic anemias—mechanisms and management. *In Disease-a-month.* Chicago, Year Book Medical Publishers, August, 1965.
3. Waxman, S., Corcino, J., and Herbert, V.: Drugs, toxins and dietary amino acids affecting vitamin B_{12} or folic acid absorption or utilization. *Amer J Med, 48:*599–608, 1970.
4. Herbert, V.: Absorption of vitamin B_{12} and folic acid. *Gastroenterology, 54:*110–115, 1968.
5. Corcino, J., Waxman, S., and Herbert, V.: Absorption and malabsorption of vitamin B_{12}. *Amer J Med, 48:*562–569, 1970.
6. Schilling, R.F.: Intrinsic factor studies. II. The effect of gastric juice on the urinary excretion of radioactivity after the oral administration of radioactive vitamin B_{12}. *J Lab Clin Med, 42:*860, 1953.
7. Carmel, R., Rosenberg, A.H., Lau, K.-S., Streiff, R.R., and Herbert, V.: Vitamin B_{12} uptake by human small bowel homogenate and its enhancement by intrinsic factor. *Gastroenterology, 56:*548–555, 1969.
8. Sullivan, L.W., Herbert, V., and Castle, W.B.: *In vitro* assay for human intrinsic factor. *J Clin Invest, 42:*1443–1458, 1963.
9. Gottlieb, C., Lau, K.-S., Wasserman, L.R., and Herbert, V.: Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated B_{12} binding capacity, antibody to IF, and serum unsaturated B_{12} binding capacity. Blood, *25:*875–884, 1965.
10. Herbert, V., Streiff, R.R., and Sullivan, L.W.: Notes on vitamin B_{12} absorption; autoimmunity and childhood pernicious anemia; relation of intrinsic factor to blood group substance. *Medicine, 43:*679–687, 1964.
11. Retief, F.P., Gottlieb, C.W., and Herbert, V.: Mechanism of vitamin B_{12} uptake by erythrocytes. *J Clin Invest, 45:*1907–1915, 1966.
12. Corcino, J., Krauss, S., Waxman, S., and Herbert, V.: Release of vitamin B_{12} binding protein by human leukocytes *in vitro. J Clin Invest, 49:*2250–2255, 1970.

13. Chikkappa, G., Corcino, J., Greenberg, M., and Herbert, V.: Correlation between various blood white cell pools and the serum B_{12}-binding capacities. *Blood, 37*:142–151, 1971.
14. Fahey, J.L.: Antibodies and immunoglobulins, I and II. *JAMA, 194*:71, 255, 1965.
15. Gilbert, H.S., Krauss, S., Pasternack, B., Herbert, V., and Wasserman, L.R.: Serum vitamin B_{12} content and unsaturated vitamin B_{12} in binding capacity (UBBC) in myeloproliferative disease. Value in differential diagnosis and as parameters of disease activity. *Ann Int Med, 71*:719–729, 1969.
16. Herbert, V.: Diagnostic and prognostic values of measurement of serum vitamin B_{12}-binding proteins. *Blood, 32*:305–312, 1968.
17. Athens, J.W., Haab, O.P., Raab, S.O., Boggs, D.R., Ashenbrucker, H., Cartwright, G.E., and Wintrobe, M.M.: Leukokinetic studies. XI. Blood granulocyte kinetics in polycythemia vera, infection, and myelofibrosis. *J Clin Invest, 44*:778, 1965.
18. Boggs, D.R.: *White Cell Manual.* Dane Boggs, University of Pittsburgh, 1968.
19. Herbert, V. Recent developments in cobalamin (B_{12}) metabolism. Symposium: *The Cobalamins. In* Arnstein, H.R.V., and Wrighton, R.J., (Eds.): London J. & A. Churchill, 1971.
20. Bernstein, L.H., Gutstein, S., Weiner, S., and Efron, G.: The absorption and malabsorption of folic acid and its polyglutamates. *Amer J Med, 48*:570, 1970.
21. Butterworth, C.W., Baugh, C.M., and Krumdieck, C.: A study of folate absorption and metabolism in man utilizing carbon-14 labeled polyglutamates synthesized by the solid phase method. *J Clin Invest, 48*:1131, 1969.
22. Gerson, C.D., Hepner, G.W., Brown, N., Sohen, N., Herbert, V., and Janowitz, H.D.: Inhibition by diphenylhydantoin (Dilantin) of folic acid absorption in man. *J Clin Invest, 49*:33a, 1970 (Abstract).
23. Gerson, C.D., Cohen, N., Hepner, G.W., Brown, N., Herbert, V., and Janowitz, H.D.: Folic acid absorption in man: enhancing effect of glucose. *Gastroenterology, 54*:August, 1971.
24. Corcino, J.J., Waxman, S., and Herbert, V.: Uptake of tritiated folates by human bone marrow cells in vitro. *Brit J Haemat, 20*:503, 1971.
25. Metz, J., Zalusky, R., and Herbert, V.: Folic acid binding by serum and milk. *Am J Clin Nutr, 21*:289–297, 1968.
26. Herbert, V., and Zalusky, R.: Interrelations of vitamin B_{12} and folic acid metabolism: Folic acid clearance studies. *J Clin Invest, 41*:1263–1276, 1962.
27. Metz, J., Kelly, A., Switt, V.C.; Waxman, S., and Herbert, V.: Deranged DNA synthesis by bone marrow from vitamin B_{12}-deficient humans. *Brit J Hoemat, 14*:575–592, 1968
28. Waxman, S., Metz, J., and Herbert, V.: Defective DNA synthesis in human megaloblastic bone marrow: Effects of homocysteine and methionine. *J Clin Invest, 48*:284–289, 1969.
29. Waxman, S., Shreiber, C., and Herbert, V.: Patterns of DNA-thymine synthesis in short-term human tumor cell cultures. *Clin Res, 18*:477, 1970.
30. Waxman, S., and Herbert, V.: Mechanism of pyrimethamine-induced megaloblastosis in human bone marrow. *New Eng J Med, 280*:1316–1319, 1969.
31. Corcino, J., Waxman, S., and Herbert, V.: Mechanism of triamterene-induced megaloblastosis. *Ann Int Med, 73*:419–424, 1970.

Biol. Brain Dysfunction 1973, 1, 373-92

Chapter 10

EFFECTS OF DEFICIENCIES OF FOLIC ACID AND VITAMIN B_{12} ON CENTRAL NERVOUS SYSTEM FUNCTION AND DEVELOPMENT

Victor Herbert and Glenn Tisman
Columbia University College of Physicians and Surgeons
and
Veterans Administration Hospital
Bronx, New York

I. INTRODUCTION

From studies in lower animals, it is believed that deficiency of folic acid during the intrauterine period may lead to an infant's being born mentally retarded.[1] The use of antimetabolites to folic acid is known to produce abortion in woman and also to produce a fetus with central nervous system and other deformities.[2,3] Work by Arakawa's group[4] in Japan suggests that congenital enzyme defects in folate metabolism result in infants born with mental retardation, dilation of the cerebral ventricles, and intracerebral calcification. There is as yet no clear evidence that folate deficiency in adults produces histologically demonstrable damage to the central nervous system. However, folate deficiency in adults is associated with irritability and forgetfulness.[5] Furthermore, it has been suggested, most recently by Reynolds,[6] that anticonvulsants act in part by interfering with folate metabolism and that the supplying of folic acid increases fit frequency in patients whose convulsions would otherwise have been better controlled by anticonvulsant medication. This chapter will review what

is known about the mechanisms whereby deficiency of folic acid may produce these alleged effects.

Vitamin B_{12} is known to be required for myelin synthesis; when there is a deficiency of vitamin B_{12}, myelin deteriorates.[7–10] This deterioration results eventually in the classical "combined systems disease," caused by deterioration of the posterior and lateral columns of the spinal cord, and multiple associated neurological deficits. Lack of folic acid never causes demyelination in the adult; lack of vitamin B_{12} always eventually produces demyelination. The biochemical basis of this demyelination is unknown; this chapter will explore some possibilities arising from current knowledge of vitamin B_{12} function in man.

It is known that folate is selectively concentrated in cerebrospinal fluid[11,12] and vitamin B_{12} is selectively excluded therefrom.[13,14] From these facts, it has been speculated that the central nervous system is protected against dysfunction due to folic acid deficiency because it has a selective ability to concentrate folic acid when that vitamin is in short supply, even at the expense of other tissues which also require the vitamin.[11] It has been further speculated that, in the case of vitamin B_{12} deficiency, other tissues may be able to concentrate the vitamin at the expense of the central nervous system and that this may play a role in the frequency of neurological damage with vitamin B_{12} deficiency.[15] These speculations are not yet supported by adequate documentation.

II. BIOCHEMICAL ROLES OF COENZYME FORMS OF FOLIC ACID AND VITAMIN B_{12}

The roles of vitamin B_{12} and folic acid in human metabolism have been extensively reviewed elsewhere.[16–22] Reduced coenzyme forms of folate are required in most biochemical processes in man in which a one-carbon transfer occurs. Vitamin B_{12} coenzymes are required in folate metabolism, in methyl group transfers, in isomerase reactions, and in a number of other reactions whose biochemistry awaits elucidation. Table 1 summarizes these roles, and Fig. 1 is a flow chart of folate metabolism.

III. CENTRAL NERVOUS SYSTEM DAMAGE DUE TO FOLIC ACID DEFICIENCY

A. Developmental Damage to Fetus Related to Use by Mother of Antifolates

Antifols are known to produce abortion in animals and have been used for the same purpose by women.[2,3] They act at dihydrofolate reductase (step 7, Fig. 1). This practice is highly dangerous because the dose lethal to the fetus is close to the dose lethal to the mother. Antifols have the potential for obliterating

TABLE 1. BIOCHEMICAL ROLES IN MAN OF COENZYMES OF FOLIC ACID AND VITAMIN B_{12}

A. Folate coenzymes in one-carbon transfers
  1. Metabolism of purines, pyrimidines, and their derivatives
    a. Purine nucleotides
    b. Pyrimidine nucleotides
      (1) Thymine deoxyribonucleotide
      (2) Deoxycytidylate hydroxymethyltransferase
  2. Metabolism of amino acids
    a. Serine and glycine
    b. Phenylalanine and tyrosine
    c. Histidine
  3. Initiation of peptide chain biosynthesis
  4. Lipid metabolism
  5. Biosynthesis of methyl groups
    a. Choline
    b. Methionine
      (1) B_{12}-independent synthesis
      (2) B_{12}-dependent synthesis
B. Vitamin B_{12} coenzymes
  1. B_{12} in folate metabolism
    a. Methionine biosynthesis from homocysteine
    b. B_{12}-dependent cell uptake of folates
    c. Other?
  2. Methyl B_{12} in methyl group transfers
    a. Methionine synthesis (methyltetrahydrofolate–homocysteine methyltransferase mediated)
    b. Other methyl group transfers?
  3. 5′-Deoxyadenosyl B_{12} in isomerase reactions
    a. Propionate oxidation (methylmalonyl CoA mutase mediated)
    b. Other isomerase reactions?
  4. Other roles of B_{12}
    a. Nucleic acid synthesis
    b. Lipid metabolism
    c. Protein and amino acid metabolism
    d. As a reducing agent
    e. Other?

one-carbon-unit metabolism and therefore have the potential for destroying any proliferating tissue, including that of the central nervous system. Thus multiple malformations of the central nervous system have been found in fetuses of mothers taking antifols.

B. Developmental Damage to Newborn Related to Congenital Defects in Folate Absorption or Utilization

Profound mental retardation and punctate calcification of the basal ganglia may occur in association with congenital defects in absorption or in utilization of folate.[4,23]

1207

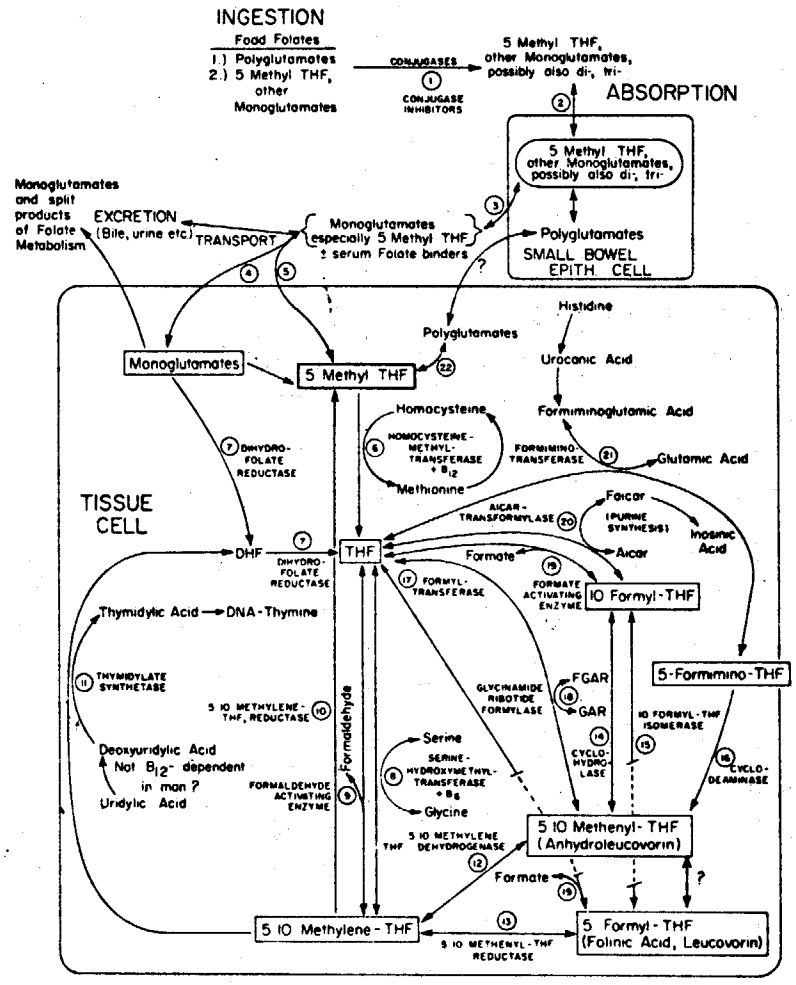


Fig. 1. Flow chart of folate metabolism in man. Circled numbers identify individual steps in folate metabolism. THF, tetrahydrofolate; DHF, dihydrofolate.

Congenital malabsorption of folate (step 2, Fig. 1), which appears to be an isolated defect in the gastrointestinal absorption of physiological amounts of folic acid, may also be accompanied by a defect in the transport of folic acid from the plasma into the cerebrospinal fluid.[23] This diagnostic possibility arises when the clinical presentation is with the combination of mental retardation, megaloblastic anemia, low serum folate level, and convulsions (which, however, may not start until the ninth year of life, or even later). The diagnosis is confirmed by demonstrating unequivocally defective absorption of folic acid. Two siblings with this disorder had ataxia[24]; a girl followed for 20 years had

athetotic movements.[23] In this latter patient, fit frequency decreased during folate depletion and increased on folic acid administration. This was consonant with the observations of Reynolds,[6] who suggested a relationship between the antifolate effects of antiepileptic drugs and their therapeutic action, as well as the report by Chanarin *et al.*[25] that folic acid had a convulsant effect in a patient with anticonvulsant-induced megaloblastic anemia. These observations suggest that folate depletion decreases and folate administration increases fit frequency in subjects with underlying convulsive disease, irrespective of whether the folate depletion results from inadequate ingestion, absorption, or utilization or from increased excretion or requirement.

However, it should be noted that Luhby and Cooperman[24] reported that seizures of the two siblings they studied were reduced rather than aggravated by folic acid therapy.

Table 2 presents the differences in the clinical syndromes of the two siblings studied by Luhby and colleagues as compared to the 20-year-old girl studied by Lanzkowsky and colleagues.

Two separate inborn errors in folate utilization have been reported.[4] The common clinical features were mental retardation and abnormalities in electroencephalograms and pneumoencephalograms, which showed cortical atrophy and ventricular dilation. These infants included four with formiminotransferase deficiency syndrome (step 21, Fig. 1) and one with methyltetrahydrofolate transmethylase deficiency (step 6, Fig. 1). It is important to note that in all of these five cases of tissue level folate deficiency, serum folate was elevated rather than reduced, presumably because the folate unable to be used in the tissues accumulated in red cells and serum as 5-methyl THF.

Table 3 presents the clinical and biochemical findings in formiminotransferase deficiency syndrome.

Walters[26] has reported a patient with dihydrofolate reductase deficiency (step 7, Fig. 1) who, despite the expected presence of megaloblastic anemia due

TABLE 2. DIFFERENCES IN THE TWO REPORTED CLINICAL SYNDROMES OF CONGENITAL MALABSORPTION OF FOLATE[a]

Clinical feature	Luhby *et al.*	Lanzkowsky *et al.*
Mental retardation	Moderate	Profound
Central nervous system degeneration	Progressive	Stationary
Physical development	Moderate retardation	Normal
Movements	Ataxia	Athetotic
Cerebral calcification	Absent	Present
Seizures	Reduced by folic acid	Aggravated by folic acid

[a] From Lanzkowsky.[23]

TABLE 3. CLINICAL AND BIOCHEMICAL FINDINGS IN FORMIMINOTRANSFERASE DEFICIENCY SYNDROME[a]

Data	Case 1	Case 2	Case 3	Case 4
Sex	Female	Female	Male	Male
Age at admission	8 months	1 year, 7 months	3 months	6 months
Mental and physical development	Retarded	Retarded	Retarded	Normal?
Electroencephalogram	Abnormal	Abnormal	Abnormal	Abnormal
Pneumoencephalogram				
Cortical atrophy	+	+	+	+
Ventricular dilation	+	++ (autopsy)	+	
Liver function				
SGOT	Normal	Normal	Normal	Normal
Thymol turbidity	Negative	Positive	Negative	Negative
Zinc sulfate	Negative		Negative	Negative
Serum cholesterol (mg/100 ml)	Normal		64–80	182
Histological findings in liver			Deposition of hemosiderin in hepatic cells	Deposition of hemosiderin in hepatic cells
Blood pictures	Slight tendency to hypersegmentation of neutrophils	Normocytic anemia	Megaloblastic and sideroblastic anemia	Normal
Serum folate (*L. casei*) (mμg/ml)	10–300	72–228	53–100	86–138
Urinary excretion of FIGLU after an oral dose of L-histidine (μmoles/8 hr)	13–66	859 μmoles/24 hr	100–175	68–87
Formiminotransferase activity of the liver	Decreased	Decreased	Decreased	Decreased

[a] From Arakawa.[4]

o folate deficiency, had no neurological abnormalities. Arakawa[4] suggested hat the microcephaly and dilation of cerebral ventricles in the infants with ormiminotransferase or methyltetrahydrofolate transferase deficiency might esult from impaired purine biosynthesis of the growing brain due to defective ctivity of the tetrahydrofolate-dependent enzymes. His group produced icrocephaly and dilation of cerebral ventricles (not accompanied by gross alformation) in the offspring of rats by intraperitoneal administration of -mercaptopurine in a dose of 20 mg/kg on the eleventh day of gestation of ams. For further discussion of the pathogenesis of brain dysfunction in relaon to disorders of purine metabolism, see Chapter 7 of this book.

Arakawa's group has also described three cases of congenital cyclohyrolase deficiency (step 14, Fig. 1) manifested by mental retardation with

microcephaly, hyperfolatemia with formiminoglutamate in the urine, and a striking decrease in cyclohydrolase activity in erythrocytes and liver biopsy.[1] However, they are currently reinvestigating that syndrome to determine if it is in fact a separate entity.[4]

These findings suggest that inborn errors in folate metabolism may be a cause of mental retardation.[1] It is not yet known whether mental retardation may result from inadequate delivery of folate to the fetus due to maternal deficiency during the gestational period or from folate deficiency of the infant during the period of maturation of brain function during the first 2 years of life. Arakawa and colleagues report that maturation patterns of electroencephalographic frequency bands were delayed at the ages of 3–6 months in infants fed exclusively on breast milk from mothers with low serum folate levels, as compared to infants fed exclusively on breast milk from mothers with normal serum folate levels.[4] Other studies suggest that folate is concentrated from serum into maternal breast milk.[27] The question has been raised as to whether the diet of all pregnant women should be supplemented with 0.1–0.3 mg of folic acid daily to reduce the prevalence of mental retardation which could be associated with folate deficiency, regardless of whether such deficiency is from nutritional inadequacy, congenital malabsorption, or malutilization of folate.[1] Regardless of the validity of this position, a good case can be made for giving all pregnant women such a dose of folic acid in a daily dietary supplement.[19,28]

Whether there is a link between the defective folate metabolism and mental retardation associated with dilation of the ventricles in the infants studied by Arakawa[4] is unknown. However, a similar accumulation of serum folate activity as 5-methyl THF occurs with vitamin B_{12} deficiency[19] (step 6, Fig. 1), and rats raised on a diet deficient in vitamin B_{12} give birth to offspring with congenital hydrocephalus.[29] The prevalence of such hydrocephalus is strikingly increased by the inclusion in the diet of a folate antagonist[29] and may be largely prevented by supplementing the diet with folic acid.[30]

To our knowledge, studies have yet to be carried out to determine whether, in humans, folate or vitamin B_{12} deficiency *in utero*, regardless of cause, may produce mental retardation with dilation of the cerebral ventricles. The possibility demands study.

C. Functional Damage to Adults Related to Nutritional Folate Deficiency

Evidence so far adduced suggests that brain development continues for approximately the first 2 years of postnatal life and that malnutrition during the vulnerable period prior to 2 years of age may result in permanent brain damage. This subject is discussed in greater detail in Chapter 8 of this book. Brains of children malnourished during this vulnerable period have shown at autopsy small size, decreased cell number, selective cell loss in cerebellum com-

1207

d with other portions of the brain (the cerebellum normally grows more dly), and reduced myelin lipid.

Presently, there is no solid evidence that any histological or anatomical n damage, not already present during the vulnerable period, may occur on as a result of folate deficiency.

Folate deficiency occurring after the vulnerable period appears not to luce morphological damage to nerve tissue but is associated with sleepless-, forgetfulness, and irritability[5,31] and even paranoid behavior.[32] These ptoms disappear dramatically within 24 hr after starting therapy. Several ps[33,34] have reported subnormal serum folate in a high percentage (up to) of patients admitted to the hospital with a diagnosis of depression. How-, depression itself usually reduces appetite, producing secondary dietary te deficiency. Thus folate deficiency is probably a secondary result rather a cause of the depression in such patients. A substantial percentage of hiatric patients are reported to have folate deficiency[35,36]; such folate ciency appears more likely related to bizarre eating habits than to the cause he psychiatric problems.

There are countless cases of adult folate deficiency in the world literature, as yet only one has been reported[37] with autopsy findings of spinal cord, bellar, and cerebral damage. The autopsy findings in that case very likely lted from the coincidental presence of a disease unrelated to folate defi-cy. There have been several reports of peripheral neuropathy or myelopathy ssociation with adult folate deficiency, but here, also, the evidence[38] sug-s coincidence rather than cause and effect. However, another possible lanation is that the patients suffered from malnutrition, which produced ary deficiency of folate (causing the folate deficiency) and also dietary ciency of other nutrients, including thiamine (with the other dietary de-ncies causing the neurological damage). Alternatively, neurological disease reduce appetite, producing secondary folate deficiency. In some cases, rapeutic responses seemingly to folate may actually be due to improved etite, resulting in improved supply of other nutrients in which the patient y have been deficient.

Anticonvulsants and Folate Deficiency—Folic Acid Alleged Undesirable or Epileptic Patients

The structural relationship between some anticonvulsants and folic acid been recognized for more than a decade,[39] and the similarity of the five-mbered ring of diphenylhydantoin (dilantin, phenytoin) to that of 5,10-thylene tetrahydrofolate was pointed out in 1962[40] (cf. Fig. 2 and Table 4). that time, it was also pointed out[40] that low serum folate levels were fre-ent in patients taking diphenylhydantoin as anticonvulsant therapy. This has

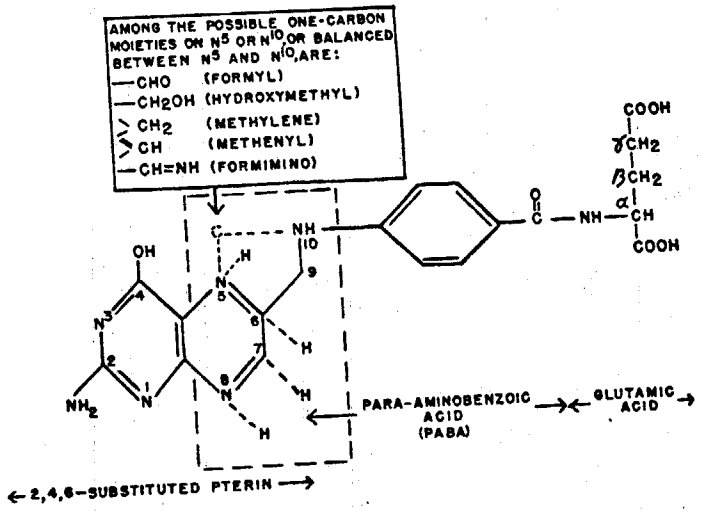


Fig. 2. Folic acid structure. The area in the broken rectangle is the "active center" in one-carbon transfers. The broken lines outline the basic one-carbon acceptor (5,6,7,8-tetrahydrofolic acid) and the various one-carbon-donating coenzymes derived from it. Note the structural similarity between the five-membered ring in the "active center" and the "common denominator structure" of anticonvulsants depicted in Table 4. (From Herbert and Zalusky.[40])

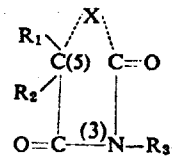
been repeatedly confirmed, with low serum folate reported in 30–90% of epileptic patients.[41–43]

Reynolds *et al.*[6,43–46] agree that the barbiturate derivatives and diphenylhydantoin may act as mild antifolates because of structural similarities to folates and further suggest that such antifolate action may relate to the mechanism of seizure control by these agents. In a recent review of this subject, Chanarin[42] has noted that megaloblastic anemia in such patients is often associated with neuropsychiatric complications which may have preceded the anemia.

The finding[11,14] that cerebrospinal fluid folate levels are generally about three times serum folate levels is now well established.[12,47,48] Reynolds *et al.*[48] found that mean cerebrospinal fluid folate was only 11 ng/ml in 60 drug-treated epileptic patients as compared to 21 ng/ml in 51 control subjects; this lower cerebrospinal fluid level was expected in view of the low serum level, despite the fact[11] that spinal fluid folate falls more slowly than serum folate as deficiency develops.

The mechanism whereby anticonvulsant therapy may produce folate deficiency is uncertain, but the best current evidence is that such deficiency occurs only in patients who have borderline folate stores to begin with and that such borderline stores are further reduced by interference with folate absorption

TABLE 4. COMMON DENOMINATOR STRUCTURE OF ANTICONVULSANTS, MAJOR DRUG CLASSES, ANTIEPILEPTIC USES, AND DOSAGES[a]



Substituents						
R_1	R_2	R_3	X	Nuclei and drugs	Typical uses[b]	Typical dosage[c] (g)
			—CO—NH—	Barbiturates		
C_6H_5	C_2H_5	H	"	Phenobarbital	GM	0.2
C_6H_5	C_2H_5	CH_3	"	Mephobarbital	GM	0.5
C_2H_5	C_2H_5	CH_3	"	Metharbital	GM	0.4
C_6H_5	C_2H_5	H	"	Primidone[d]	GM, PS	1.5
			—NH—	Hydantoins		
C_6H_5	C_6H_5	H	"	Diphenylhydantoin	GM,PS	0.5
C_6H_5	H	C_2H_5	"	Ethotoin	GM	2.0
C_6H_5	C_2H_5	CH_3	"	Mephenytoin	GM, PS	0.6
			$—NH_2$	Acetylureas[e]		
C_6H_5	H, H	H	"	Phenacemide	PS	1.5
C_6H_5	C_2H_5, H	H	"	Pheneturide	PS	1.2
			—O—	Oxazolidimediones		
CH_3	CH_3	CH_3	"	Trimethadione	PM	0.9
C_2H_5	CH_3	CH_3	"	Paramethadione	PM	0.9
			$—CH_2—$	Succinimides		
C_6H_5	H	CH_3	"	Phensuximide	PM	2.0
C_6H_5	CH_3	CH_3	"	Methsuximide	PM	0.9
C_2H_5	CH_3	H	"	Ethosuximide	PM	1.0

[a] From chapter by J. E. P. Toman *in* "The Pharmacological Basis of Therapeutics," 4th ed. (L. S. Goodman and A. Gilman, eds.), p. 207, Macmillan, New York, 1970
[b] GM, grand mal, focal cortical seizures, etc.; PS, psychomotor epilepsy and related disorders; PM, petit mal.
[c] Total daily dosage in adults.
[d] Carbonyl oxygen of urea moiety of phenobarbital is replaced by two hydrogen atoms.
[e] Straight-chain compounds.

brought about by the diphenylhydantoin therapy. Although earlier evidence suggested that diphenylhydantoin may reduce folate absorption by inhibiting intestinal folate conjugase,[49,50] later work has not supported this possibility.[51,52] The most recent studies indicate that diphenylhydantoin directly interferes with folate monoglutamate absorption (step 2, Fig. 1), perhaps by blocking intestinal ATPase.[53,54] In preliminary results in our laboratory, Momtazi has observed[54a] inhibition by diphenylhydantoin of uptake of folic acid by individual rat small bowel epithelial cells (step 5, Fig. 1). Separate studies suggest that diphenylhydantoin may block delivery of methylfolate to human bone marrow

cells.[55] Diphenylhydantoin has been reported to block delivery of radioactive folate to brain tissue,[56] where it is known to inhibit synaptosome ATPase.[56–58]

Although serum vitamin B_{12} levels may be lower than in a series of control subjects, they tend to be within normal limits in drug-treated epileptic patients.[59] Serum B_{12} levels tend to fall in epileptic patients treated with folic acid,[60] just as such levels tend to fall in nonepileptic patients treated with folic acid.[40]

It has recently been suggested that folate may be required for adequate utilization of vitamin B_{12},[61] just as vitamin B_{12} is known to be required for adequate utilization of folate.[5,11,16,18–21,61a]

It is not yet certain that either folic acid or vitamin B_{12} in fact increases fit frequency in drug-treated epileptic patients, as has been alleged.[43,45] Snaith *et al.*[62] noted no change in fit frequency in 14 patients treated with folic acid for 2–6 months. If, in fact, folate deficiency reduces fit frequency, this may relate to the report that uridine and uridine monophosphate are both effective anticonvulsants in animals.[63] The utilization of these two agents is partly blocked by folate antagonists.[58] Thus it is conceivable that folate antagonists (perhaps including diphenylhydantoin) may produce an accumulation of uridine and its phosphorylated nucleotide, which could have anticonvulsant action. Relief of such blockade by provision of folic acid could eliminate this accumulation, thereby precipitating seizure activity. It is an interesting fact that Piccoli *et al.*[64] report that cerebral concentration of uridine monophosphate increases seven- to eight-fold during arrest of a convulsive seizure. Detailed discussion of brain nucleotides and the excitatory process may be found in Chapter 7 of this book and also in a paper by Bonavita and Piccoli.[65]

Another currently speculative role of folic acid in relation to brain function is its structural relationship to the pteridine coenzyme involved in the hydroxylation of tyrosine and tryptophan, which are involved in catecholamine and serotonin production.[66]

Hunter *et al.*[67] reported that folic acid therapy produced mental changes, sleep disturbance, and gastrointestinal symptoms in normal volunteers. Since the symptoms were completely subjective and no placebo controls were carried out, the study lacks credibility. In a subsequently studied group, Gibberd *et al.*[68] found no toxicity in association with folate therapy. In general, the overwhelming evidence is that folate therapy in man is nontoxic,[19] except for the possibility that it may increase fit frequency in patients susceptible to convulsions by opposing in some way the stabilizing influence on virtually all excitable membranes of diphenylhydantoin.[69]

According to recent work,[70] patients suffering from diphenylhydantoin toxicity (nystagmus, ataxia, and confusion) may get relief within 24 hr (either

1202

id improvement or disappearance of symptoms) on treatment with 10–15 mg folic acid by mouth. In most cases, serum diphenylhydantoin levels did not suggesting that catabolism of diphenylhydantoin (by parahydroxylation) usually not accelerated by folic acid. However, Kutt *et al.*[71] reported patient whose serum diphenylhydantoin level dropped on folic acid therapy, sumably because of increased parahydroxylation. Thus it may be that the ease in fit frequency observed in some but not other patients after folic acid apy is related to whether or not in the particular patient parahydroxylation iphenylhydantoin is increased by folic acid.

There is no sound evidence that either vitamin B_{12} or folic acid is of value enile dementia.[72]

Severe Neurological Damage in Adults Associated with Intrathecal Methotrexate Therapy

Kay *et al.*,[73] in a recent letter to the editor of *Lancet*, report seven patients, d 3–24, who developed neurological signs and dementia during or shortly r a period of intrathecal methotrexate therapy for acute lymphoblastic emia. The main signs were confusion, irritability, somnolence, ataxia, and entia, with major epileptic fits in two cases and progression to coma and h in one. In all cases, these signs appeared after all evidence of the men- al leukemia (headache, vomiting, cerebrospinal fluid free of cells) had ppeared. In the one fatal case, necropsy showed patchy areas of necrosis in poral and parietal lobes of the cerebrum; microscopy of these lesions showed t appeared to be infarcts with fibrinoid necrosis of small blood vessels. ere the neurological damage was recognized, its progress was arrested by inistering folinic and folic acids, with partial recovery. A possibly similar has been reported by Pinkel *et al.*[74]

CENTRAL NERVOUS SYSTEM DAMAGE DUE TO VITAMIN B_{12} DEFICIENCY

Developmental Damage to Newborn Related to Congenital Defects in Vitamin B_{12} Utilization

As is true for folic acid, vitamin B_{12} is metabolically active only after it been reduced. The two active reduced coenzyme forms in man are 5′-de- denosyl-B^{12} and methyl-B_{12}.[20,21] Mudd *et al.*[75,76] described a male infant was lethargic and ate poorly from the time of birth. He lived only 7½ weeks. cle tone and spontaneous movements were poor. He deteriorated pro- sively and had generalized convulsions on the days prior to death. Necropsy ved a small brain with diffuse changes including focal softening of white

matter: The infant had a generalized derangement in vitamin B_{12} metabolism associated with homocystinemia, cystathioninemia, hypomethioninemia, and methylmalonic aciduria. There was defective activity of the two vitamin B_{12} dependent enzymes, methyltetrahydrofolate transferase and methylmalonyl-CoA mutase; the latter defect proved correctable in tissue culture of the infant's skin fibroblasts by adding a high concentration of hydroxo-B_{12}. Thus the primary defect may well have been inability either to transport vitamin B_{12} into cells or to convert trivalent B_{12a} to monovalent B_{12s}, which is the final common precursor of the two common enzyme forms of vitamin B_{12} required by man. The bone marrow of the infant did not show megaloblastic changes; presumably, megaloblastic changes did not develop because the infant was so severely ill or perhaps had not lived long enough. The same abnormalities in the metabolism of sulfur-containing amino acids seen in this disorder of coenzyme activation would be expected in the *apo* methyltetrahydro folate transmethylase deficiency described by Arakawa (see previously) but were not mentioned by him. This point requires further investigation.

Several infants have now been described with congenital methylmalonic aciduria. This appears to be two disorders, one of which is vitamin B_{12} responsive and the other vitamin B_{12} unresponsive.[76–78] Retardation of development of central nervous system function was present in most of the six described cases of vitamin B_{12} responsive methylmalonic aciduria but has been reversible on treatment. The disorder appears to be a defect in the metabolism of the coenzyme 5′-deoxyadenosyl-B_{12}. This defect appears to be overcome by large doses (at least 0.2 mg daily) of vitamin B_{12}. As pointed out by Mahoney and Rosenberg,[76] the requirement for pharmacological amounts of the vitamin in the absence of any evidence of deficiency has defined these patients as being vitamin B_{12} *dependent* in contrast to being vitamin B_{12} *deficient*. The defect in metabolism of 5′-deoxyadenosyl-B_{12} appears to be a defect in biosynthesis of this coenzyme, presumably an abnormality of the deoxyadenosyl transferase.

Identical clinical and laboratory findings have been obtained for the seven patients described with vitamin B_{12} unresponsive methylmalonic aciduria. Here the defect is in methylmalonyl-CoA mutase apoenzyme, and therefore supplying pharmacological amounts of coenzyme is without value.[76]

Both forms of congenital methylmalonic aciduria fit an autosomal recessive mode of inheritance, with both sexes involved. There was no parental consanguinity.

Treatment for both forms of congenital methylmalonic aciduria, in addition to pharmacological doses of vitamin B_{12} for those patients with vitamin B_{12} responsive disease, consists of limiting protein intake and vigorously treating acidosis when it appears.

legaloblastic anemia is neither seen nor expected in congenital methyl-
ic aciduria, since there is no defect in either the methyl-B_{12} coenzyme
the methylfolate–homocysteine transmethylase coenzyme. The central
is system disturbances appear to be related to ketosis and disturbed pro-
etabolism.[76]

uctural and Functional Damage Due to Nutritional Vitamin B_{12} ficiency at Any Age

ecause the fetus will take such nutrients as it needs even from a deficient
r, deficiency of vitamin B_{12} (or folate) on a nutritional basis has rarely
oted in newborns. Characteristically, at birth the concentrations of vi-
B_{12} and folate in cord blood are two to five times those in maternal
[8,79] Infants born of vegetarian mothers may suffer from vitamin B_{12}
ncy.[80] More commonly, clinical vitamin B_{12} deficiency with the classical
ological and neurological damage results from long-standing nutritional
quacy, due either to a diet devoid of animal protein or to a gastric or
nal disorder resulting in malabsorption of vitamin B_{12}.[81] The classical
logical damage of vitamin B_{12} deficiency is a variably patchy demyeli-
n, involving particularly frequently the spinal cord.[8,10] The biochemical
anism whereby vitamin B_{12} is required for synthesis of myelin is still
own, although it almost must be in synthesis of either the lipid or the
n (or both) which constitute myelin.[19] It has been speculated that the
ependent isomerization of propionate–methyl malonate is the site of the
,[82] but demyelination does not occur in congenital methylmalonyl-CoA
se deficiency.[76] The recent report[83] of two abnormal fatty acid peaks
romatography of nerve tissue from B_{12}-deficient subjects requires further
ation—the results may very likely prove to be plasmalogen[84] and
no relation to the biochemical lesion of vitamin B_{12} deficiency. Thus
ochemical basis is still unknown for the characteristic demyelination which
inevitable result of severe vitamin B_{12} deficiency, regardless of cause, and
damages all myelinated axons including those in the brain, the posterior
ateral columns of the spinal cord, and the peripheral nerves.[7–10]
The symptoms resulting from this demyelination have been repeatedly
ibed and reviewed.[7,8]
The classical association of subacute combined degeneration (combined
ms disease, posterolateral sclerosis, funicular degeneration) with vitamin
eficiency is well known.[85,86] It should be noted, however, that the disease
lly starts insidiously and not subacutely, combined lesions are often
t, and lesions of the peripheral nerves occur much more frequently and
earlier than lesions of the central nervous system.[87–90]
The use of the term "subacute combined degeneration" to include all

of those changes in the nervous system which may occur as a result of vitamin B_{12} deficiency should probably be discontinued.[87,91] The nervous system changes would be more accurately described by direct reference to the actual involvement (i.e., peripheral nerve, spinal cord, or cerebral damage due to vitamin B_{12} deficiency[91,92]) or, in general terms, as vitamin B_{12} neuropathy.

Paresthesia, especially numbness and tingling in the hands and feet, is the most frequent neurological symptom of vitamin B_{12} deficiency.[87,93,94] Other signs are often present: diminution of vibration and position sense, unsteadiness, poor muscular coordination with ataxia, moodiness, mental slowness, poor memory, confusion, agitation and depression, delusions, hallucinations, psychosis (usually with paranoid ideation), and central scotomata, sometimes with dim vision due to optic atrophy or tobacco amblyopia.[87,92,93,95–98] The wide variety of sensory and motor changes tend to be symmetrical, especially if maintained for a period of weeks or months[99]; however, this is not invariable.

In the authors' experience, loss of position sense in the index toes and loss of vibration sense, as tested by a 256 vibrations/sec tuning fork, usually precede by many months any evidence of loss of position sense in the big toes or of vibration sense with a 128 vibrations/sec tuning fork.[8]

It is always difficult to be certain that a psychosis or damage to the nervous system is derived purely from vitamin B_{12} deficiency. The major criterion is response to vitamin B_{12} therapy. Unless such response is complete, it may be impossible to determine how much of the failure to return to complete normality is due to irreversible damage related to the length of time the patient was deficient in vitamin B_{12} and how much is due to other causes. This problem frequently arises in the approximately 10% of patients with pernicious anemia who also have diabetes mellitus,[99,100] either of which is capable of producing severe peripheral neuropathy. Parenthetically, the possibility of abnormal metabolism of vitamin B_{12} in diabetic subjects remains unestablished.[101]

The most frequent initial symptoms are weakness, dyspnea, paresthesia, and sore tongue, any or all of which may precede the onset of overt anemia by years.

A number of studies have suggested that screening of a peripheral blood film for evidence of megaloblastosis and/or determination of serum vitamin B_{12} levels should be carried out routinely in psychiatric patients[8,102–105] because of the frequency of vitamin B_{12} deficiency in psychiatric patients, especially the elderly,[102] ranging from 0.88% of 1012 patients in one series[103] to 15.4% of another group[104] of 396 psychiatric patients.

Confusion, forgetfulness, and paranoid ideation have been considered among the most prominent psychiatric phenomena in patients with vitamin B_{12} deficiency.[8] In a prospective controlled study,[105] in which the status of two

1202

(one B_{12}-deficient and the other not) of psychiatric patients was com-
impairment of memory appeared to be clearly related to vitamin B_{12}
ncy, with improvement often within 24 hr of the start of B_{12} therapy
urn to normal within 2 weeks. Although depression was also common,
1 improved with knowledge of the good prognosis, even prior to the
B_{12} therapy. Clear delineation of cause and effect relationships between
tric stigmata and vitamin B_{12} deficiency awaits greater knowledge of
hemistry and the biochemical roles of vitamin B_{12}. The answers may lie
in the roles of vitamin B_{12} in nucleoprotein and protein synthesis.

OWLEDGMENTS

ne studies of the authors have been supported in part by USPHS Grant
5163 and in part by Health Research Council of the City of New York
Scientist Award I-683 and a Veterans Administration Medical Inves-
ship to Dr. Herbert.

RENCES

Herbert, Inborn errors in folate metabolism—a cause of mental retardation? *Ann. Med.* **68**:956–958, 1968.
B. Thiersch, Therapeutic abortions with folic acid antagonist, 4-aminopteroylglutamic id (4-amino P.G.A.) administered by oral route, *Am. J. Obstet. Gynecol.* **63**:1298–1304, 52.
B. Thiersch, The control of reproduction in rats with the aid of antimetabolites and ly experiences with antimetabolites as abortifacient agents in man, *Acta Endocrinol.* :37–45, 1956 (Suppl. 28).
Arakawa, Congenital defects in folate utilization, *Am. J. Med.* **48**:594–598, 1970.
Herbert, Experimental nutritional folate deficiency in man, *Trans. Ass. Am. Physicians* :307–320, 1962.
H. Reynolds, Mental effects of anticonvulsants, and folic acid metabolism, *Brain* **91**: 7–214, 1968.
Victor and A. Lear, Subacute combined degeneration of the spinal cord: Current ncepts of the disease process. Value of serum vitamin B_{12} determinations in clarifying me of the common clinical problems, *Am. J. Med.* **20**:896, 1956.
Herbert, "The Megaloblastic Anemias," Grune & Stratton, New York, 1959.
E. Oxnard and W. T. Smith, Neurological degeneration and reduced serum vitamin $_2$-levels in captive monkeys, *Nature* **210**:507, 1966.
S. Pant, A. K. Asbury, and E. P. Richardson, Jr., The myelopathy of pernicious anemia: neurological reappraisal, *Acta Neurol. Scand.* **44**:1–36, 1968 (Suppl. 35).
Herbert, Studies of folate deficiency in man, *Proc. Roy. Soc. Med. (London)* **57**:377–4, 1964.
Levitt, P. F. Nixon, J. H. Pincus, and J. R. Bertino, Transport characteristics of ates in cerebrospinal fluid; a study utilizing doubly labeled 5-methyltetrahydrofolate d 5-formyltetrahydrofolate, *J. Clin. Invest.* **50**:1301–1308, 1971.
Sobotka, H. Baker, and O. Frank, Vitamin levels in normal cerebrospinal fluid, *roc. Soc. Exptl. Biol. Med.* **103**:801–802, 1960.

14. V. Herbert and R. Zalusky, Selective concentration of folic acid activity in cerebrospinal fluid, *Fed. Proc.* **20**:453, 1961.
15. V. Herbert and L. W. Sullivan, Activity of coenzyme B_{12} in man, *Ann. N. Y. Acad. Sci.* **112**:855–870, 1964.
16. F. M. Huennekens, Folate and B_{12} coenzymes, *in* "Biological Oxidations" (T. P. Singer, ed.), pp. 439–513, Interscience Publishers, New York, 1968.
17. R. L. Blakley, "The Biochemistry of Folic Acid and Related Pteridines," John Wiley & Sons, Inc., New York, 1969.
18. H. Weissbach and R. T. Taylor, Roles of vitamin B_{12} and folic acid in methionine synthesis, *Vitamins & Hormones* **28**:415–440, 1970.
19. V. Herbert, Drugs effective in megaloblastic anaemias: Vitamin B_{12} and folic acid, *in* "The Pharmacological Basis of Therapeutics" (L. S. Goodman and A. Gilman, ed.), 4th ed., pp. 1414–1444, Macmillan, New York, 1970.
20. V. Herbert (guest ed.), Symposium on vitamin B_{12} and folate, *Am. J. Med.* **48**:539–617, 1970.
21. V. Herbert, Recent developments in cobalamin metabolism, *in* "The Cobalamins" (H. R. V. Arnstein and R. J. Wrighton, eds.), pp. 2–16, J. & A. Churchill, Ltd., London, 1971.
22. V. Herbert, Folic acid and vitamin B_{12}, *in* "Modern Nutrition in Health and Disease" (R. S. Goodhart and M. Shils, ed.), 5th ed., Lea & Febiger, Philadelphia, 1972.
23. P. Lanzkowsky, Congenital malabsorption of folate, *Am. J. Med.* **48**:580–583, 1970.
24. A. L. Luhby and J. M. Cooperman, Congenital megaloblastic anemia and progressive central nervous system degeneration. Further clinical and physiological characterization and therapy of syndrome due to inborn error of folate transport, American Pediatric Society, Inc., Annual Meeting, Atlantic City, April 1967.
25. I. Chanarin, J. Laidlaw, L. W. Loughridge, and D. L. Mollin, Megaloblastic anemia due to phenobarbitone, the convulsant action of therapeutic doses of folic acid, *Brit. Med. J.* **1**:1099, 1960.
26. T. F. Walters, Congenital megaloblastic anemia responsive to N^5-formyltetrahydrofolic acid administration, *J. Pediat.* **70**:686, 1967.
27. J. Metz, R. Zalusky, and V. Herbert, Folic acid binding by serum and milk, *Am. J. Clin. Nutrition* **21**:289–297, 1968.
28. FAO/WHO Expert Group, "Requirements of Ascorbic Acid, Vitamin D, Vitamin B_{12} Folate, and Iron," WHO Technical Report Series, No. 452, 1970 (available for $1.25 from World Health Organization Distribution and Sales, Geneva, Switzerland).
29. J. C. Woodard and P. M. Newberne, Relation of vitamin B_{12} and one-carbon metabolism to hydrocephalus in the rat, *J. Nutrition* **88**:375, 1966.
30. A. G. Hogan, B. L. O'Dell, and J. R. Whitley, Maternal nutrition and hydrocephalus in newborn rats, *Proc. Soc. Exptl. Biol. Med.* **74**:293, 1950.
31. B. Hoogstraten, J. Cuttner, and B. Natovitz, Sequence of recovery from multiple manifestations of folic acid deficiency, *J. Mount Sinai Hosp. N.Y.* **31**:10–16, 1964.
32. C. E. Butterworth, Jr., Personal communication.
33. R. W. Strachan and J. G. Henderson, Dementia and folate deficiency, *Quart. J. Med.* **36**:189–204, 1967.
34. E. H. Reynolds, J. M. Preece, J. Bailey, and A. Coppen, Folate deficiency in depressive illness, *Brit. J. Psychiat.* **117**:287–292, 1970.
35. M. W. Carney, Serum folate values in 432 psychiatric patients, *Brit. Med. J.* **4**:512–516, 1967.
36. R. Hunter, M. Jones, T. G. Jones, and D. M. Matthews, Serum B_{12} and folate concentrations in mental patients, *Brit. J. Psychiat.* **113**:1291–1295, 1967.
37. H. A. Hansen, P. Nordquist, and P. Sourander, Megaloblastic anemia and neurologic disturbances combined with folic acid deficiency, *Acta Med. Scand.* **176**:243–251, 1964.
38. H. C. Grant, A. V. Hoffbrand, and D. G. Wells, Folate deficiency and neurological disease, *Lancet* **2**:763–767, 1965.

. H. Girdwood, Folic acid, its analogs and antagonists, *Advan Clin. Chem.* 3:235–297, 960.

. Herbert and R. Zalusky, Interrelations of vitamin B_{12} and folic acid metabolism: olic acid clearance studies, *J. Clin. Invest.* 41:1263–1276, 1962.

. A. Klipstein, Subnormal serum folate and macrocytosis associated with anticonvulsant rug therapy, *Blood* 23:68–86, 1964.

Chanarin, "The Megaloblastic Anemias," Blackwell, Oxford, F. A. Davis Co., Philalphia, 1969.

H. Reynolds, Iatrogenic disorders in epilepsy, *in* "Modern Trends in Neurology" Williams, ed.), pp. 271–286, Butterworths, London, 1970.

H. Reynolds, G. Milner, D. M. Matthews, and I. Chanarin, Anticonvulsant therapy, egaloblastic haemopoiesis and folic acid metabolism, *Quart. J. Med.* 35:521–537, 1966.

H. Reynolds, I. Chanarin, G. Milner, and D. M. Matthews, Anticonvulsant therapy, lic acid and vitamin B_{12} metabolism and mental symptoms, *Epilepsia (Amsterdam)* 261–270, 1966.

H. Reynolds, Effects of folic acid on the mental state and fit frequency of drug-treated ileptic patients, *Lancet* 1:1086–1088, 1967.

. G. Wells and H. Casey, *Lactobacillus casei* C. S. F. folate activity, *Brit. Med. J.* 834–837, 1967.

H. Reynolds, J. Preece, and I. Chanarin, Folic acid and anticonvulsants, *Lancet* 1: 64–1265, 1969.

H. Rosenberg, H. A. Godwin, R. R. Streiff, and W. B. Castle, Impairment of intestinal conjugation of dietary folate. A possible explanation of megaloblastic anemia asciated with phenytoin therapy, *Lancet* 2:530–532, 1968.

V. Hoffbrand and T. F. Necheles, Mechanism of folate deficiency in patients receiving enytoin, *Lancet* 2:528–530, 1968.

M. Baugh and C. L. Krumdieck, Effects of phenytoin on folic acid conjugases in man, *ncet* 2:519–521, 1969.

H. Bernstein, S. Gutstein, S. Weiner, and G. Efron, The absorption and malabsorption folic acid and its polyglutamates, *Am. J. Med.* 48:570–579, 1970.

D. Gerson, N. Cohen, G. W. Hepner, N. Brown, V. Herbert, and H. D. Janowitz, lic acid absorption in man: Enhancing effect of glucose, *Gastroenterology* 61:224–227, 71.

W. Hepner, L. M. Aledort, D. C. Gerson, N. Cohen, V. Herbert, and H. D. Janowitz, ibition of intestinal ATPase by diphenylhydantoin and acetazolamide, *Clin. Res.* 382, 1970.

Momtazi and V. Herbert, Studying mechanisms of intestinal absorption using susisions of vibration-obtained individual small bowel epithelial cells: folate absorption, . *J. Clin. Nutrit.* 25:449–450, 1972.

Tisman and V. Herbert, Inhibition by human serum, dilantin (diphenylhydantoin), l methotrexate (MTX), and enhancement by 2-deoxyglucose of ^{3}H-pteroylglutamic d (^{3}HPGA) and ^{3}H-5-methyltetrahydrofolate (M-THF) uptake by human bone marrow s *in vitro*, *Clin. Res.* 19:433, 1971 (Abst.).

M. Woodbury and J. W. Kemp, Some possible mechanisms of action of anti-epileptic gs, *in* "Pharmakopsychiatrie Neuro-Psychopharmakologie. Advances in Theoretical Clinical Research" (H. Coper, M. Engelmeier, K. Heinrich, A. Herz, H. Hippins, and Kielholz, eds.), pp. 201–226, Georg Thieme Verlag, Stuttgart, 1970.

W. Festoff and S. H. Appel, Effect of diphenylhydantoin on synaptosome sodium–assium–ATPase, *J. Clin. Invest.* 47:2752, 1968.

Vaxman, J. Corcino, and V. Herbert, Drugs, toxins and dietary amino acids affecting min B_{12} or folic acid absorption or utilization, *Am. J. Med.* 48:599–608, 1970.

. Malpas, G. H. Spray, and L. J. Witts, Serum folic acid and vitamin B_{12} levels in convulsant therapy, *Brit. Med. J.* 1:955–957, 1966.

60. E. H. Reynolds, I. Chanarin, G. Milner, and D. M. Matthews, Anticonvulsant therapy, folic acid and vitamin B_{12} metabolism and mental symptoms, *Epilepsia (Amsterdam)* 7:261–270, 1966.

61. R. J. Harrison, Vitamin B_{12} levels in erythrocytes in anaemia due to folate deficiency, *Brit. J. Haematol.* 20:623–628, 1971.

61a. V. Herbert, G. Tisman, L. T. Go, and L. Brenner, B_{12}-dependence of cell uptake of serum folate: an explanation for high serum folate and cell folate depletion in B_{12} deficiency, *Clin. Res.* 20:489, 1972.

62. R. P. Snaith, S. Mehta, and A. H. Raby, Serum folate and vitamin B_{12} in epileptics with and without mental illness, *Brit. J. Psychiat.* 116:179–183, 1970.

63. V. Bonavita, P. Monaco, and E. Tripi, Analisi degli effetti centrali di nucleotidi purinici e pirimidinici e loro derivati, *Acta Neurol.* 19:215–222, 1964.

64. F. Piccoli, R. Camarda, and V. Bonavita, Purine and pyrimidine nucleotides in the brain of normal and convulsant rats, *J. Neurochem.* 16:159–169, 1969.

65. V. Bonavita and F. Piccoli, Brain nucleotides and excitatory processes, *in* "Advances in Experimental Medicine and Biology" (R. Paoletti, ed.), Vol. 13, pp. 355–373, Plenum Press, New York, 1971.

66. E. H. Reynolds, J. M. Preece, J. Bailey, and A. Coppen, Folate deficiency in depressive illness, *Brit. J. Psychiat.* 117:287–292, 1970.

67. R. Hunter, J. Barnes, H. F. Oakeley, and D. M. Matthews, Toxicity of folic acid given in pharmacologic doses to healthy volunteers, *Lancet* 1:61–64, 1970.

68. F. B. Gibberd, A. Nicholls, J. F. Dunne, and D. M. Chaput de Saintonge, Toxicity of folic acid, *Lancet* 1:360–361, 1970.

69. J. H. Pincus, I. Grove, B. B. Marino, and G. E. Glaser, Studies on the mechanism of action of diphenylhydantoin, *Arch. Neurol.* 22:566–571, 1970.

70. R. R. Streiff, B. J. Wilder, and R. Hammer, Folic acid and diphenylhydantoin toxicity, *in* "Proceedings of a Symposium on the Pharmacology of Antiepileptic Drugs" (J. Penry, ed.), National Institutes of Health Monograph, to be published June 1972, by Raven Press.

71. H. Kutt, W. Winters, and F. H. McDowell, Depression of parahydroxylation of diphenylhydantoin by antituberculosis chemotherapy, *Neurology* 16:594–602, 1966.

72. D. M. Shaw, D. A. Macsweeney, A. L. Johnson, R. O'Keefe, D. Naidoo, D. M. Macleod, S. Jog, J. M. Preece, and J. M. Crowley, Folate and amine metabolites in senile dementia: A combined trial and biochemical study, *Psychol. Med.* 1:166–171, 1971.

73. H. E. M. Kay, P. J. Knapton, J. P. O'Sullivan, D. G. Wells, R. F. Harris, E. M. Innes, J. Stuart, F. C. M. Schwartz, and E. N. Thompson, Severe neurological damage associated with methotrexate therapy, *Lancet* 2:542, 1971.

74. D. Pinkel, K. Hernandez, L. Borella, C. Holton, R. Aur, G. Samoy, and C. Pratt, Drug dosage and remission duration in childhood lymphocytic leukemia, *Cancer* 27:247–256, 1971.

75. S. H. Mudd, H. L. Levy, and R. H. Abeles, A derangement in B_{12} metabolism leading to homocystinemia, cystathioninemia and methylmalonic aciduria, *Biochem. Biophys. Res. Commun.* 35:121–126, 1969.

76. M. J. Mahoney and L. E. Rosenberg, Inherited defects of B_{12} metabolism, *Am. J. Med.* 48:584–593, 1970.

77. G. Morrow, III, L. A. Barness, V. H. Auerbach, A. M. DiGeorge, T. Ando, and W. L. Nyhan, Observations on the coexistence of methylmalonic acidemia and glycinemia, *J. Pediat.* 74:680, 1969.

78. G. Morrow, III, L. A. Barness, G. J. Cardinale, R. H. Abeles, and J. G. Flaks, Congenital methylmalonic acidemia: Enzymatic evidence for two forms of the disease, *Proc. Natl. Acad. Sci.* 63:191, 1969.

79. H. Baker, O. Frank, I. Pasher, H. Ziffer, and H. Sobotka, Pantothenic acid, thiamine and folic acid levels at parturition, *Proc. Soc. Exptl. Biol. Med.* 103:321–323, 1960.

80. M. Jadhav, J. K. G. Webb, and S. J. Baker, Vitamin B_{12} deficiency in Indian infants, *Lancet* **1**:720, 1963.
81. J. J. Corcino, S. Waxman, and V. Herbert, Absorption and malabsorption of vitamin B_{12}, *Am. J. Med.* **48**:562–569, 1970.
82. E. V. Cox, D. Robertson-Smith, M. Small, and A. M. White, The excretion of propionate and acetate in vitamin B_{12} deficiency, *Clin. Sci.* **35**:123, 1968.
83. E. P. Frenkel, Studies on mechanism of the neural lesion of pernicious anemia, *J. Clin. Invest.* **50**:33a, 1971.
84. A. J. Marcus, H. L. Ullman, L. B. Safier, and H. S. Ballard, Platelet phosphatides. Their fatty acid and aldehyde composition and activity in different clotting systems, *J. Clin. Invest.* **41**:2198–2212, 1962.
85. K. C. Smithburn and L. G. Zerfas, The neural symptoms and signs in pernicious anemia, *Arch. Neurol. Psychiat.* **25**:1110, 1931,
86. H. H. Hyland, G. O. Watts, and R. F. Farquharson, Course of subacute combined degeneration of spinal cord, *Can. Med. Ass. J.* **65**:295, 1951.
87. J. Richmond and S. Davidson, Subacute combined degeneration of the spinal cord in non-addisonian megaloblastic anaemia, *Quart. J. Med.* **27**:517, 1958.
88. S. Davidson, Clinical picture of pernicious anemia prior to introduction of liver therapy in 1926 and in Edinburgh subsequent to 1944, *Brit. Med. J.* **1**:241, 1957.
89. J. G. Greenfield and E. A. Carmichael, The peripheral nerves in cases of subacute combined degeneration of the cord, *Brain* **58**:483, 1935.
90. W. M. Van Der Scheer and H. C. Koek, Peripheral nerve lesions in cases of pernicious anemia, *Acta Psychiat Neurol.* **13**:61, 1938.
91. E. C. C. Jewesbury, Subacute combined degeneration of the cord and achlorhydric peripheral neuropathies without anaemia, *Lancet* **2**:307, 1954.
92. J. M. Holmes, Cerebral manifestation of vitamin B_{12} deficiency, *Brit. Med. J.* **2**:1394, 1956.
93. M. M. Wintrobe, "Clinical Hematology," Philadelphia, Lea & Febiger, 1956.
94. W. B. Castle, The anemias, *in* "A Textbook of Medicine" (R. L. Cecil and R. F. Loeb, eds.), 10th ed., W. B. Saunders, Co., Philadelphia, 1959.
95. C. C. Ungley, Treatment with vitamin B_{12} in haematologic disorders, *in* "Vitamin B_{12} and Intrinsic Factor" (H. C. Heinrich, ed.), Ferdinand Enke Verlag, Stuttgart, 1957.
96. J. M. Heaton, A. J. A. McCormick, and A. G. Freeman, Tobacco amblyopia: A clinical manifestation of vitamin B_{12} deficiency, *Lancet* **2**:286, 1958.
97. H. E. Hamilton, P. P. Ellis, and R. F. Sheets, Visual impairment due to optic neuropathy in pernicious anemia. Report of a case and review of the literature, *Blood* **14**:378, 1959.
98. C. E. C. Harris, Snares and delusions in the recognition of "pernicious anemia," *Connecticut Med. J.* **12**:543, 1958.
99. Von W. Gassmann and J. Schneeweiss, Diabetes mellitus und perniziose Anamie, *Acta Haematol.* **16**:396, 1956.
100. G. Panzram, Pernicious anemia and diabetes mellitus, *Z. Ges. Inn. Med. Leipzig* **15**:239, 1960.
101. H. Keen and R. Smith, Vitamin B_{12} and the course of diabetic retinopathy, *Lancet* **1**:849, 1956.
102. R. Shulman, A survey of vitamin B_{12} deficiency in an elderly psychiatric population, *Brit. J. Psychiat.* **113**:241–251, 1967.
103. G. J. Henderson, R. W. Strachan, J. Swanson Beck, A. A. Dawson, and M. Daniel, The antigastric-antibody test as a screening procedure for vitamin B_{12} deficiency in psychiatric practice, *Lancet* **2**:809–813, 1966.
104. E. Edwin, K. Holten, K. R. Norum, A. Schrumpf, and O. E. Skaug, Vitamin B_{12} hypovitaminosis in mental disease, *Acta Med. Scand.* **177**:689–699, 1965.
105. R. Shulman, Psychiatric aspects of pernicious anaemia: A prospective controlled investigation, *Brit. Med. J.* **3**:266–270, 1967.

J. Nutr. 92(2): 261-266

Percutaneous Absorption of Vitamin B_{12} in the Rat and Guinea Pig

E. E. HOWE, C. L. DOOLEY, R. F. GEOFFROY [1]
AND CHARLES ROSENBLUM [1]
Merck Institute for Therapeutic Research and Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey

ABSTRACT The percutaneous absorption of vitamin B_{12} by the rat and guinea pig was investigated. Growth experiments in weanling rats indicated that the vitamin was efficiently absorbed in an active form. Studies with ^{57}Co-labeled cyanocobalamin demonstrated that absorption was more efficient from an ethanol solution than from water or dimethyl sulfoxide and was independent of the quantity of vitamin applied to a given area. The skin was found to act as a reservoir absorbing large amounts of cyanocobalamin and releasing it slowly to other tissues. Absorption was similar in the 2 species studied.

Vitamin B_{12} is very difficultly absorbed from the gastrointestinal tract despite the fact that of all the essential nutrients it alone has a known specific mechanism to facilitate its absorption. There are reports that the vitamin is absorbed sublingually (1) and by nasal inhalation (2), but none indicate whether it is absorbed into or through the skin.

Because of the reports that dimethyl sulfoxide (DMSO) enhances the percutaneous absorption of many organic molecules, experiments were conducted in the laboratory rat to determine whether DMSO would bring about the absorption of vitamin B_{12}. Somewhat unexpectedly, it was observed that the vitamin was well absorbed through the skin from either a DMSO, an ethanolic or aqueous solution. The present report describes the results obtained in demonstrating this phenomenon, some of the factors which influence it, and its occurrence in the guinea pig as well as in the rat.

EXPERIMENTAL

Growth experiments. The first experiments were performed to determine whether cyanocobalamin applied to the skin in DMSO would stimulate growth of weanling rats consuming a vitamin B_{12}-deficient diet. Male rats of the Holtzman strain were separated into groups of eight, housed individually and allowed free access to food and water. The percentage composition of the diet was: soybean meal, 60; glucose, 23.9; salt mixture (3), 4; partially hydrogenated fat,[2] 10; cod liver oil, 2; thyroid powder,[3] 0.1; and a vitamin addendum [4] lacking only vitamin B_{12}. With such a diet it had been established previously that 0.1 µg cyanocobalamin/rat/day usually produced a maximal growth response (4). On the assumption that only a small fraction of the topically applied vitamin would be absorbed, 5 µg of cyanocobalamin in 0.03 ml of DMSO or water were applied daily in experiment 1 (table 1) to a 6-cm^2 shaved area on the backs of the animals. They were weighed at 5-day intervals and finally at 28 days when they were killed.

In experiments 2 and 3 (table 2) only 0.1 µg of cyanocobalamin in 0.03 ml of solution was applied to the rat, the experiments being identical in all other respects.

Tracer experiments with radioactive vitamin B_{12}. To quantitate the effect more accurately and to extend the initial observations, experiments were performed using vitamin B_{12}-^{57}Co. In experiment 4 (table 3) absorption from water, 90% DMSO and ethanol were measured using 2 concentrations, 0.025 µg and 2.5 µg cyanocobalamin in 0.03 ml of solution and 2 contact periods, 2 and 5 days. The solutions, each containing several tenths of a microcurie of radioactivity per dose, were

Received for publication January 14, 1967.

[1] Merck, Sharp and Dohme Research Laboratories.
[2] MFB, Hunt-Wesson Company, Fullerton, California.
[3] Armour Laboratories, Kankakee, Illinois.
[4] Micronutrients per 100 g of food: (in milligrams) thiamine·HCl, 1.0; riboflavin, 2.0; pyridoxine·HCl, 1.0; Ca pantothenate, 10.0; niacinamide, 10.0; inositol, 5.0; choline, 100.0; p-aminobenzoic acid, 30.0; biotin, 0.03; folic acid, 0.2; α-tocopherol, 14.2; and menadione, 14.2.

520

TABLE 1

Effect of 5 μg cyanocobalamin applied to skin on growth of weanling rats (exp. 1)

Diet	Topical application	Avg 28-day wt gain [1]	P value
Basal		156 ± 14.1 [2]	—
Basal + 0.1% thyroid powder		134 ± 23.8	—
Basal + 0.1% thyroid powder + 10 μg cyanocobalamin/kg diet		182 ± 36.2	0.01
Basal + 0.1% thyroid powder	5 μg cyanocobalamin in 0.03 ml DMSO [3]	188 ± 23.2	0.01
Basal + 0.1% thyroid powder	5 μg cyanocobalamin in 0.03 ml H_2O	185 ± 17.0	0.01
Basal + 0.1% thyroid powder	0.03 ml DMSO [3]	113 ± 24.5	—
Basal + 0.1% thyroid powder + 10 μg cyanocobalamin/kg diet	0.03 ml DMSO	185 ± 25.4	0.01

[1] Eight rats/group.
[2] SD.
[3] DMSO indicates dimethyl sulfoxide.

applied to a 6-cm² shaved area on the backs of male Holtzman rats weighing about 200 g and the applied areas were covered by gauze bandages held in place by adhesive tape circling the bodies of the animals. The rats were housed individually in metabolism cages to permit the collection of urine and feces, and were allowed unrestricted access to laboratory ration and water. After the designated time they were killed, the skin was excised from the area of application and the bandage and skin section were washed with portions of detergent solution.[5] Combined skin and bandage washings were diluted to 100 ml with water, as were urine samples, and the fecal samples were suspended in 100 ml water for assay. All radiometric measurements were made by gamma-ray scintillation counting. Urine, feces, washings and whole carcasses were measured in a Tobor large-volume counter, and the washed skin samples were measured in the Autogamma instrument for small volume.

In rat experiment 5 absorption of cyanocobalamin from ethanolic solution was compared at 4 concentrations (0.025, 0.25, 2.5 and 25 μg/0.03 ml) at 3 contact times; 2 hours, 2 days and 5 days. Experiment 6 was designed to investigate the role of the skin as a depot or reservoir of vitamin B_{12} after topical application. Four groups of 5 rats each were treated as described previously with 0.03 ml of ethanolic solution containing 2.5 μg of radioactive cyanocobalamin. The animals were killed at 5, 10, 19 and 40 days and appropriate measurements made. Urine and feces of all surviving animals were collected daily.

The purpose of the seventh and final experiment was to show that the observed absorptive process is not peculiar to the rat. Ten female guinea pigs of approximately 300 g in weight were subjected to experimental procedures similar in all details to those of experiments 6 with the following exceptions. One animal died on the sixth day and was examined at the time of death, 4 were killed on day 13 and the remaining five were killed on day 24.

RESULTS

The average 28-day weight gains in the growth experiments (exps. 1–3) are shown in tables 1 and 2 together with standard deviation. Also reported are probability values of differences, based on comparisons with basal diet + 0.1% thyroid powder, calculated by the method of analysis of variance. Growth responses obtained (table 1) with daily topical application of 5 μg of cyanocobalamin were significant ($P = 0.01$) and equivalent to ad libitum feeding of 10 μg/kg diet. Even the low dose of 0.1 μg/day when applied topically to the skin caused a maximal growth response. The DMSO and aqueous solutions were equally active.

Group average absorption results as percentage of dose in experiment 4 are re-

[5] 75 ml water containing 5 drops of PHisoHex (Winthrop Laboratories, New York).

TABLE 2

Effect of 0.1 μg cyanocobalamin applied to skin on growth of weanling rats

Diet	Application to skin	Avg 28-day wt gain [1]		P value	
		Exp. 2	Exp. 3	Exp. 2	Exp. 3
Basal+0.1% thyroid powder		139±19.4 [2]	116±22.3	—	—
Basal+0.1% thyroid powder	0.1 μg cyanocobalamin in 0.03 ml DMSO,[3] topical	157±22.8	151±12.2	—	0.01
Basal+0.1% thyroid powder	0.1 μg cyanocobalamin in 0.03 ml H_2O, topical	160± 9.9	142±22.7	—	0.05
Basal+0.1% thyroid powder	0.1 μg cyanocobalamin in aqueous solution, subcutaneous	165±15.5	162±21.2	0.05	0.01
Basal+0.1% thyroid powder+ 10 μg cyanacobalamin/kg diet		152±31.8	164±12.8	—	0.01

[1] Eight rats/group.
[2] SD.
[3] DMSO indicates dimethyl sulfoxide.

TABLE 3

Effect of solvent on percutaneous absorption of cyanocobalamin-^{57}Co in the rat (exp. 4)

Application			Percutaneous absorption of cyanocobalamin-^{57}Co [1]				
Vitamin B_{12}	Vehicle	Days	Skin	Carcass	Urine	Feces	Total absorption
μg			avg % of dose	avg % of dose	avg % of dose	avg % of dose	avg % of dose
0.025	H_2O	2	2.23± 0.56 [2]	3.02±5.42	0.06±0.04	0.57±1.21	5.87± 6.16
		5	2.27± 0.88	0.19±0.07	0.06±0.05	0.13±0.03	2.65± 0.94
0.021	EtOH	2	35.3 ±12.3	0.56±0.28	0.04±0.03	0.10±0.04	36.0 ±12.53
		5	21.6 ±10.7	0.80±0.38	0.08±0.06	0.20±0.08	22.7 ±10.78
0.021	90% DMSO	2	3.52± 0.60	0.10±0.07	0.03±0.02	0.09±0.02	3.74± 0.63
		5	2.39± 0.41	0.37±0.26	0.06±0.05	0.18±0.06	3.0 ± 0.58
2.5	H_2O	2	7.0 ± 3.3	5.95±3.63	0.04±0.03	1.38±1.46	14.4 ± 6.64
		5	8.53± 4.0	0.40±0.29	0.05±0.05	0.21±0.11	9.2 ± 4.30
2.1	EtOH	2	20.7 ± 7.7	0.51±0.58	0.03±0.02	0.13±0.17	21.4 ± 8.16
		5	29.3 ± 7.6	2.69±1.87	0.16±0.10	2.39±2.60	34.6 ±11.09
2.2	90% DMSO	2	5.57± 6.07	4.69±5.77	0.08±0.11	1.75±2.49	12.1 ± 8.52
		5	2.64± 0.79	1.07±1.85	0.08±0.09	0.88±1.72	4.7 ± 3.52

[1] Six rats/group.
[2] SD.

corded in table 3. These data show absorption from all 3 vehicles but that absorption from ethanol is definitely superior to that from water or DMSO which were again essentially equal. The apparent lower total absorption observed at 5 days as compared with 2 days is probably the result of variations between animals (note high incidence of large standard deviations). Such variability was not encountered frequently in later experiments. This may also be due to variable retention of vitamin by the bandage.

The data obtained in the performance of experiment 5 are compiled in table 4 and show that the dose-response (total absorption) curves are approximately of a log-log type, characteristic of an adsorption process. This is true of all 3 time-intervals studied; and the slopes of the log-log curves are about equal. Initial absorption was rapid, and remained essentially constant between 2 hours and 2 days, but increased approximately 2.5-fold between 2 and 5 days. Individual absorption was variable but under the conditions of the experiment does not appear to be a function of the quantity of the vitamin applied.

Table 5 is a compilation of group average values obtained in experiment 6 expressed as percentage of dose. These figures show that the skin acts as a storage depot for vitamin B_{12}, gradually releasing the vitamin for use in other body tissues. This is shown by 1) the gradual decrease in skin radioactivity with time; 2) the concomitant increase in carcass activity; and 3) the continuous elimination of radioactivity in urine plus feces. Thus increases in elimination of radioactivity in excreta and increases in carcass concentrations occur at the expense of a parallel decrease in skin radioactivity. Excretion during the first 5 days is negligible, but increases thereafter reached a maximum in about 20 days and were still high 40 days after topical application. Animals varied considerably in the permeability and retentivity of their skin to radioactive vitamin B_{12}. In many cases high skin retentivity was accompanied by low urinary output.

The results of experiment 7 are summarized in table 6. Despite great variability between animals, the carcass and excreta activities of the guinea pig increased

TABLE 4

Effect of concentration and time on percutaneous absorption by the rat of cyanocobalamin-^{57}Co applied in ethanolic solution (exp. 5) [1]

Conc	Duration	Percutaneous absorption					
		Skin	Urine	Fèces	Carcass	Total absorption	
μg	*hours*	*avg % of dose*	*avg % of dose*	*avg % of dose*	*avg % of dose*	*% of dose*	*μg*
0.025	2	14.8 ± 6.3 [2]	—	—	1.15 ± 1.53	15.9 ± 5.78	0.0040 ± 0.0015
0.25	2	8.3 ± 2.3	—	—	0.10 ± 0.04	8.4 ± 2.28	0.021 ± 0.006
2.5	2	8.2 ± 1.6	—	—	0.22 ± 0.04	8.4 ± 1.54	0.21 ± 0.04
25	2	9.1 ± 2.2	—	—	1.20 ± 1.20	10.3 ± 1.87	2.6 ± 0.47
	days						
0.025	2	19.4 ± 10.6	0.14 ± 0.07	0.77 ± 0.51	2.47 ± 1.17	22.8 ± 11.28	0.0057 ± 0.0028
0.25	2	3.5 ± 1.7	0.12 ± 0.07	0.16 ± 0.11	0.43 ± 0.20	4.2 ± 1.78	0.01 ± 0.004
2.5	2	7.7 ± 2.5	0.22 ± 0.24	0.58 ± 0.54	1.43 ± 0.91	9.9 ± 3.50	0.25 ± 0.09
25	2	5.0 ± 2.0	0.19 ± 0.11	1.04 ± 0.30	2.88 ± 1.99	9.1 ± 3.85	2.3 ± 0.97
0.025	5	27.5 ± 5.3	0.31 ± 0.53	1.83 ± 2.90	2.57 ± 3.57	32.2 ± 12.06	0.0080 ± 0.0030
0.25	5	9.9 ± 5.3	1.49 ± 1.56	4.62 ± 4.64	4.51 ± 1.48	20.4 ± 6.61	0.056 ± 0.018
2.5	5	13.0 ± 5.1	0.82 ± 0.72	2.84 ± 2.34	5.1 ± 4.66	21.7 ± 10.46	0.54 ± 0.26
25	5	18.5 ± 7.8	0.31 ± 0.17	4.49 ± 3.45	3.46 ± 1.37	26.8 ± 10.31	6.7 ± 2.58

[1] Four rats/group.
[2] SD.

TABLE 5

Percutaneous absorption of cyanocobalamin-^{57}Co (2.5 μg) showing depot effect (exp. 6) [1]

Duration of experiment	Percutaneous absorption of cyanocobalamin-^{57}Co												
	Urine and feces, days								Skin	Carcass	Wash + bandage	Total
	1–5	5–10	10–14	14–19	19–24	24–28	28–34	34–40	Total				
days	*avg % of dose*				*avg % of dose*			*avg % of dose*					
5	0.09 ±0.03 [a]	—	—	—	—	—	—	—	0.09 ±0.03	26.0 ± 4.9	0.62 ±0.25	65.8 ± 8.1	92.5 ± 5.9
10	0.08 ±0.04	0.54 ±0.80	—	—	—	—	—	—	0.62 ±0.81	19.1 ±11.8	1.01 ±1.04	75.9 ±13.2	96.6 ± 5.8
19	0.17 ±0.10	0.33 ±0.13	0.44 ±0.39	0.41 ±0.33	—	—	—	—	1.35 ±0.76	3.66 ± 1.60	1.19 ±0.67	87.8 ± 4.8	94.1 ± 4.7
40	0.06 ±0.05	0.28 ±0.37	1.28 ±1.78	1.50 ±1.86	0.96 ±0.94	0.77 ±0.59	0.77 ±0.67	0.65 ±0.56	6.25 ±6.15	3.26 ± 4.11	3.19 ±1.60	79.4 ±10.28	92.1 ± 5.2

[1] Five rats per group.
[a] SD.

at the expense of a diminishing skin radioactivity, just as observed with the rat. This behavior indicates that the skin of the guinea pig also serves as a depot source for vitamin B_{12}. Values for skin plus bandage washings are recorded in tables 5 and 6 as an indication of the radioactivity accountability achieved in these experiments. Although somewhat variable, an average of 96% of the activity is accounted for.

Preliminary experiments with radioactive hydroxocobalamin applied to the rat indicated considerably higher percutaneous absorption of cyanocobalamin.

DISCUSSION

Cyanocobalamin appears to be readily absorbed through the skin. This is evident from the growth study as well as from the tracer measurements.

Furthermore the stimulation of growth of rats receiving a vitamin B_{12}-deficient diet shows that the topical vitamin is absorbed in a form active for growth production. It was unexpected that the percentage absorbed remained fairly constant over the wide range of concentrations used.

In the growth studies we were concerned that although the most inaccessible area of the back was used as the site of application, some of the vitamin might be transferred to the paws of the rat and thence to its mouth. Since in experiments 2 and 3 the quantity applied was that previously shown to be the minimum required to produce maximal growth when given parenterally (4) it did not appear likely that oral ingestion was an important factor.

In view of an earlier report (5) that 50% of a subcutaneous dose of vitamin B_{12} was excreted in the urine and 6% in the feces, the reversed ratio of these parameters in the present experiment may require a word of explanation. In the former experiment in which the vitamin was administered parenterally, the vitamin probably appeared immediately in the blood stream in high concentration and consequently was excreted to a large extent by the kidney. However, during slow percutaneous absorption, blood levels probably never become excessively high, but over a period of time the vitamin is excreted in the bile and appears in the feces.

TABLE 6

Percutaneous absorption of cyanocobalamin-^{57}Co (2.5 μg) from ethanol solution by the guinea pig (exp. 7)

Guinea pig no.	Percutaneous absorption of cyanocobalamin-^{57}Co					
	Skin	Carcass	Urine + feces	Total retention [1]	Wash + bandage	Total
	% of dose	*% of dose*	*% of dose*	*% of dose*	*% of dose*	*% of dose*
(10) [2]	(7.1)	(0.17)	(0.07)	(7.4)	(87.4)	(94.8)
1 [3]	13.2	0.40	0.15	13.8	83.6	97.4
2	6.7	0.51	0.18	7.4	91.6	99.0
3	4.61	2.47	2.07	9.1	90.1	99.2
4	17.9	0.73	0.25	18.9	92.6	111.5
Avg ± SD	10.6 ± 6.1	1.03 ± 0.97	0.66 ± 0.94	12.3 ± 5.17	89.5 ± 4.05	101.8 ± 6.53
5 [4]	5.62	4.84	9.7	20.2	77.5	97.7
6	1.29	2.25	3.41	6.95	86.5	93.5
7	2.43	0.57	0.18	3.18	96.0	99.2
8	1.20	0.99	0.34	2.53	101.8	104.3
9	2.32	0.73	0.26	3.31	98.7	102.0
Avg ± SD	2.57 ± 1.80	1.88 ± 1.78	2.78 ± 4.10	7.2 ± 7.45	92.1 ± 9.97	99.3 ± 4.14

[1] Sum of skin, carcass and urine + feces values.
[2] Died on sixth day, not included in average.
[3] Nos. 1–4 killed on day 13.
[4] Nos. 5–9 killed on day 24.

Hyman (1) has reported that absorption of the vitamin does not occur sublingually in pernicious anemia patients but Monto and associates (2) observed absorption by such patients by the nasal route. It would be unexpected indeed if intrinsic factor were found to play a part in percutaneous absorption.

The observation that large quantities of the vitamin pass rapidly into the skin from whence it is slowly released to other tissues presents possibilities for a long-lasting pharmaceutical preparation if subsequent clinical experiments show that these results are translatable to man. The literature on the effect of massive parenteral doses of vitamin B_{12} on certain peripheral disorders such as trigeminal neuralgia is voluminous and controversial. Clear-cut cases of vitamin B_{12} deficiency, i.e., those responding to physiological doses of the vitamin, have been recorded in which the symptoms were not those of classic vitamin B_{12} deficiency (6, 7).[6,7,8] Hence it is possible that any or all effects of vitamin B_{12} may be due to correction of nutritional deficiencies rather than to pharmacological effects. The large amounts of the vitamin required may be due to poor utilization or to inefficient transport to the site where needed. For this reason it is possible that topical application in the treatment of a disease such as trigeminal neuralgia may be more effective than parenteral administration.

LITERATURE CITED

1. Hyman, G. A. 1960 Sub-lingual absorption of vitamin B_{12}. Clin. Res., *8*: 210.
2. Monto, R. W., J. W. Rebuck and M. J. Breman 1953 Crystalline B_{12} inhalation in pernicious anemia. Amer. J. Med. Sci., *225*: 113.
3. Hegsted, D. M., R. C. Mills, C. A. Elvehjem and E. B. Hart 1941 Choline in the nutrition of chicks. J. Biol. Chem., *138*: 459.
4. Emerson, G. A. 1949 Growth promoting activity of vitamin B_{12} in rats receiving thyroid substance. Proc. Soc. Exp. Biol. Med., *70*: 392.
5. Rosenblum, C., B. F. Chow, G. P. Condon and R. S. Yamamoto 1952 Oral versus parenteral administration of Co^{60}-labeled vitamin B_{12} to rats. J. Biol. Chem., *198*: 915.
6. Bakke, H. 1960 A seven-year study of vitamin B_{12} deficiency exclusive of macrocytic anemia: preliminary report. J. Amer. Geriatrics Soc., *8*: 903.
7. Kane, F. J. 1963 Psychosis with masked pernicious anemia. North Carolina Med. J., *24*: 245.

[6] Smith, A. D. M. 1960 Megaloblastic madness. Brit. Med. J., *2*: 1840 (in Correspondence).

[7] Freeman, A. G., and J. M. Heaton 1961 Megaloblastic madness. Brit. Med. J., *1*: 128 (in Correspondence).

[8] The Royal College of Physicians and Surgeons of Canada. Canad. Med. Assoc. J., *84*: 396, 1961 (in Medical Meetings).

Arch. Biochem. Biophys. 142(1): 231-237

1429

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 142, 231-237 (1971)

Studies on Vitamin B_{12} Metabolism in HeLa Cells[1]

S. S. KERWAR, CARLOS SPEARS, BRIAN McAUSLAN AND HERBERT WEISSBACH

Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Received August 10, 1970; accepted October 19, 1970

In the present report some aspects of vitamin B_{12} metabolism in HeLa cells and other tissue culture lines have been investigated. Mangum *et al.* (1) previously reported the presence of higher levels of N^5-methyl-H_4-folate-homocysteine transmethylase in tissue culture cells grown in the presence of vitamin B_{12}. The present experiments indicate that under the growth conditions used, HeLa cells do not contain sufficient vitamin B_{12} to satisfy the cobamide requirement of the transmethylase, and the increase in enzymatic activity that is observed when the cells are grown in the presence of vitamin B_{12} results from the conversion of the apoenzyme form of the enzyme to active holoenzyme. No effect of the vitamin on the oxidation of propionate to CO_2 was observed in these cells suggesting that the amount of 5′-deoxyadenosyl-B_{12} (DBCC) present was sufficient so that the methylmalonyl CoA isomerase reaction was not rate limiting. Extracts of HeLa cells readily convert hydroxy-B_{12} to DBCC in the presence of ATP and a reducing system. The optimum conditions for the enzymatic synthesis of DBCC are described.

In mammalian cells grown in tissue culture vitamin B_{12}[2] is required for the biosynthesis of methionine (1) and the isomerization of methylmalonyl-CoA to succinyl-CoA (2, 3). Recently Mangum *et al.* (1) made the observation that the activity of N^5-methyl-H_4-folate-homocysteine transmethylase (B_{12} transmethylase) increased several fold when vitamin B_{12} was added to the culture medium. These studies did not ascertain whether the observed increase in enzymatic activity was due to induction of enzyme synthesis by the vitamin or the conversion of the apoenzyme form of the transmethylase to the holoenzyme. The results reported here establish that HeLa cells grown in the presence of the vitamin synthesize the apoenzyme form of the transmethylase, and the increase in enzymatic activity observed by Mangum *et al.* (1) during growth of cells with vitamin B_{12} is due to *in vivo* conversion of apoenzyme to holoenzyme. In contrast, the vitamin had no effect on the oxidation of propionate suggesting that these cells have sufficient levels of DBCC so that the methylmalonyl-CoA isomerase reaction is not rate limiting. Although several laboratories (4-7) have shown the presence of DBCC in animal tissues and tissue culture cells, the biosynthesis of the coenzyme in animal cells has not been demonstrated previously. The present report also describes an enzymatic system in HeLa extracts capable of synthesizing DBCC from hydroxy-B_{12} and ATP.

MATERIALS AND METHODS

HeLa S_3 cells were grown in suspension in Eagle's medium supplemented with 5% calf serum. When vitamin B_{12} was added to the growth medium, its concentration was 0.5 mg/l. Cells were harvested, washed with 0.1 M Tris buffer, pH 8.0,

[1] A preliminary report of this work has been presented in *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* 29, 473 (1970).

[2] The following abbreviations have been used: Vitamin B_{12}, cyano-cobalamin; DBCC, 5,6-dimethyl-benzimidazolylcobamide-5′-deoxyadenosine or vitamin B_{12} coenzyme; vitamin B_{12a}, hydroxy-cobalamin; H_4-folate, tetrahydrofolate.

526

suspended in 3 vol of 1×10^{-3} M potassium phosphate buffer (KPO_4 buffer), pH 7.4, and sonicated for 30 sec in a Bronwill sonicator. The homogenate was centrifuged at 12,000*g* for 60 min. Unless otherwise stated, the supernatant fraction was used as the source of enzyme.

N^5-Methly-H_4-folate-homocysteine transmethylase (B_{12} transmethylase). The incubation system for the assay of methionine contained in a total volume of 0.2 ml, enzyme, 0.1 M KPO_4 buffer, pH 7.4; 5×10^{-5} M *S*-adenosylmethionine, 2.5×10^{-4} M L-homocysteine, 1.5×10^{-4} M DL-N^5-methyl-^{14}C-H_4-folate (specific activity 1.6×10^6 cpm/μmole), 0.2 M 2-mercaptoethanol and 5×10^{-5} M of either methyl-B_{12} or propyl-B_{12}. After 30 min at 37°, 0.8 ml of cold water was added, and the amount of methionine formed was determined by a Dowex 1-Cl^- method (8). Under these conditions, systems containing propyl-B_{12} measure the amount of holoenzyme present in the extract, whereas those containing methyl-B_{12} measure both apoenzyme and holoenzyme (9). The amount of holoenzyme present is expressed as percentage ratio of activity obtained with propyl-B_{12} as compared to that observed with methyl-B_{12}.

In vivo effect of vitamin B_{12} derivatives on the formation of holoenzyme. The ability of various alkyl-B_{12} derivatives to form holoenzyme *in vivo* was determined as follows: 200 ml of a HeLa cell suspension (10^5 cells) in a culture medium lacking vitamin B_{12} was supplemented with 20 μg of the cobamide to be tested. Cells were allowed to grow for 3 hr in the dark and harvested. The cell pellet was washed with 10 ml of 0.1 M Tris buffer, pH 8.0, and suspended in 1 ml of KPO_4 buffer. After sonication for 10 sec using a Bronwill sonifier, the homogenate was centrifuged at 12,000*g* for 60 min, and the supernatant fraction was assayed for B_{12} transmethylase activity. All manipulations were carried out in the dark.

Oxidation of labeled propionate and succinate. Oxidation of propionate and succinate by HeLa cells was assayed as follows: 0.2 ml of a washed HeLa cell suspension in 0.1 M KPO_4 buffer, pH 7.4 (approximately 2×10^7 cells) was incubated with either 0.25 μmoles of 1-^{14}C-sodium propionate or 1,4-^{14}C-succinic acid (1.8×10^7 cpm/μmole) in a total volume of 0.5 ml using a stoppered side-arm vessel. When DBCC was present, its concentration was 4×10^{-6} M. A filter strip containing 50 μl of NCS solubilizer (Amersham Searle) was suspended in the reaction vessel to absorb $^{14}CO_2$ liberated during the reaction. The reaction vessels were sealed and incubated at 37°. The reaction was stopped by the addition of 0.5 ml of 10% TCA present in the side arm of the vessel, and the mixture was incubated at 37° for an additional 15 min. The sealed vessel was opened, and the filter strip was removed and suspended in 10 ml of a naphthalene dioxane counting fluid (10) and assayed for radioactivity.

Enzymatic synthesis of DBCC. Cell-free extracts of HeLa cells, or a 0-50% $(NH_4)_2SO_4$ fraction prepared from a 12,000*g* supernatant fraction, were tested for their ability to synthesize DBCC from hydroxy-B_{12} and ATP. The incubation system contained in a volume of 0.50 ml: 2.0 μM hydroxy-B_{12}, 100 mM KPO_4 buffer, pH 7.4; 1.0 mM ATP, 12 μM FAD, 2.0 mM $MgCl_2$, 60 mM DTT, and 40 mM 2-mercaptoethanol. After the addition of 0.05 ml enzyme, the tubes were sealed with rubber serum caps and flushed with hydrogen for 5 min at 0° with the excess hydrogen permitted to escape through an exhaust needle outlet in the cap. The outlet needle was then removed, and the reaction mixture (under hydrogen pressure throughout the incubation) was incubated at 37° in the dark, and subsequent manipulations were done in the dark. The reaction was terminated by immersing the tubes in boiling water for 5 min. The incubation mixture was diluted to 10 ml with water and clarified by centrifugation for 5 min at full speed in a clinical centrifuge. The clear supernatant was assayed for DBCC using dioldehydrase as described by Abeles *et al.* (11).

N^5-Methyl-^{14}C-H_4-folate was synthesized from ^{14}C-formaldehyde and H_4-folate by the method of Keresztesy and Donaldson (12), and alkyl cobamides were synthesized by the procedure of Smith *et al.* (13). Dioldehydrase was purified from sonicates of *A. aerogenes*[3] by the method of Lee and Abeles (14). DBCC was a gift of Dr. L. Mervyn, Glaxo Laboratories, England, and holoenzyme synthetase and apotransmethylase from *E. coli* B were prepared by the method of Brot and Weissbach (15). Protein concentrations were determined either by the method of Lowry *et al.* (16) or by a spectrophotometric method (17). All radiochemical assays were performed with a Beckman LS-100 liquid scintillation counter using a naphthalene-dioxane counting fluid as described by Bray (10).

RESULTS AND DISCUSSION

Effect of vitamin B_{12} on the transmethylase activity of HeLa cells. In previous studies with *E. coli*, an assay was developed to measure the relative amounts of apoenzyme and holoenzyme in extracts and the conversion of the apoenzyme form of the transmethylase to the holoenzyme species (9). This meth

[3] We are grateful to Dr. R. H. Abeles for supplying us with sonicates of *A. aerogenes* cells, and to Dr. N. Brot for many helpful discussions and continued interest.

was based on the ability of propyl-B_{12} to satisfy the cobamide requirement of holoenzyme, whereas methyl-B_{12} functioned with both apoenzyme and holoenzyme. A similar assay has been used in the experiments here to study the nature of the enzyme present in HeLa cells. Extracts of cells grown with or without vitamin B_{12} showed similar transmethylase activity when methyl-B_{12} was used in the *in vitro* assay. However, extracts of cells grown with the vitamin had a propyl-B_{12}/methyl-B_{12} ratio of 1, while a propylB_{12}/methyl B_{12} ratio of 0.1 was obtained with extracts from cells grown without the vitamin (Table I). These results indicated than when HeLa cells were cultured in the absence of the vitamin, the B_{12} transmethylase was present almost entirely in the apoenzyme form, and that the addition of vitamin B_{12} to the growth medium resulted in the *in vivo* conversion of apoenzyme to holoenzyme. It was concluded, therefore, that the vitamin B_{12} stimulation of transmethylase activity previously observed by Mangum *et al.* (1) was not due to induction of enzyme synthesis by the vitamin but was related to the conversion of the transmethylase apoenzyme to holoenzyme.

The requirements of the HeLa transmethylase (a reducing system, homocysteine and a slight stimulation by *S*-adenosylmethionine) are similar to those observed in crude extracts of *E. coli* and mammalian liver (18, 19) (Table II).

Similar to what has been seen in studies with *E. coli* (20), hydroxy-B_{12}, DBCC, and methyl-B_{12} were able to form holoenzyme

TABLE I

NATURE OF METHIONINE SYNTHETASE IN HeLa CELLS[a]

Additions to growth medium	Cofactor used in *in vitro* assay	mμmoles methionine/mg/hr	% Holoenzyme (Propyl B_{12}/Methyl B_{12}) × 100
None	Propyl B_{12}	0.6	9
	Methyl B_{12}	6.6	
Vitamin B_{12a}	Propyl B_{12}	7.6	98
	Methyl B_{12}	7.7	

[a] Details of the experiments are described in the text and the assay for the amount of holoenzyme present has been described previously (15).

TABLE II

COFACTOR REQUIREMENTS FOR METHIONINE SYNTHESIS IN HeLa CELL EXTRACTS[a]

	mμmoles/mg/hr	
	Apoenzyme	Holoenzyme
Complete	6.6	8.8
− Homocysteine	0.3	0.6
− *S*-Adenosylmethionine	5.0	7.6
− Reducing system	0.0	0.0
− Methyl-B_{12} + propyl-B_{12}	0.6	7.7

[a] Details of the incubation are described in the text. The reducing system consisted of 0.2 M 2-mercaptoethanol and 5×10^{-5} M cobamide (15). Thirty microliters of extract (400 μg of apoenzyme protein and 580 μg of holoenzyme protein) were used for these experiments.

when HeLa cells were incubated with these cobamides *in vivo* (Table III). These derivatives are thought to be the predominant forms of the vitamin found in animal tissues.

Studies with DBCC in HeLa cells; Role of DBCC in methionine synthesis. Previous *in vitro* studies (15) with *E. coli* extracts have shown that the transmethylase apoenzyme can be converted to holoenzyme in the presence of DBCC if the system is supplemented with a specific reducing system consisting of DPNH and a protein fraction of *E. coli* extracts that precipitates between 60–90% saturation with $(NH_4)_2SO_4$.[4] It has been proposed that DBCC first binds to the transmethylase apoenzyme to yield an enzyme–DBCC complex. Cleavage of the carbon–cobalt bond of the enzyme-bound DBCC could yield active holoenzyme. Since *in vivo* formation of holoenzyme could be demonstrated with HeLa cells grown in the presence of DBCC (Table III), attempts were made to demonstrate a similar reaction *in vitro*. The results of a typical experiment are shown in Table IV. Unlike the results with bacterial extracts, DBCC was not able to satisfy the cobamide requirement

[4] While this manuscript was in preparation, Rosales, Ritari, and Sakami (*Biochem. Biophys. Res. Commun.* **40**, 271, 1970) have reported the separation of holoenzyme synthetase of *E. coli* into two components both of which are required for holoenzyme formation from apoenzyme and DBCC.

TABLE III

EFFECT OF VITAMIN B_{12} DERIVATIVES ON THE *In Vivo* FORMATION OF HOLOENZYME[a]

Additions to growth medium	% Holoenzyme
None	6
Hydroxy-B_{12}	43
Methyl-B_{12}	44
DBCC	39

[a] Where indicated, 20 μg of each cobamide were added to 200 ml of cell suspension and growth was continued for 3 hr. Other details are described in the text.

TABLE IV

EFFECT OF DBCC ON THE ACTIVITY OF HELA APOTRANSMETHYLASE

Conditions	mμmoles methionine/mg/hr
+Methyl-B_{12}	5.1
−Methyl-B_{12} + Propyl-B_{12}	0.5
−Methyl-B_{12} + DBCC	0.4
−Methyl-B_{12} + DBCC + DPNH	0.5
−Methyl-B_{12} + DBCC + DPNH + *E. coli* holoenzyme synthetase[a]	0.3

[a] Under the conditions of the assay, *E. coli* holoenzyme synthetase catalyzed complete conversion of *E. coli* apoenzyme (25–45% ammonium sulfate fraction) to holoenzyme in the presence of DPNH (15). The concentration of cobamides used in the assay was 5×10^{-5} M and that of DPNH was 5×10^{-3} M. Details of the assay and other experimental conditions are described in the text.

when extracts of HeLa cells were supplemented with either DPNH or TPNH. In addition, holoenzyme synthetase [a 60–90% $(NH_4)_2SO_4$ fraction from *E. coli*] could not catalyze holoenzyme formation from HeLa apoenzyme and DBCC in the presence of DPNH. Other reductants such as DTT, 2-mercaptoethanol, reduced lipoic acid, $FADH_2$ and ascorbic acid were also tested but found to be ineffective. Thus, although DBCC could function *in vivo* in the formation of holoenzyme, its ability to form holoenzyme *in vitro* could not be demonstrated under the conditions tested.

Studies on the oxidation of propionate in HeLa cells. HeLa cells grown in the absence of vitamin B_{12} appeared vitamin B_{12}-deficient as evidenced by the fact that there was not sufficient vitamin to satisfy the cobamide requirement *in vivo* for the transmethylase reaction. A vitamin B_{12}-deficient animal cell would be a useful tool for metabolic studies, and therefore attempts were made to establish this point. If HeLa cells were vitamin B_{12} deficient, one would expect that the oxidation of propionate should be affected in cells grown in the absence of added vitamin since this conversion involves the DBCC-dependent, methylmalonyl-CoA isomerase. Previous studies by Rosenberg *et al.* (2) have shown that in B_{12} deficiency, oxidation of propionate is decreased as compared to normal cells.

The oxidation of propionate by cells grown in the absence of the vitamin was compared with cells grown in medium supplemented with the vitamin. As seen in Table V, there was no significant difference between the two systems. It should be also noted in Table V that the oxidation of succinate, which was used as a control for the propionate studies, showed no difference between cells grown with or without the vitamin. Thus, the amount of vitamin B_{12} or DBCC present in calf serum used in the growth medium appears to be sufficient to saturate the isomerase requirement for the oxidation of propionate. This could be due to either a much lower requirement for DBCC to saturate the isomerase as compared to the transmethylase enzyme, or that

TABLE V

OXIDATION OF PROPIONATE AND SUCCINATE BY HELA CELLS[a]

Growth conditions	Time (min)	Additions	μμmoles CO_2 liberated	
			Propionate	Succinate
$-B_{12}$	30	None	120	880
		+DBCC	120	—
	60	None	180	180[illegible]
		+DBCC	190	[illegible]
$+B_{12}$	30	None	131	7[illegible]
		+DBCC	120	
	60	None	210	2[illegible]
		+DBCC	210	

[a] Details of the assay are described in the [illegible]

he amount of isomerase containing DBCC is ot rate limiting in propionate oxidation, ven though the isomerase is not completely n the holoenzyme form. Hence, cells grown vithout vitamin B_{12} are B_{12} deficient in the ransmethylase reaction but appear normal with regard to propionate metabolism. We have not eliminated the possibility that propionate may be converted to CO_2 via a B_{12} independent pathway, although to our knowledge there is no evidence for such a reaction in mammalian systems.

DBCC synthesis in HeLa extracts. Since HeLa cells grown in the presence or absence of added vitamin B_{12} were able to oxidize propionate, it appeared that these cells obtained sufficient DBCC from the medium or could synthesize DBCC from the vitamin. Although the enzymatic synthesis of DBCC has been studied in some detail in bacterial systems (22, 23), and in fact, several laboratories have shown the presence of DBCC in animal tissues and tissue culture cells (4–7), the *in vitro* synthesis of this coenzyme by animal cell extracts has not been demonstrated. Using extracts of HeLa cells grown in the absence of vitamin B_{12}, it was possible to show the presence of an enzyme which catalyzes DBCC synthesis from hydroxy-B_{12} and ATP as measured by the dioldehydrase reaction. The enzyme was present in the soluble fraction after centrifuging the cell homogenate at 12,000*g* for 30 min. Using a 0–50% $(NH_4)_2SO_4$ precipitable fraction, the characteristics of the reaction were examined. As seen in Fig. 1, the amount of DBCC synthesis was linear with time (up to 60 min) and with enzyme concentration. Table VI shows the cofactor requirements for DBCC synthesis using a 0–50% $(NH_4)_2SO_4$ fraction. The reaction was dependent on the presence of ATP, hydroxy-B_{12}, FAD and a reducing system. A slight stimulation of the reaction was obtained when Mg^{2+} was present. As seen in Figs. 2 and 3, DBCC synthesis was maximal when the amounts of hydroxy-B_{12} and ATP added to the incubation were 1.0 mμmole and 0.25 μmoles, respectively.

As mentioned above, the assay for DBCC formation was based on the ability of the

TABLE VI

COFACTOR REQUIREMENTS FOR DBCC SYNTHESIS

Omissions	mμmoles DBCC
None	0.12
−FAD	0.03
−Hydroxy-B_{12}	0
−ATP	0
−Mg	0.09
−(DTT + 2-mercaptoethanol)	0
−Enzyme	0
None[a]	0

[a] This sample was exposed to light for 15 min (150 W, 20 cm) before aliquots were removed for DBCC assay with dioldehydrase. The incubations were for 30 min and contained 0.05 ml of a 0–50% $(NH_4)_2SO_4$ fraction (1.24 mg of protein). Other details are described in the text.

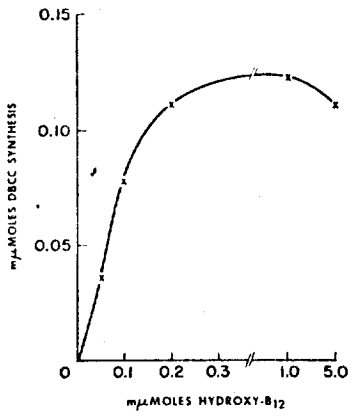


FIG. 2. Effect of hydroxy-B_{12} concentration on DBCC synthesis. 0.05 ml of a 0–50% $(NH_4)_2SO_4$ fraction (1.24 mg protein) was used, and incubations (0.55 ml) were for 30 min at 37°.

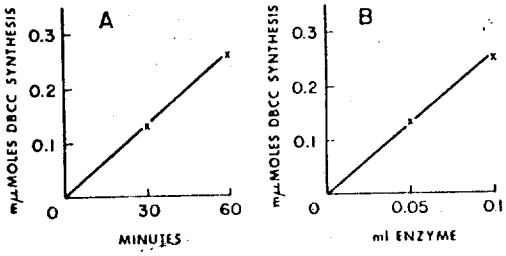


FIG. 1. A. Time course study of DBCC synthesis using 0.05 ml of a 0–50% $(NH_4)_2SO_4$ fraction (1.24 mg protein). B. Effect of enzyme concentration on DBCC synthesis using a 30-min incubation.

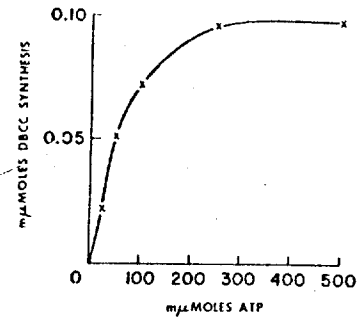


FIG. 3. Effect of ATP concentration on DBCC synthesis. 0.05 ml of a 0-50% $(NH_4)_2SO_4$ fraction (1.24 mg protein) was used, and incubations (0.55 ml) were for 30 min at 37°.

coenzyme to stimulate the dioldehydrase reaction, but to provide more evidence that coenzyme synthesis took place, advantage was taken of the light sensitivity of DBCC. As seen in Table VI, light exposure of the reaction mixture after incubation inactivated the material which stimulated the dioldehydrase reaction. Thus far preliminary attempts to further purify the enzyme responsible for DBCC synthesis have been unsuccessful.

Previous studies with experimental vitamin B_{12} deficiency in animals have established that the ability of the animal to synthesize methionine from methyl-H_4-folate is greatly diminished. Since the regeneration of H_4-folate from methyl-H_4-folate is dependent on the B_{12} transmethylase reaction (18), it would be expected that in vitamin B_{12} deficiency the regeneration of H_4-folate from its methyl derivative would be diminished. How this experimental metabolic lesion affects other H_4-folate enzymes is not known. Since HeLa cells grown in tissue culture appear B_{12} deficient in the transmethylase reaction, these cells might serve as a model animal system in which to study the metabolic interrelationship among folic acid, vitamin B_{12} and one-carbon metabolism.

The present experiments have also described for the first time a cell-free system from animal cells capable of converting hydroxy-B_{12} to its coenzyme form. A defect in DBCC synthesis has recently been considered by Rosenberg *et al.* (2) and Mudd *et al.* (3) as the basis for the increased excretion of methylmalonic acid observed in certain patients with methylmalonic aciduria. Of interest was the finding that cultured fibroblasts of certain patients suffering from methylmalonic aciduria had much lower levels of DBCC than normal cells (2). Based on the present results, it should be possible to assay the amount of DBCC synthetase in tissue culture cells to determine whether the metabolic abnormality in these patients is due to a defective or missing enzyme.

REFERENCES

1. MANGUM, J. H., MURRAY, B. K., AND NORTH, J. A., *Biochemistry* **8**, 3496 (1969).
2. ROSENBERG, L. E., LILLIJEQUIST, A. L., HSIA, Y. E., AND ROSENBLOOM, I. M., *Biochem. Biophys. Res. Commun.* **37**, 607 (1969).
3. MUDD, S. H., LEVY, H. L., AND ABELES, R. H., *Biochem. Biophys. Res. Commun.* **35**, 121 (1969).
4. WEISSBACH, H., TOOHEY, J. I., AND BARKER, H. A., *Proc. Nat. Acad. Sci. U.S.A.* **45**, 521 (1959).
5. TOOHEY, J. I., AND BARKER, H. A., *J. Biol. Chem.* **236**, 560 (1961).
6. CARDINALE, G. J., DREYFUS, P. M., AULD, P., AND ABELES, R. H., *Arch. Biochem. Biophys.* **131**, 92 (1959).
7. PERLMAN, D., AND GUIFFRE, N. A., *N. Y. Acad. Sci.* **112**, 831 (1964).
8. WEISSBACH, H., PETERKOFSKY, A., REDFIELD, B., AND DICKERMAN, H., *J. Biol. Chem.* **238**, 3318 (1963).
9. WEISSBACH, H., REDFIELD, B., AND DICKERMAN, H., *Biochem. Biophys. Res. Commun.* **17**, 17 (1964).
10. BRAY, A. G., *Anal. Biochem.* **1**, 279 (1960).
11. ABELES, R. H., MYERS, C., AND SMITH, T. A., *Anal. Biochem.* **15**, 192 (1966).
12. KERESZTESY, J. C., AND DONALDSON, K. O., *Biochem. Biophys. Res. Commun.* **5**, 286 (1960).
13. SMITH, E. L., MERVYN, L., JOHNSON, A. W., AND SHAW, N., *Nature (London)* **194**, 175 (1962).
14. LEE, H. A., JR., AND ABELES, R. H., *J. Biol. Chem.* **238**, 2367 (1967).
15. BROT, N., AND WEISSBACH, H., *J. Biol. Chem.* **241**, 2024 (1966).
16. LOWRY, O., ROSEBROUGH, N., FARR, A., AND RANDALL, R., *J. Biol. Chem.* **193**, 265 (1951).

17. WARBURG, O., AND CHRISTIAN, W., *Biochem. Z.* **310**, 384 (1941).
18. ELFORD, H. L., KATZEN, H. M., ROSENTHAL, S., SMITH, L. C., AND BUCHANAN, J. M., *in* "Transmethylation and Methionine Biosynthesis" (S. K. Shapiro and F. Schlenk, eds.). Univer. of Chicago Press, Chicago (1965).
19. LOUGHLIN, R. E., ELFORD, H. L., AND BUCHANAN, J. M., *J. Biol. Chem.* **239**, 2888 (1964).
20. WEISSBACH, H., REDFIELD, B., DICKERMAN, H., AND BROT, N., *J. Biol. Chem.* **240**, 856 (1965).
21. WEISSBACH, H., REDFIELD, B., AND PETERKOFSKY, A., *J. Biol. Chem.* **236**, 40 (1961).
22. VITOLS, E., WALKER, G. A., AND HUENNEKENS, F. M., *J. Biol. Chem.* **241**, 1455 (1966).

Archives of Disease in Childhood, 1970, 45, 385.

Treatment of Neuroblastoma with Vitamin B12

A Report to the Working Party* of the Medical Research Council

M. J. S. LANGMAN†
From University Department of Medicine, General Hospital, Nottingham

Langman, M. J. S. (1970). *Archives of Disease in Childhood*, 45, 385. **Treatment of neuroblastoma with vitamin B12.** A retrospective study of treatment given to 108 children with neuroblastoma failed to confirm that vitamin B12 therapy is beneficial. 43 of 47 children whose treatment did not include vitamin B12 are known to have died, compared with 56 of 61 children who received the vitamin in addition to other treatments. 2 children who received vitamin B12 as their only treatment were alive and well six years later, but this apparently favourable response could well be ascribed to spontaneous remission of the tumour.

In 1960 a Medical Research Council Working Party was set up to examine the suggestion that vitamin B12 might be of value in treating neuroblastoma (Bodian, 1959). It was agreed that a controlled clinical trial could not be started because such a course would have involved the denial to one group of patients of a potentially valuable yet apparently harmless treatment. Data were, therefore, collected retrospectively from various centres in the United Kingdom so that the results of treatment in children with neuroblastoma who had or who had not received vitamin B12 could be compared.

Method

Questionaries asking for clinical details of children treated for neuroblastoma were sent to 55 hospitals which agreed to co-operate in the study. The answers to the questionaries, amplified by later inquiries about follow-up results, form the basis of this report.

Results

Reports on the clinical course of 134 patients were received but 26 were unsuitable for analysis. In 7 patients a definite diagnosis of neuroblastoma could not be established, in 12 patients information about general treatment was inadequate, and in 3 it was unclear whether vitamin B12 had been given; the remaining 4 died before any medical treatment could be administered.

Sixty-five patients had received treatment with vitamin B12 at some time during their illness and 43 had never been given any such treatment. However, 4 of the treated group had received vitamin B12 only by mouth, and as it has been claimed that to be effective vitamin B12 must be given in large doses (Bodian, 1963), these 4 were transferred to the control group. The 61 remaining treated patients had all received at least 2,000 μg. vitamin B12 by injection during the course of their illness, but at varying intervals after the diagnosis of the tumour. The inclusion in the treated group of individuals who had been given vitamin B12 as a late secondary treatment would clearly have biased the results, and therefore the main comparison was restricted to the 35 whose treatment with vitamin B12 had been started less than one month after the diagnosis had been made and the 47 who received either no vitamin B12 or only oral treatment. In 17 of the remaining 26 the dates of administration of the drug were uncertain, and in 9 it had been given at least one month after diagnosis of the disease.

Table I gives the numbers of children who are known to have died and the number who were still alive when last followed up, and their survival time after the diagnosis of neuroblastoma. Only 9 (8%) of the whole group were still alive at the time of the last inquiry; they included 4 (9%) of the 47 who had not received vitamin B12, and 2 (6%) of those 35 who had received early treatment with

Received 27 October 1969.

*Members of the working party were: Professor W. Gaisford, Professor D. A. G. Galton, Professor Sir Alexander Haddow, Professor Sir Austin Bradford Hill, Professor Sir A. Moncrieff, Dr. Edith Paterson, Professor R. W. Scarff, Professor Sir Brian Windeyer, the late Mr. G. H. Macnab and the late Dr. M. Bodian.

†Formerly member of Scientific Staff, M. R. C. Statistical and Gastroenterological Research Units.

TABLE I

Survival Time and Final Outcome in Patients Treated with or without Vitamin B12

Treatment Group	Results	Survival Time (mth.)				Survival Time (mth.) in Those Living >3 years
		0–12	13–24	25–36	37 or more	
No vitamin B12						
Dead	43	30	8	2	3	41, 70, 76
Alive	4	—	—	—	4	72, 76, 120, 120
Early vitamin B12						
Dead	33	27	4	1	1	39
Alive	2	—	—	—	2	51, 120
Late vitamin B12						
Dead	9	5	2	1	1	39
Alive	0	—	—	—	—	—
Vitamin B12 given but dates uncertain						
Dead	14	8	3	3	—	—
Alive	3	—	—	—	3	39, 72, 79
Total	108	70	17	7	14	

vitamin B12. In addition 3 (18%) were still alive of the 17 who had been given vitamin B12 though at uncertain dates. If all in this group had properly been included in the early vitamin treated group, the survival rate in that group would have been raised to 10% but it would still have been immaterially different from that in the group who did not receive vitamin B12.

Tables II and III give details of the comparability of the patients in respect of age, presence or absence of metastases when first seen, and other treatments given. There were no clear differences except that when compared with the group of patients treated early with the vitamin a slightly higher proportion of those who did not receive the vitamin were treated by operation, suggesting that they had localized disease. This difference could not, however, account for the poor general results obtained with vitamin treatment. Of those children living for at least five years 2 received no treat-

TABLE II

Comparability in Age and in Presence or Absence of Metastases when First Seen

Metastases	No Vitamin B12	Early Vitamin B12	Late Vitamin B12	Vitamin B12 Given but Dates Uncertain
Present	33	26	6	12
Absent	12	5	1	2
Not known	2	4	2	3
Total	47	35	9	17
Ages (yr.)				
<1	4	4	—	2
1–2	11	10	—	3
2–3	5	5	2	4
3–4	4	3	1	—
4 and over	23	13	6	8
Total	47	35	9	17

TABLE III

Comparability in Other Treatments Given

Treatment	No Vitamin B12	Early Vitamin B12	Late Vitamin B12	Vitamin B12 Given but Dates Uncertain
Surgery	6 (1)	1	—	3
Radiotherapy	15 (1)	10	3	5
Chemotherapy/steroids	3	2	—	—
Radiotherapy and excision	11 (2)	6	5	3 (1)
Radiotherapy, excision, and chemotherapy/steroids	—	—	—	1
Excision and chemotherapy	2	—	—	1
Radiotherapy and chemotherapy/steroids	1 (1)	5	—	—
None	9 (1)	11 (1)	1	4 (1)
Total	47 (6)	35 (1)	9	17 (2)

The numbers of individuals surviving at least five years included in each group are given in parentheses.

ment other than vitamin B12, one further patient was given no specific treatment at all and, in general, none of the combinations of treatments appears to have been particularly successful.

Discussion

The results of this investigation have failed to confirm that vitamin B12 is of value in the treatment of neuroblastoma, and the findings are in agreement with those obtained in a similar retrospective study of American experience (Sawitsky and Deposito, 1965), based on 103 patients with neuroblastoma treated with or without vitamin B12. It was then concluded that the vitamin did not confer any therapeutic benefit, the outcome of treatment being thought to be influenced more by the natural behaviour of the tumour than by the individual remedies prescribed.

Though exact details of vitamin B12 dosage could not always be obtained from the records in this retrospective study, it did not seem likely that inadequate dosage could explain the poor results of vitamin treatment. Most of the treated patients had received frequent injections each of 1 mg. or more of the vitamin, and the intensity of initial treatment did not appear to be related to the length of survival. 2 of the 16 patients given vitamin B12 as their only treatment were still alive five years later, as was 1 of the 9 patients who received no specific treatment, and their survival could be ascribed to slow growth of the tumour or to spontaneous regression which sometimes occurs in the absence of specific treatment. The poor general response of patients with neuroblastoma is clearly illustrated by the survival curve shown in the Fig. Half of all the patients were dead within six months and four-fifths within two years.

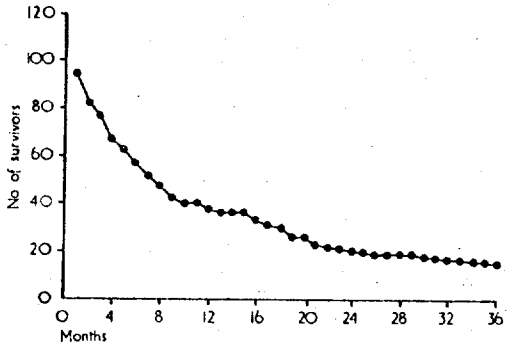


FIG.—*Survival curve for the total group of 108 patients initially treated.*

REFERENCES

Bodian, M. (1959). Neuroblastoma. *Pediatric Clinics of North America*, **6**, 449.

—— (1963). Neuroblastoma: an evaluation of its natural history and the effects of therapy with particular reference to treatment by massive doses of vitamin B12. *Archives of Disease in Childhood*, **38**, 606.

Sawitsky, A., and Deposito, F. (1965). A survey of American experience with vitamin B12 therapy of neuroblastoma. *Journal of Pediatrics*, **67**, 99.

Correspondence to Dr. M. J. S. Langman, Department of Medicine, General Hospital, Nottingham NG1 6HA.

Nature (London) 1969, 224 (5221), 806 (Eng)

Alcohol-induced Malabsorption of Vitamin B_{12} in Man

INTOXICATION with alcohol is frequently associated with diarrhoea, and disturbances of intestinal function have been reported to be common in chronic alcoholics studied shortly after drinking-bouts[1-5]. Because the normal diet of alcoholics is characteristically poor, it is not clear whether the transient malabsorption frequently found can be attributed to a direct toxic effect of ethanol on the small intestine, or to a nutritional deficiency. We therefore studied the effects of the experimental ingestion of ethanol in man on vitamin B_{12} absorption under controlled metabolic ward conditions in the absence of nutritional deficiency.

Our experimental design was similar to that of previous studies[6,7] in which toxic effects of ethanol on liver and bone marrow in man were demonstrated. Four asymptomatic, haematologically normal adult male volunteers with normal liver biopsies and a history of chronic alcoholism were studied. Throughout each of three study periods (pre-ethanol control, ethanol and post-ethanol control), they received daily multivitamin supplements including pharmacological doses of folic acid[7]. Protein intake comprised 12·5 per cent of total calories in two, and 25 per cent in two others. Ethanol was substituted isocalorically for carbohydrate for 3 to 8 weeks, the intake having the same calorie value. The maximal daily dose varied from 158 to 253 g ethanol.

Vitamin B_{12} absorption was studied during control and ethanol periods by the method of Schilling[8]. ^{57}Co-cyanocobalamin (0·75 μg; specific activity 1 μCi/μg) was given orally with intrinsic factor concentrate. The urinary excretion of ^{57}Co-vitamin B_{12} during ethanol testing periods was less than that of any of the control values for each subject (Table 1). Comparison of control and ethanol values revealed an average decrease in B_{12} excretion during the alcohol period of 41·5 per cent (range 22·9–52·7 per cent) for 24 h urines, and 34·7 per cent (range 17·1–47·5 per cent) for 48 h ($P < 0·005$ for 24 h and $P < 0·01$ for 48 h). In two subjects the 24 h excretion during the ethanol period fell below what we consider as normal (7 per cent or greater). Urine volumes did not differ between periods. In subject 1, faecal radioactivity was determined for two weeks after three of the Schilling tests, and was higher during the ethanol period (pre-ethanol 17·7 per cent of administered dose; ethanol 41·3 per cent; post-ethanol 34·4 per cent). Serum vitamin B_{12} levels[9], and urinary indican excretion[10], remained normal throughout the study.

Our results indicate that the administration of ethanol for two weeks or more along with a proper intake of protein and vitamin interferes with the absorption of vitamin B_{12} by the human ileum. That impairment of ileal function occurred was further suggested by the presence of striking ultrastructural abnormalities (dilated endoplasmic reticulum and focal cytoplasmic degradation) in an ileal biopsy obtained from subject 2 on the fifty-first ethanol day. Light microscopic histological findings were normal. (These changes will be reported in detail by B. Rybak, E. Rubin, J. L. and C. S. L. elsewhere.) Whether the "toxic" effect of ethanol is limited to B_{12} assimilation or to ileal function in general remains to be determined. In animals, depression of active jejunal transport of amino-acids by ethanol has been reported[11,12].

Table 1. PER CENT URINARY EXCRETION OF ^{57}CO-VITAMIN B_{12} DURING CONTROL PERIODS AND ETHANOL ADMINISTRATION IN HUMAN VOLUNTEERS

Subject	Urinary ^{57}Co-vitamin B_{12} excretion (per cent) Control (pre- and/or post ethanol) 24 h	48 h	Ethanol 24 h	48 h	Days on ethanol when tested (per cent)	Maximal daily dose (total calories intake)
1	42·4	51·4	27·4	33·0	13	46
	40·7	50·6	11·9	20·6	26	
2	12·1	16·2	5·9	9·1	21	62
	12·1	16·8	6·7	11·0	31	
	11·2	17·5				
3	17·0	21·0	13·1	17·4	21	69
4	10·5	15·4	5·9	10·2	34	63

This work was supported by grants from the U.S. Public Health Service. We thank Mrs Nancy Shea and Miss Nancy Lowe for technical assistance.

JOHN LINDENBAUM

Department of Medicine,
Columbia University, and
Harlem Hospital Center.

CHARLES S. LIEBER

Department of Medicine,
Mount Sinai School of Medicine, and
Section of Liver Disease and Nutrition,
Bronx Veterans Administration Hospital,
New York.

Received July 14; revised August 29, 1969.

1 Roggin, G. M., Kater, R. M. H., Tobon, F., and Iber, F. L., *Ann. Int. Med.*, 70, 1070 (1969).
2 Small, M., Longarini, A., and Zamcheck, N., *Amer. J. Med.*, 27, 575 (1959).
3 Halstead, C. H., Griggs, R. C., and Harris, J. W., *J. Lab. Clin. Med.*, 69, 116 (1967).
4 Tomasulo, P. A., Kater, R. M. H., and Iber, F. L., *Amer. J. Clin. Nutr.*, 21, 1340 (1968).
5 Thomson, A., Baker, H., and Leevy, C. M., *Amer. J. Clin. Nutr.*, 21, 537 (1968).
6 Lieber, C. S., and Rubin, E., *Amer. J. Med.*, 44, 200 (1968).
7 Lindenbaum, J., and Lieber, C. S., *New Engl. J. Med.*, 281, 333 (1969).
8 Schilling, R. F., *J. Lab. Clin. Med.*, 42, 860 (1953).
9 Lau, K., Gottlieb, C., Wasserman, L. R., and Herbert, V., *Blood*, 26, 542 (1965).
10 Carson, G., and Walsh, J., *Clin. Chim. Acta*, 7, 657 (1962).
11 Chang, T., Lewis, J., and Glazko, A. J., *Biochim. Biophys. Acta*, 135, 1033 (1967).
12 Israel, U., Salazar, I., and Rosenmann, E., *J. Nutrit.*, 96, 499 (1968).

J. Allerg. 34: 382-387

TUBERCULIN TYPE OF HYPERSENSITIZATION TO CRYSTALLINE VITAMIN B-12 (CYANOCOBALAMIN) INDUCED IN GUINEA PIGS

Murray M. Lipton and Alex J. Steigman,** Louisville, Ky.*

EXPERIMENTALLY induced sensitization to central nervous system tissue can cause extensive changes in the CNS of guinea pigs and is referred to as experimental allergic encephalomyelitis. The precise dynamics of the events is not elucidated.[1] One possibility is that certain normal metabolic pathways are specifically interrupted which leads to the CNS changes observed.

The subacute combined degeneration of human spinal cord associated with improper vitamin B_{12} metabolism has no experimental counterpart. An effort is described of an attempt to interfere with normal vitamin B_{12} metabolism in the guinea pig by inducing sensitization to vitamin B_{12}. No CNS changes were observed but, in the course of the study, a tuberculin-type cutaneous sensitization to crystalline vitamin B_{12} was developed. Crystalline vitamin B_{12} purified from Streptomyces fermentation† (as ordinarily used in human therapy) and crystalline vitamin B_{12} prepared from liver‡ were used.

There have been several reports of hypersensitivity to vitamin B_{12} in human subjects injected either with *crude* extracts or concentrates prepared from liver or fermentation of Streptomyces. In 1950 Young and associates[2] reported a case of anaphylactic hypersensitivity in a person sensitive to purified liver extracts following the administration of vitamin B_{12} concentrate made from streptomycin broth. This individual did not react to crystalline vitamin B_{12}.

Bedford[3] reported that 14 of 100 individuals, skin tested with concentrates made from Streptomyces fermentation liquors, showed positive skin reaction and that 6 showed side effects after intramuscular injection. When these individuals were tested with crystalline material prepared from Streptomyces fermentation only 2 showed positive skin tests and none had side effects following intramuscular administration. None of the individuals showed sensitvity

From the Kentucky Child Health Foundation Laboratory, Department of Pediatrics, University of Louisville School of Medicine, Louisville, Ky.

This investigation was supported in whole by U. S. Public Health Service Research Grant No. NB 00982 from the National Institute for Neurological Diseases and Blindness.

Received for publication March 25, 1963.

*Career Development Award of the National Institute of Allergy and Infectious Diseases, Grant No. 5-K3-AI-14920.

**Present address: P. O. Box 1035, Evanston, Illinois.

†Obtained from Dr. Harry J. Robinson of the Merck Institute for Therapeutic Research.

‡Obtained from Dr. Bacon Chow (Johns Hopkins University).

to purified vitamin B_{12} prepared from liver. Those individuals who had received large amounts of antibiotics in the past were sensitive to vitamin B_{12} prepared from Streptomyces. Bedford, therefore, attributed the sensitization to by-products rather than to vitamin B_{12} itself.

Rostenberg and Perkins[4] demonstrated a tuberculin-type skin sensitization to crystalline vitamin B_{12} in an individual who was sensitive to cobalt and nickel. Ungley[5] reported the occurrence of either the immediate type or atopic type of sensitization in 3 individuals to crystalline vitamin B_{12}, the source of which was not given. The fact that the sensitization was transferred to a nonsensitive normal subject, in what appeared to be a Prausnitz-Küstner transfer by serum, rules out the delayed type of sensitization. He stated that in the following 4 years with the use of crystalline vitamin B_{12} no other cases were encountered. Traina[6] reported the inability (in a very limited attempt) to produce anaphylactic shock in guinea pigs injected subcutaneously with many very small doses of crystalline vitamin B_{12}. Traina also mentions the anti-anaphylactic effect of vitamin B_{12}.

MATERIALS AND METHODS

Hartley strain guinea pigs of both sexes, weighing from 500 to 650 grams, were used throughout the experiments. They were fed Purina guinea pig chow and alfalfa hay, and were given water ad libitum.

In the first experiment, crystalline vitamin B_{12} prepared from Streptomyces fermentation was used for the initial injection as follows: a solution of the crystals in sterile distilled water or 0.85 per cent sodium chloride solution was emulsified with complete Freund's adjuvants as described previously.[7] The emulsion for the initial injection contained aqueous vitamin B_{12}, killed dried *Mycobacterium tuberculosis*, Arlacel A, and light mineral oil. Each animal was injected intracutaneously at seven sites, six on the back with 0.05 ml. of emulsion and 0.1 ml. on the ventral aspect of the neck. Each animal received a total of 2.0 mg. of crystalline vitamin B_{12} and 0.8 mg. of killed tubercle bacilli in a total volume of 0.4 ml. of emulsion. Two control groups of animals were as follows: a group of 8 normal uninjected animals and a group of 8 animals injected with complete Freund's adjuvants without vitamin B_{12}.

Animals were weighed and observed for a period of 28 days after immunization. At this time all animals were skin tested by the intracutaneous injection of an aqueous (distilled water) solution of crystalline vitamin B_{12} in the right flank. Three different concentrations of crystalline vitamin B_{12} were used; 200 gammas, 20 gammas and 2 gammas each, in a volume of 0.1 ml. The skin test sites were observed for a period of 72 hours; measurements were made of the erythema as well as of the type and degree of swelling after 5, 24, 48, and 72 hours.

The second experiment included two groups of 8 animals each. One group received crystalline vitamin B_{12} prepared from Streptomyces fermentation in complete Freund's adjuvants as outlined above. The second group received only complete Freund's adjuvant and served as controls. At 13 days all 16

animals were skin tested on the right side with 100 gammas of the vitamin B_{12} prepared from Streptomyces. At 20 days and at 27 days these animals also received on the same side (right) two skin test doses of 100 gammas and 10 gammas of vitamin B_{12}. The skin test sites were examined and measured as in the first experiment. Three of the experimental animals which showed the highest degree of sensitization as judged by skin test reaction at 27 days were skin tested on the previously uninjected side (left) with 40 gammas of crystalline vitamin B_{12} derived from *liver* in a volume of 0.2 ml. Three animals injected with Freund's complete adjuvants alone were similarly skin tested at the same time.

The remaining 5 animals in each group were skin tested at 40 days with 20 gammas of crystalline vitamin B_{12} from liver in a volume of 0.1 ml. These skin tests were done on the previously untested left side.

RESULTS

The results are summarized in Tables I and II. Table I shows that all animals injected originally with crystalline vitamin B_{12} (Streptomyces) in Freund's complete adjuvants developed skin sensitization to 200 gammas of crystalline vitamin B_{12} injected intracutaneously. Only 2 of the 8 animals showed positive tests to 20 gammas of vitamin B_{12} and none of these 8 animals reacted to 2 gammas of vitamin B_{12}. No reaction was observed after 5 hours. Skin reactions reached their peak between 24 and 48 hours and were still visible in most instaces 72 hours after injection. This indicated a tuberculin or delayed type of hypersensitivity. All 8 of the control animals injected with com-

TABLE I. SKIN REACTIONS IN 8 GUINEA PIGS SENSITIZED AND SKIN TESTED WITH CRYSTALLINE VITAMIN B_{12} (CYANOCOBALAMIN) DERIVED FROM STREPTOMYCES FERMENTATION*

AMOUNT†	TIME OF READING OF REACTION (HOURS AFTER INJECTION OF TEST DOSE)			
	5	24	48	72
200	Neg.	WDdP-Ind. 8‡	WDP-Ind. 7	WDP-Ind. 8
20	Neg.	WDP-Ind. 6	IDP-Ind. 7	WDP-Ind. 7
200	Neg.	WDR-Ind. 10	WDdP-Ind. 10	WDP-Ind. 10
20	Neg.	WDP-Ind. 5 × 4	P-Nod.4	P Nod. 3
200	Neg.	WDR-Ind. 10 × 8	WDP-Ind. 10 × 7	ID-P-Ind. 8
20	Neg.	Neg.	Neg.	Neg.
200	Neg.	WDR-Ind. 9	WDdP-Ind. 9 × 6	IDP-Ind. 7
20	Neg.	Neg.	Neg.	Neg.
200	Neg.	WDdP-Ind. 9 × 6	WDdP-Ind. 7	IDP-Ind. 8 × 5
20	Neg.	Neg.	Neg.	Neg.
200	Neg.	WDP-Ind. 8	WDR-Ind. 10	IDP-Ind. 7
20	Neg.	Neg.	Neg.	Neg.
200	Neg.	IDdP-Ind. 7	IDP-Ind. 5	IDP-Nod. 5
20	Neg.	Neg.	Neg.	Neg.
200	Neg.	WDP-Ind. 7 × 5	IDdP-Ind. 7 × 5	IDP-Nod. 5
20	Neg.	Neg.	Neg.	Neg.

Legend: WD—Well defined; ID—ill defined; Nod.—nodule; Ind.—induration (thickness 1 to 3 mm.); P—pink; R—red; dP—dark pink.

**Controls* injected with *adjuvants alone* or *uninjected* (8 per group) were *negative* with occasional ill-defined nodules 2 to 3 mm. in diameter.

†Test dose (gammas) in 0.1 ml. volume.

‡Dimensions are in millimeters and one number is diameter. Two numbers are 2 dimensions if reaction was irregular.

plete Freund's adjuvants and the 8 uninjected controls showed no skin reaction to the injection of 200 gammas of crystalline vitamin B_{12}.

Table II shows the results of using crystalline vitamin B_{12} prepared from Streptomyces fermentation for the initial injection and skin tests and that derived from liver for final skin tests. Not presented in the table are the skin test reactions observed at 13 days and at 20 days following the intracutaneous injection of crystalline vitamin B_{12} prepared by Streptomyces fermentation. The group injected with crystalline vitamin B_{12} combined with Freund's adjuvants showed weak but positive skin reactions when tested at 13 days and stronger reactions at 20 days. Skin tests done at 27 days with Streptomyces crystalline vitamin B_{12} are presented in the table. These reactions ranged from well-defined pink and indurated zones measuring 14 mm. in diameter to a negative reaction seen in one animal which was tested with 100 gammas of crystalline vitamin B_{12}. Four of the 8 animals showed sensitization at the 10-gamma skin site. The animals treated at 35 days with vitamin B_{12} derived from liver and injected with 40 gammas in a 0.2 ml. volume were highly positive. All of the remaining 5 animals skin tested at 40 days showed reactions to 20 gammas in 0.1 ml. of crystalline liver vitamin B_{12}.

TABLE II. SKIN REACTIONS IN GUINEA PIGS SENSITIZED WITH CRYSTALLINE VITAMIN B_{12} (STREPTOMYCES) IN COMPLETE FREUND ADJUVANTS AND SKIN TESTED WITH CRYSTALLINE VITAMIN B_{12} DERIVED FROM STREPTOMYCES AND FROM LIVER*

TEST DOSE SOURCE† AMOUNT	TEST DAY	TIME OF READING OF REACTION (HOURS AFTER INJECTION OF TEST DOSE)		
		24	48	72
Strep. 100	27	WDP-Ind. 14‡	WDP-Ind. 14	WDP-Ind. 14
Strep. 10	27	P-Ind. 9	WDP-Ind. 12	WDP-Ind. 12
Liver 40	35	WDdP-Ind. 16	WDdP-Ind. 15	WDdP-Ind. 14
Strep. 100	27	P-Ind. 14 × 10	WDP-Ind. 11	WDP-Ind. 15
Strep. 10	27	P-Ind. 6	Neg.	Neg.
Liver 40	35	WDP-Ind. 11	WDP-Ind. 12	WDP-Ind. 12
Strep. 100	27	WDP-Ind. 12	WDP-Ind. 12	WDP-Ind. 12
Strep. 10	27	WDP-Ind. 12	WDP-Ind. 12	WDP-Ind. 12
Liver 40	35	WDP-Ind. 15	WDP-Ind. 11	WDP-Ind. 9
Strep. 100	27	P-Ind. 19	P-SS 10	P-SS 10
Strep. 10	27	Neg.	Neg.	Neg.
Liver 20	40	WDP-Ind. 13	WDP-Ind. 10	P-Ind. 10
Strep. 100	27	P-S 12 × 8	P-S 9	Neg.
Strep. 10	27	P-Ind. 13 × 10	P-Ind. 10	Neg.
Liver 20	40	P-Ind. 11	P-Ind. 8	P-Ind. 9
Strep. 100	27	Neg.	Neg.	Neg.
Strep. 10	27	Neg.	Neg.	Neg.
Liver 20	40	WDP-Ind. 12	P-Ind. 8	P-Ind. 6
Strep. 100	27	WDP-Ind. 14	WDP-Ind. 12	WDP-Ind. 12
Strep. 10	27	P-Nod. 5	P-Nod. 4	Neg.
Liver 20	40	P-Ind. 11	WDP-Ind. 9	P-Nod. 6
Strep. 100	27	P-Ind. 11 × 8	P-Ind. 6	Neg.
Strep. 10	27	Neg.	Neg.	Neg.
Liver 20	40	WDP-Ind. 14 × 11	WDP-Ind. 9	P-Nod. 6

*Legend: See Table I. SS—[illegible]
†8 controls injected with complete Freund's adjuvants and vitamin B_{12} were negative.
‡Strep.—Vitamin B_{12} derived from Streptomyces. Liver—Vitamin B_{12} derived from liver.
§See Table I.

No reactions were visible at 5 hours following the injection of the skin test doses of crystalline vitamin B_{12} derived from Streptomyces or from that derived from liver. The reactions reached their peak in 24 to 48 hours indicating a delayed type of hypersensitivity to the vitamin B_{12}.

The control animals which received complete Freund's adjuvants alone showed negative skin tests, although all of these animals had received the same series of skin test injections of aqueous vitamin B_{12} as the experimental animals.

DISCUSSION

The original purpose of our experiments, i.e., to produce a disease in guinea pigs similar to subacute degeneration of the cord in man, was not realized. This may have been due to an inadequate immunization or sensitization and the relative resistance of the guinea pig to vitamin B_{12} deficiency. However, in the course of these experiments we demonstrated that the guinea pigs could be sensitized to a vitamin which is essential for the growth and maintenance of most mammals. The sensitization developed in these experiments was to crystalline vitamin B_{12} itself rather than to contaminants which might be present in its preparation. This was demonstrated by the use of crystalline vitamin B_{12} prepared from Streptomyces fermentation for sensitization with cross skin test reactions to vitamin B_{12} prepared from liver. Due to the difficulty of obtaining crystalline vitamin B_{12} prepared from liver, this material could only be used for skin testing after the sensitization was produced by the use of crystalline vitamin B_{12} prepared from Streptomyces.

These experiments were carried out by the use of complete Freund's adjuvants and no effort was made to produce sensitization by any other means. In the course of the experiments, however, the control animals were skin tested several times and did not develop sensitization although skin testing was favorable for the production of delayed hypersensitization.

The sensitization was demonstrated to be of the delayed or tuberculin type by temporal sequence of the skin test reaction. No attempt was made to transfer this sensitization to other animals by cells. Unreported here was the unsuccessful attempt to demonstrate neutralizing antibodies to vitamin B_{12} by the use of serum from a highly sensitive animal in a test for vitamin B_{12}. The inability to produce pathological changes may be due to a lack of circulating antibody. The ability of cells of an animal sensitized with a delayed type of sensitization to neutralize a pharmacological substance, such as crystalline vitamin B_{12} or any other physiologically active material, had not been demonstrated.

The significance of sensitization of guinea pigs to crystalline vitamin B_{12} in regard to the clinical application of vitamin B_{12} in humans is unknown. It only points to the possibility that one can sensitize to crystalline vitamin by artifical means. It should be kept in mind that sensitization is possible and therefore that the best method of injection should be one which does not favor sensitization.

SUMMARY

Crystalline vitamin B_{12} (cyanocobalamin), emulsified with Freund's adjuvants including killed tubercle bacilli and injected intracutaneously, is capable

of sensitizing guinea pigs to crystalline vitamin B_{12}. The specificity of sensitization to vitamin B_{12} was ascertained by sensitizing with cyanocobalamin derived from Streptomyces fermentation liquor and by a skin test with crystallin vitamin B_{12} derived from liver. The sensitization induced is of the tuberculin type and reaches its peak in 24 to 48 hours after the skin test injection.

REFERENCES

1. Proceedings of the Symposium on Experimental "Allergic" Encephalomyelitis and Its Relation to Other Diseases of Man and Animals, edited by M. W. Kies and E. C. Alvord, Jr., Springfield, Ill., 1959, Charles C Thomas, Publisher.
2. Young, W. C., Ulrich, C. W., and Fouts, P. J.: Sensitivity to Vitamin B-12 Concentrate, J. A. M. A. 143: 893-894, 1950.
3. Bedford, P. D.: Side Effects of a Preparation of Vitamin B-12, Brit. M. J. 1: 690-691, 1952.
4. Rostenberg, A., Jr., and Perkins, A. J.: Nickel and Cobalt Dermatitis, J. ALLERGY 22: 466-474, 1951.
5. Ungley, C. C.: The Chemotherapeutic Action of Vitamin B-12, Vitamins & Hormones 13: 137-202, 1955.
6. Traina, V.: Toxicity Studies on Vitamin B-12 in Albino Mice, Arch. Path. 49: 278-279, 1950.
7. Lipton, M. M., and Freund, J.: The Efficacy of the Intracutaneous Route of Injection and the Susceptibility of the Hartley Strain of Guinea Pigs in Experimental Allergic Encephalitis, J. Immunol. 70: 326-329, 1953.

Mycopathologia 21: 289-314 30 Dec 63

EFFECT OF WATER SOLUBLE VITAMINS AND THEIR ANALOGUES ON GROWTH OF CANDIDA ALBICANS II. VITAMIN B_{12}, THIAMINE, OXYTHIAMINE, NEOPYRITHIAMINE, SUBSTITUTED PYRIMIDINES AND THIAZOLES

by

M. L. LITTMAN & T. MIWATANI

Bureau of Laboratories, Department of Health, New York 13, N.Y.,) U.S.A. and *The Research Institute for Microbial Diseases, Osaka University, Osaka, Japan)*

(with 12 figs.)

(2.VIII.1963)

INTRODUCTION

The vitamin requirements of *C. albicans* were studied by LITTMAN & MIWATANI (1963) using basal synthetic mineral medium with wide ranges in vitamin concentrations. Pyridoxamine was found to act as an active metabolite at 1000 μg/ml and pyridoxine at 2056 μg/ml. Both compounds were non-stimulatory to growth at 1 to 10 μg/ml. Antimetabolites of B_6 desoxypyridoxine, 4- and 5-desoxypyridoxine were non-inhibitory to growth and, in fact, were used as metabolites, from which it was concluded that the organism does not possess a requirement for B_6. However, vitamin B_6 shortened the lag phase of the organism and reversed the toxicity of 5-fluorouracil and 5-fluoro-2'-deoxyuridine, pointing to a new role of B_6 in nucleic acid metabolism of *C. albicans* (LITTMAN & MIWATANI, 1961, 1962, 1963). *C. albicans* possessed a complete dependency for biotin, a two-step stimulation of growth taking place at 0.001 to 1.0 mμg/ml and at 2 to 30 μg/ml. Desthiobiotin was growth stimulatory indicating that *C. albicans* is blocked in the biosynthesis of biotin at a step prior to desthiobiotin synthesis. Alterations in the biotin structure caused a pronounced effect upon its growth stimulatory activity for the organism. Diminished growth activity was observed with biotin sulfone and biotin diamine sulfate but not with biocytin, N-biotinyl-β-alanine, N-biotinyl-L-aspartic ethyl ester, or biotin-D-sulfoxide. Avidin and egg albumin inhibited the growth of the organism in synthetic medium. D-norbiotin, d-homobiotin, d-biotinol and dl-oxybiotin failed to produce anti-biotin effects up to 10 μM/ml. The technique

1659

of examining the effects of vitamins on *C. albicans* over wide ranges of concentration was applied to vitamin B_{12} and thiamine and its analogues. It forms the subject of this paper.

METHODS

Test organism. Five strains of *C. albicans* of human origin were employed in the vitamin studies: American Type Culture Collection (ATCC) #10231, Communicable Disease Center (CDC) #B3 and B37, and Mount Sinai Hospital strains (MS) #33 and #191. All 5 strains were identical by a variety of cultural, biochemical and serological tests as described in a previous paper (LITTMAN & MIWATANI, 1963). The culture media, preparation of cultures and methods of growth measurements were the same as those described in an earlier paper (LITTMAN & MIWATANI, 1963).

RESULTS

Vitamin B_{12}

The addition of 0.5 μg B_{12}/ml alone or with 5 μg inositol/ml to biotin basal synthetic medium in previous experiments produced heavier growth than the biotin control (LITTMAN & MIWATANI, 1963). Also, the omission of 0.5 μg B_{12} from a mixture of 12 vitamins in biotin basal synthetic medium produced a small but definite decrease in the growth of *C. albicans* from that caused by the

TABLE I.

Growth response of Candida albicans to vitamin B_{12} in biotin (1mμg/ml) basal synthetic medium. Incubated at 28° on Gyrotory shaker at 250 cycles/min. Per cent light transmission at 650 mμ, each figure representing an average of triplicate values

B_{12} Conc. μg/ml	Growth Response to Biotin + B_{12} (Vitamin Free Tube Set as Control at 100 Per Cent Light Transmission)										Growth Response to B_{12} (Biotin Containing Tube Set as Control at 100 Per Cent Light Transmission)			
	Candida albicans #MS—33					*Candida albicans* #CDC-B3					*Candida albicans* #MS-33		*Candida albicans* #CDC-B3	
	Days on Shaker					Days on Shaker					Days on Shaker		Days on Shaker	
	1	2	3	4	5	1	2	3	4	5	1	2	1	2
0	72	47	30	22	18	75	53	33	25	21	100	100	100	100
0.1	73	51	32	22	18	75	53	33	25	21	100	100	100	100
0.25	74	50	33	24	18	73	50	33	25	21	100	100	98	93
0.5	70	41	26	20	17	70	48	31	24	18	99	95	95	94
1.0	61	37	22	18	17	66	41	27	20	17	86	86	89	81
2.5	52	24	16	15	14	52	28	19	16	15	73	56	69	55
5.0	40	17	14	14	13	39	18	16	14	14	55	40	52	36
7.5	37	17	14	14	13	32	18	18	15	14	50	40	43	37
10.0	32	16	14	14	13	27	17	15	14	13	44	37	36	34
20.0	22	17	15	15	15	21	19	16	14	14	31	40	29	38
30.0	29	23	20	19	19	25	21	18	17	16	41	54	34	41
40.0	32	25	24	23	22	30	23	19	18	17	44	58	40	45
50.0	32	26	25	23	22	36	27	22	20	18	45	60	47	53

13-vitamin mixture (LITTMAN & MIWATANI, 1963). In order to determine whether *C. albicans* was indeed partially deficient for vitamin B_{12}, a series of tubes of biotin basal synthetic medium was prepared in triplicate with B_{12} concentration ascending from 0.1 to 50 μg/ml. Standard inocula of vitamin depleted cells of *C. albicans* (MS—#33 and CDC—#B3) were added and tubes were placed under constant agitation at 250 cycles/min. in the gyrotory incubator shaker at 28°). Since *C. albicans* has an absolute requirement for biotin, and this was added at 1 mμg/ml to the medium, detection and measurement of the growth stimulatory effect of B_{12} was necessarily limited to the increased growth that B_{12} furnished above that provided by biotin. For this reason photometric readings were made at each observation period with two different controls. The first control was the seeded vitamin-free culture medium set at 100 % light transmission. The reading obtained with this control

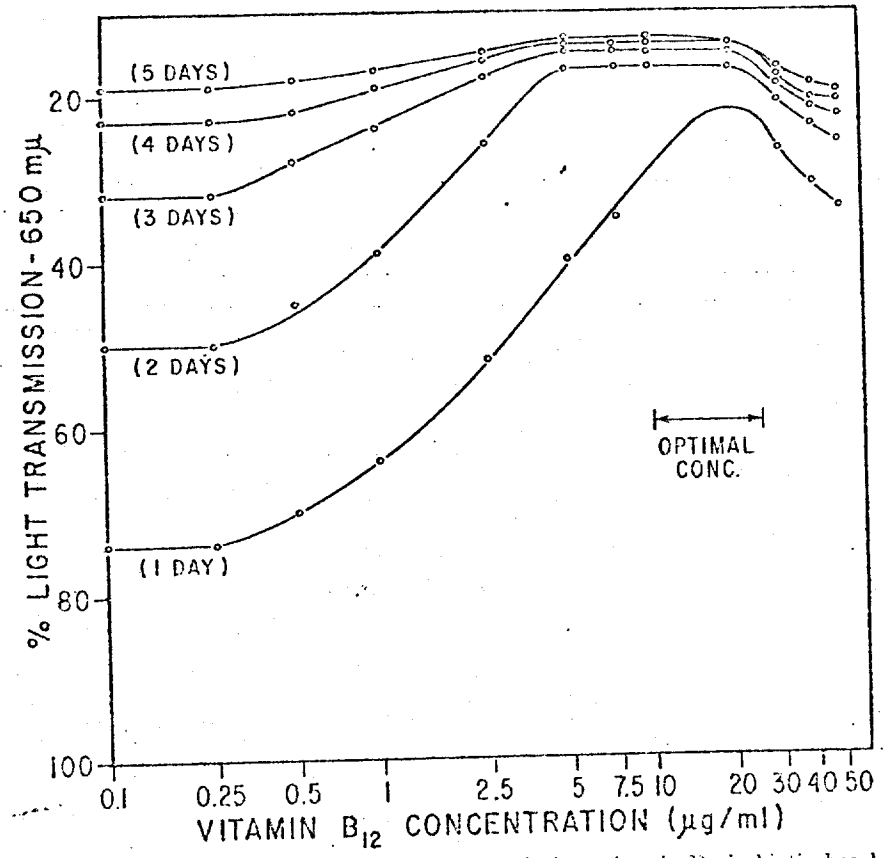


Fig. 1. Growth response of *C. albicans* (2 strains) to vitamin B_{12} in biotin basal synthetic medium. Incubated at 28° on Gyrotory shaker at 250 cycles/min. Vitamin-free seeded tube set at 100% light transmission. Each point represents an average of 6 values, 3-cycle semi-log plot.

represented the growth response to both biotin and vitamin B_{12}. The second control was seeded biotin basal synthetic medium set at 100 % light transmission. The reading obtained with this control represented growth response to B_{12} alone.

In the presence of an optimal biotin concentration (1 mμg/ml), vitamin B_{12} caused a marked stimulatory effect on the growth of both strains of *C. albicans* over the entire range of 0.1 to 50 μg B_{12}/ml (Table 1 and Fig. 1). The optimal concentration of vitamin

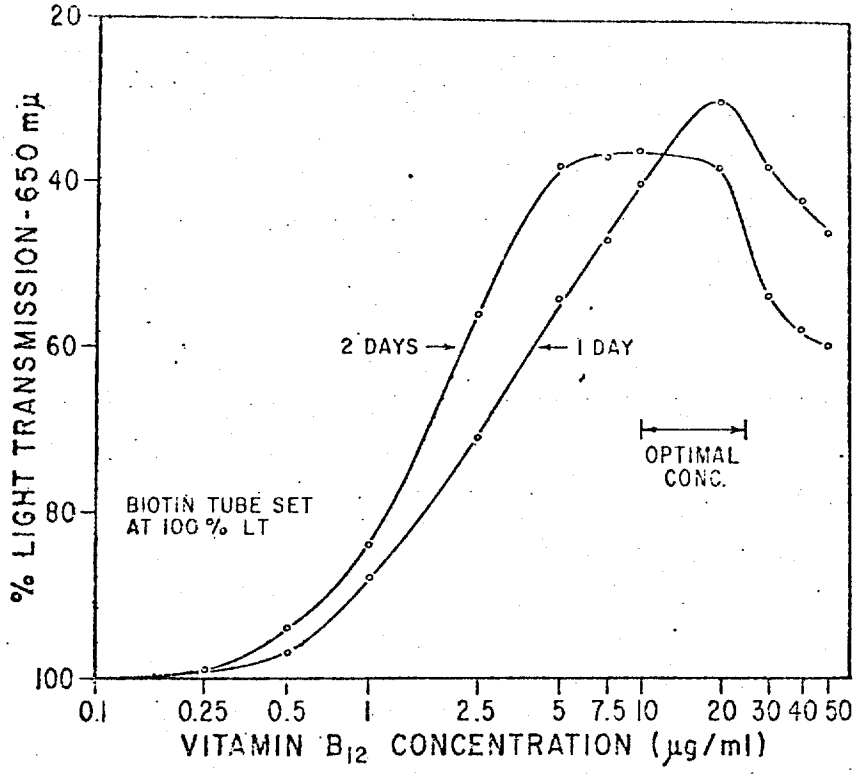


Fig. 2. Growth response of *C. albicans* (2 strains) to vitamin B_{12} in biotin basal synthetic medium. Incubated at 28° on Gyrotory shaker at 250 cycles/min. Effect of biotin cancelled by setting biotin tube at 100% light transmission. Each point represents an average of 6 values, 3-cycle semi-log plot.

B_{12} for *C. albicans* was 10 to 25 μg/ml on the first day of incubation. Rate of growth of the organism in response to vitamin B_{12} and biotin was significantly greater than with biotin alone (Fig. 1). This effect persisted for the second and third days of incubation but diminished by the fourth and fifth days, at which time the rate of growth of the organism in biotin approached that of biotin plus B_{12} (Fig. 1). The growth enhancing action of B_{12} could be more clearly demonstrated graphically when the effect of biotin was

cancelled as in Figure 2, the optimal concentration of B_{12} again proving to be 10 to 25 μg/ml. The rates of growth response of *C. albicans* to different concentrations of B_{12} in the presence of 1 mμg biotin/ml are shown in Figure 3. The highest rate of growth occurred at 20 μg B_{12}/ml. The 0.5 μg B_{12}/ml curve was only slightly higher than the biotin control but was comparable to that obtained in the addition experiment described previously (LITTMAN & MIWATANI, 1963). The marked difference between the 20 μg/ml and 0.5 μg/ml B_{12} response curves clearly illustrates the need for

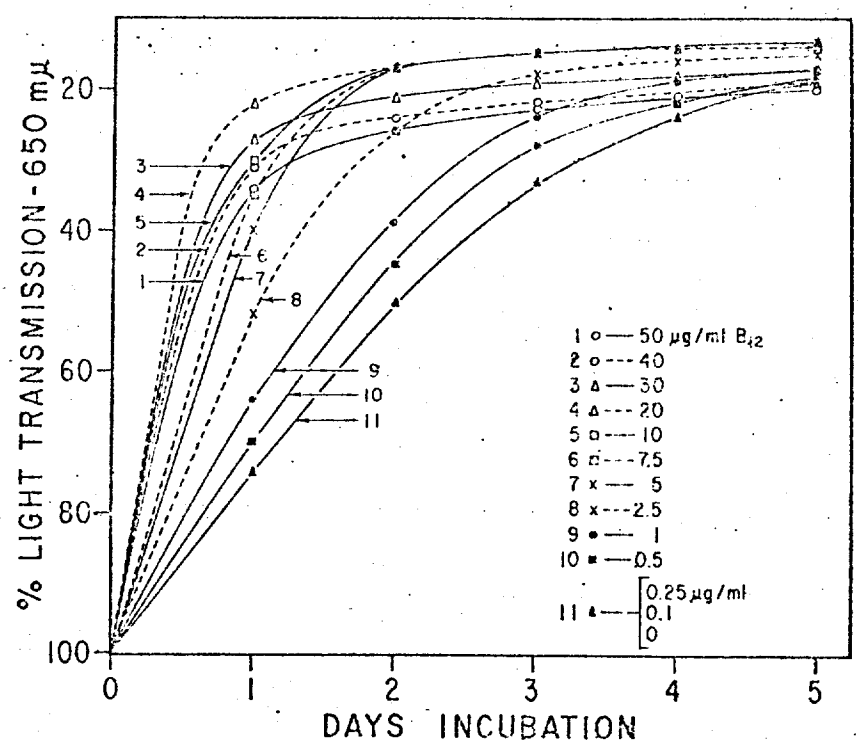


Fig. 3. Variation in growth rate of *C. albicans* (2 strains) to ascending concentrations of vitamin B_{12} in biotin (1mμg/ml) basal synthetic medium. Incubated at 28° on Gyrotory shaker at 250 cycles/min. Each point represents an average of 6 values, arithmetic plot.

performing vitamin addition and omission experiments with *optimal* rather than arbitrary low concentrations of vitamins. The foregoing data show that in addition to having an absolute requirement for biotin, *C. albicans* has also a partial dependency for vitamin B_{12}.

The role of vitamin B_{12} in the growth of C. albicans. The role of B_{12} is unknown in *C. albicans*. The present knowledge of the metabolic role of B_{12} was reviewed by SMITH (1958, 1960) and ARNSTEIN (1958). Vitamin B_{12} is believed to function in the cell in

the transport of single carbon fragments, especially the labile methyl group as in the methylation of homocysteine to methionine by *Escherichia coli* (LASCELLES & CROSS, 1955). B_{12} is thought to be responsible for keeping —SH compounds in the reduced reactive state and to be involved in protein synthesis. Vitamin B_{12} also functions at some step in DNA synthesis. This was confirmed in 1949 with radioactive phosphorus (ROBERTS, ROBERTS & ABELSON, 1949) (VON WACKER, PFAHL & SCHRÖDER, 1957) and finally narrowed down to the synthesis of deoxyribose moiety of DNA (DOWNING & SCHWEIGERT, 1956). In *L. leichmannii*, vitamin B_{12} influences several aspects of nucleic acid metabolism. Thymidine

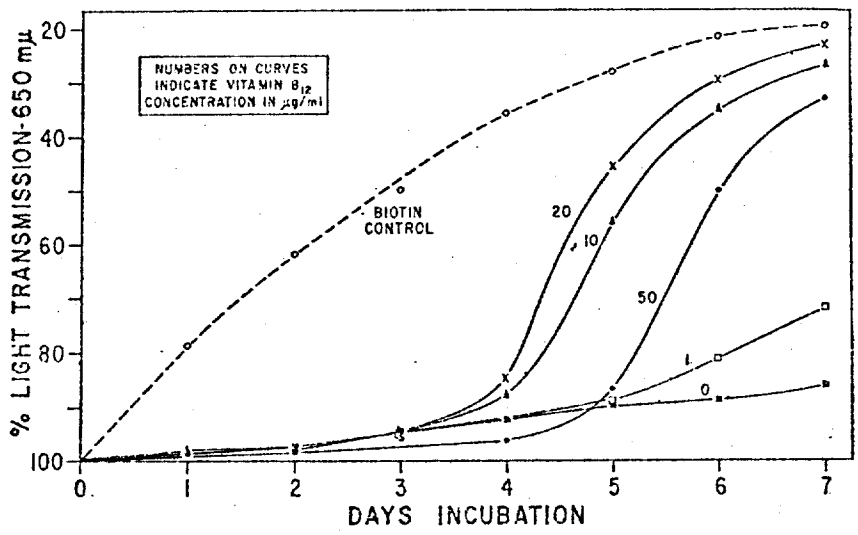


Fig. 4. The reversing effect of ascending concentrations of vitamin B_{12} on growth inhibition of cells of *C. albicans* by 0.5 μM FU/ml in basal synthetic medium at 28° in incubator shaker at 250 cycles/min. FU and vitamin B_{12} introduced together. Biotin control does not contain FU. Each point represents an average of triplicate values, arithmetic plot.

replaces B_{12} for this organism (WRIGHT, SKEGGS & HUFF, 1948). Later, it was shown with the same organism, that thymine, adenine, hypoxanthine and cytosine would also replace B_{12} (KITAY, MCNUTT & SNELL, 1949). Vitamin B_{12} is also required by *L. leichmannii* for the conversion of ribose to deoxyribose (SPELL & DINNING, 1959). Deoxyribonucleic acid thymine is reported to be in metabolic equilibrium with extracellular thymine, the exchange being catalyzed by vitamin B_{12} (DINNING & YOUNG, 1960). In view of our findings of the growth stimulatory effect on *C. albicans* of vitamin B_{12}, its known involvement in other biological systems in DNA deoxyribose synthesis (DOWNING & SCHWEIGERT, 1956) and the

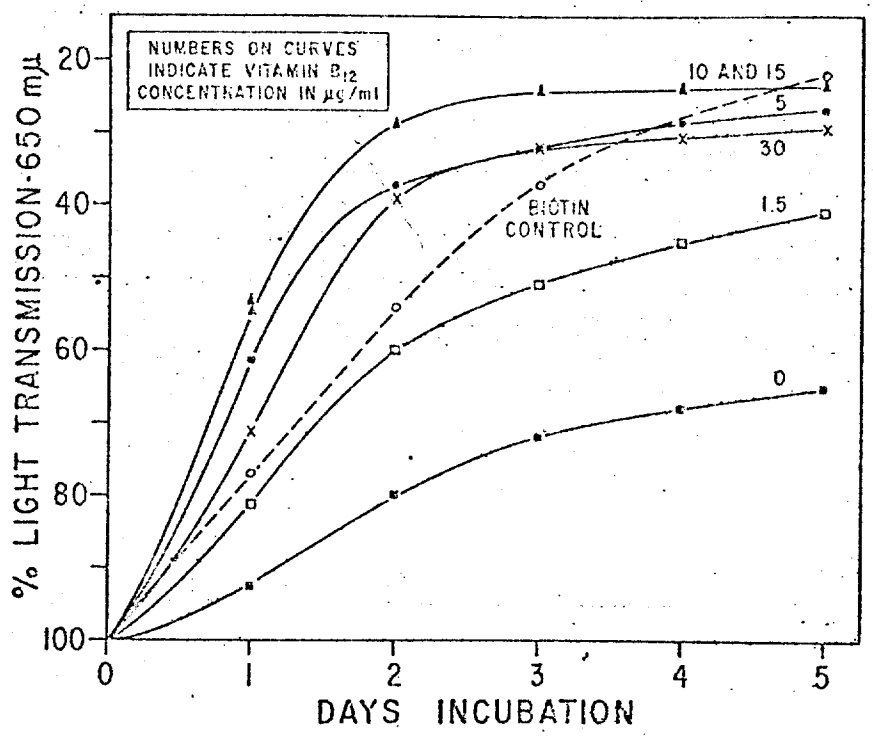


Fig. 5. The reversing effect of ascending concentrations of vitamin B_{12} on growth inhibition of *C. albicans* by 0.25 µM FU/ml in basal synthetic medium at 28° in incubator shaker at 250 cycles/min. FU and vitamin B_{12} introduced together. Biotin control does not contain FU. Each point represents an average of triplicate values, arithmetic plot.

TABLE II.

Amounts of FU required to produce half-maximal inhibition of growth of Candida albicans in the presence of varying amounts of vitamin B_{12} (incubation at 28° in New Brunswick Gyrotory Shaker, 250 cycles/minute). (LITTMAN & MIWATANI, 1962)

Vitamin B_{12} µM/ml.	ID_{50} FU µM/ml. (days incubation)				Inhibition index* (days incubation)			
	2	3	4	5	2	3	4	5
0	0.23	0.23	0.23	0.23	–	–	–	–
0.0015	0.28	0.28	0.29	0.29	33.3	31.3	40.0	36.6
0.005	0.32	0.30	0.30	0.29	18.0	14.0	13.4	11.0
0.010	0.32	0.31	0.31	0.31	9.5	8.0	7.7	7.5
0.015	0.32	0.31	0.68	0.60	6.3	5.3	29.6	24.3
0.030	0.32	0.27	0.30	0.32	3.0	1.3	2.2	2.8

* Inhibition index = (ID_{50} of FU in presence of vitamin B_{12}) minus (ID_{50} in absence of B_{12}) divided by (concentration of vitamin B_{12}).

interfering action of 5-fluorouracil on DNA and RNA biosynthesis in other systems, it was of interest to determine whether B_{12} could reverse the inhibitory action of FU on *C. albicans*. Vitamin B_{12} was therefore added in ascending concentrations (1 to 50 μg/ml) to biotin basal synthetic medium containing 0.5 μM FU/ml. FU and B_{12} were introduced together followed by seeding with standard inocula of FU-unexposed cells of *C. albicans*. As seen in Figure 4, reversal of FU inhibition by vitamin B_{12} was most pronounced at 10 and 20 μg B_{12}/ml, and at a more delayed rate at 50 μg B_{12}/ml. Reversal of inhibition reached completion by the 10th day of incubation in the 10, 20 and 50 μg B_{12}/ml tubes. The reversing action of B_{12} was more pronounced at a lower concentration of FU (0.25 μM/ml) as seen in Figure 5. Study of the amount of FU required to produce half-maximal inhibition of growth in the presence of ascending concentrations of B_{12} showed inconstant inhibition indices (Table II), indicating that the antagonism of B_{12} with FU was noncompetitive. Previous studies with *C. albicans* have demonstrated that many of the major purine and pyrimidine bases of RNA and DNA as well as derived nucleosides and nucleotides were capable of reversing FU inhibition of growth (LITTMAN & MIWATANI, 1962). Unlike nucleic acids which possess pyrimidines or purines with an imidazole ring, the nucleotide of vitamin B_{12} contains neither pyrimidine nor purine, but the base 5:6 dimethyl benziminazole. The sugar is ribose, phosphorylated at C3 and the glycoside linkage is α unlike the β linkage in the nucleic acids (SMITH, 1960). Despite these differences, we suspect that the reversing action of B_{12} on FU inhibition is due to the nucleotide and ribose moieties of B_{12}. The growth stimulatory action of B_{12} on *C. albicans* and its activity in reversing the growth inhibition of FU, suggest the utilization of B_{12} by *C. albicans* in nucleic acid biosynthesis. As with B_6, this will require confirmatory studies with labelled compounds.

Thiamine (B_1)

The omission of 5 μg thiamine/ml from a 13-vitamin mixture in previous experiments failed to lower the growth response in biotin basal synthetic medium of any of 5 strains of *C. albicans* (LITTMAN & MIWATANI, 1963). Also, the single addition of 5 μg thiamine/ml to biotin basal synthetic medium failed to produce marked growth response (LITTMAN & MIWATANI, 1963). On the basis of these results, one might conclude, albeit erroneously, that all 5 strains of *C. albicans* were self-sufficient for thiamine. However, when the organism was placed in biotin basal synthetic medium with thiamine concentrations ascending from 0 to 100 μg/ml, and incubated in the gyrotory shaker at 28°, a small but definite growth stimulatory effect was produced over the range 5 to 30 μg thiamine/ml for all 5 strains (Table III and Fig. 6). This stimulatory effect was most pronounced within the first 48 hours of incubation and persisted to

TABLE III.

Growth response of Candida albicans to thiamine in biotin (mμg/ml) basal synthetic medium. Incubated at 28° on Gyrotory shaker at 250 cycles/min. Per cent light transmission at 650 mμ, each figure representing an average of triplicate values

Thiamine Concentration μg/ml	*Candida albicans* #MS-33					*Candida albicans* #ATCC-10231					*Candida albicans* #MS-191					*Candida albicans* #CDC-B3					*Candida albicans* #CDC-B37				
	Days on Shaker					Days on Shaker					Days on Shaker					Days on Shaker					Days on Shaker				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
0	77	55	39	27	21	77	52	35	28	26	73	37	23	21	20	82	60	43	32	27	75	55	39	25	22
5	76	53	37	26	21	72	46	39	28	26	71	39	24	21	21	78	56	40	28	26	78	56	41	26	23
10	76	53	37	27	21	74	48	32	30	28	73	42	24	21	21	80	57	42	29	26	76	51	36	25	20
30	68	42	27	20	17	68	39	28	28	26	65	32	21	20	19	80	57	43	29	26	72	44	31	21	19
50	71	45	28	20	17	66	40	29	27	26	68	35	22	21	20	76	50	35	26	22	73	49	34	22	21
100	68	41	26	20	17	64	38	29	27	25	66	31	21	20	20	76	50	36	25	22	73	49	33	21	20

the third day. It became much less evident at the 4th and 5th days however (Fig. 6). It must be recalled that the stimulatory effect of thiamine took place in the presence of biotin. Since *C. albicans* has an absolute requirement for biotin, without which the organism will not grow, the growth stimulatory effect of thiamine is discernible only if it causes increased growth response above that provided by biotin. Disappearance of the stimulatory effect of thiamine by the 4th and 5th days of incubation is attributed,

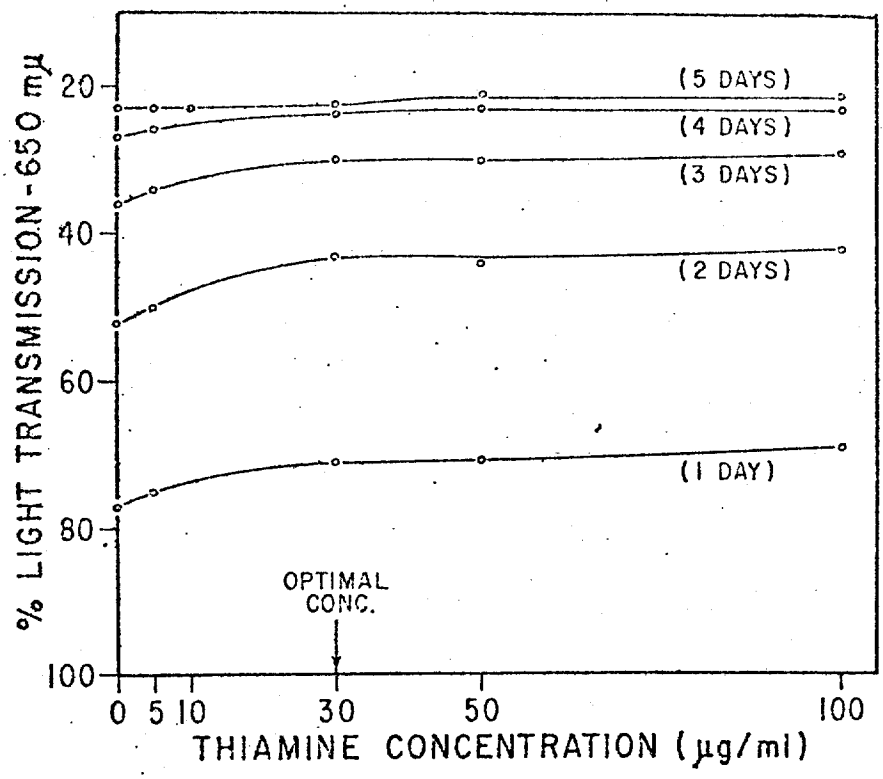


Fig. 6. Growth response of *C. albicans* to thiamine in biotin (1 mμg/ml) basal synthetic medium. Incubated at 28° on Gyrotory shaker at 250 cycles/min. Each point represents an average of 15 values, arithmetic plot.

therefore, to (1) increased thiamine biosynthesis by the organism with time or (2) the rate of growth of the organism in biotin basal synthetic medium approaches that of the thiamine enriched medium.

Effect of metabolite analogues of thiamine on the growth of C. albicans. For added evidence that *C. albicans* is partially dependent upon thiamine, metabolite analogues of thiamine that are known to interfere with the growth of thiamine-dependent species, were studied. Both *neopyrithiamine*, a metabolite analogue with thiazole-

TABLE IV.

Growth response of Candida albicans to neopyrithiamine in biotin (1 mμg/ml) basal synthetic medium. Incubated at 28° on Gyratory shaker at 250 cycles/min. Per cent light transmission at 650 mμ, each figure representing an average of triplicate values*

Neopyrithiamine Concentration μg/ml	*Candida albicans* #MS-33					*Candida albicans* #ATCC-10231					*Candida albicans* #MS-191					*Candida albicans* #CDC-B3					*Candida albicans* #CDC-B37				
	Days on Shaker					Days on Shaker					Days on Shaker					Days on Shaker					Days on Shaker				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
0	84	61	47	34	26	74	50	35	30	28	81	49	31	25	25	87	67	49	33	27	88	65	48	33	26
0.1	83	60	43	31	24	75	53	36	30	28	80	50	31	24	24	85	66	50	33	26	87	63	47	33	25
1	84	59	42	28	23	74	51	35	29	27	80	51	31	26	26	87	68	51	37	29	87	61	46	33	26
10	85	60	43	27	22	74	48	35	30	28	84	53	32	26	26	88	68	51	36	29	93	65	47	33	25
100	99	95	79	51	39	97	71	46	37	34	99	71	43	30	27	99	87	58	35	27	100	97	84	57	41
500	99	97	95	80	61	98	94	85	78	64	99	91	76	52	42	99	97	92	84	73	99	98	97	94	86
1000	99	97	95	80	61	98	96	90	86	70	99	95	84	70	57	100	98	95	87	82	100	98	98	97	92
2500	99	98	96	81	59	99	97	94	91	68	99	96	88	80	72	99	98	96	94	92	100	98	98	97	95
5000	99	98	97	87	67	99	97	94	90	75	99	96	91	84	74	100	98	96	94	91	100	99	98	98	96

*) California Corp. Biochem. Res.

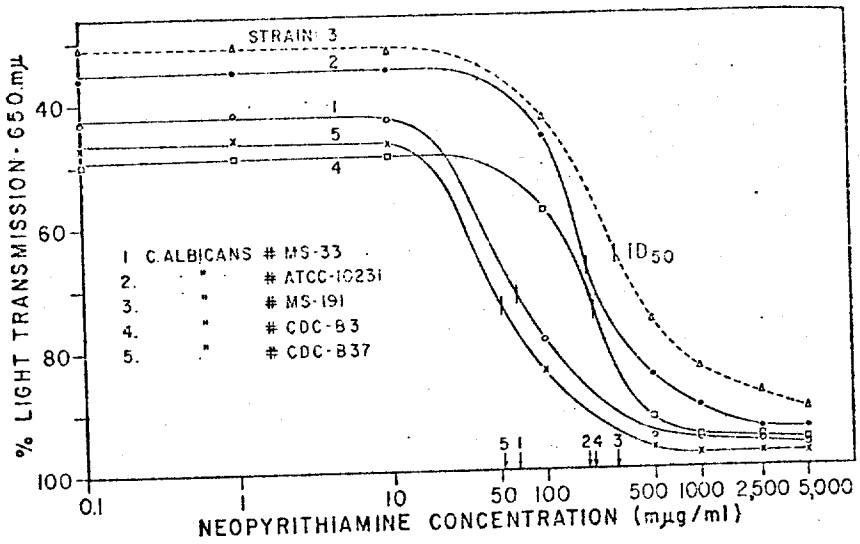


Fig. 7. Growth response of *C. albicans* to neopyrithiamine in biotin (1 mμg/ml) basal synthetic medium after 3 days incubation at 28° on Gyrotory shaker at 250 cycles/min. Each point represents an average of 3 values, 5-cycle semi-log plot.

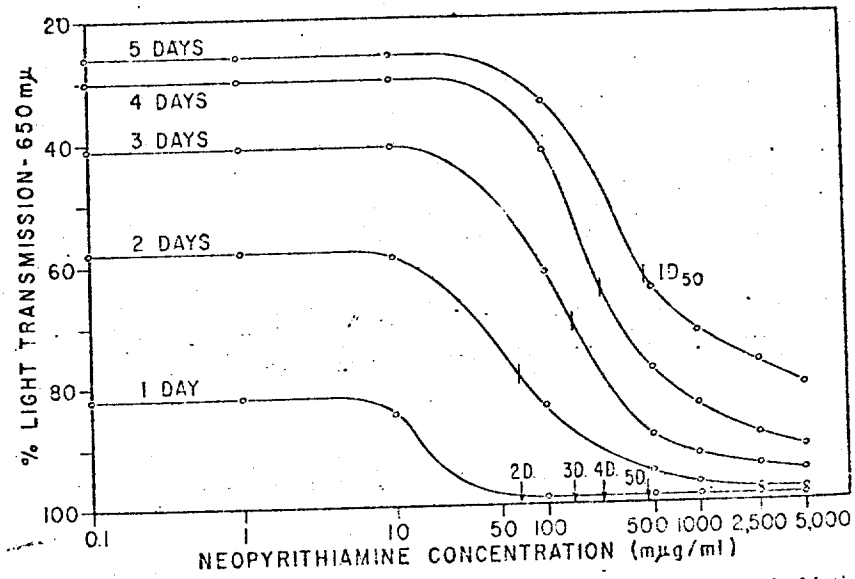


Fig. 8. Growth response of *C. albicans* (5 strains) to neopyrithiamine in biotin (1 mμg/ml) basal synthetic medium, from 1 to 5 days incubation at 28° on Gyrotory shaker at 250 cycles/min. Each point represents an average of 15 values, 5-cycle semi-log plot.

moiety modification, and oxythiamine, with pyrimidine moiety modification, were tested against *C. albicans*.

Neopyrithiamine (thiazole modification of thiamine):

Addition of neopyrithiamine to shaker cultures of *C. albicans* in biotin basal synthetic medium, in the absence of thiamine, caused a pronounced inhibitory effect on growth (Table IV and Figs. 7 and 8). By the third day of incubation, the ID_{50} of neopyrithiamine for 5 strains varied from 52 to 290 mμg/ml among strains (Fig. 7). The average inhibitory effect of neopyrithiamine on *C. albicans* is plotted in Figure 8. Average ID_{50} varied from 66 to 450 mμg

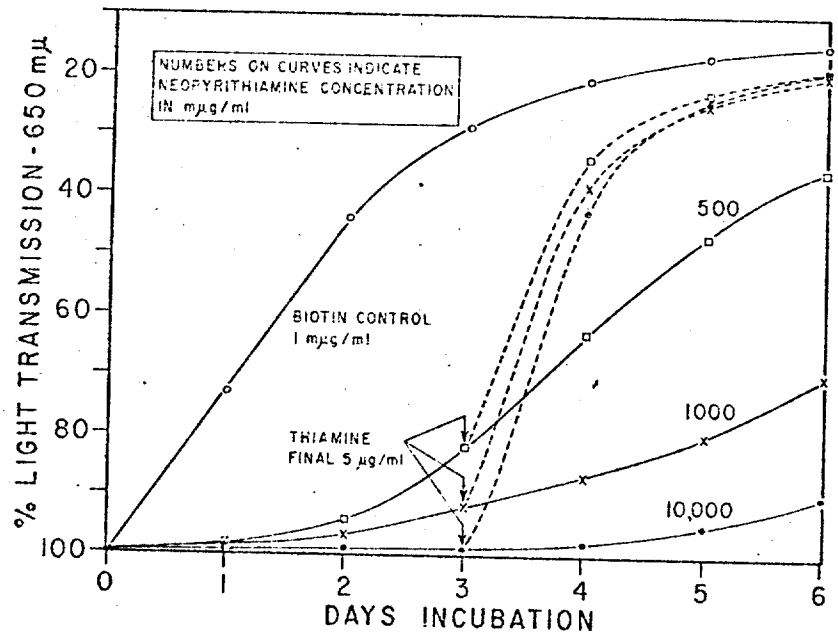


Fig. 9. The reversing effect of thiamine on the growth inhibition of *C. albicans* (CDC #B-37) by neopyrithiamine in biotin (1 mμg/ml) basal synthetic medium. Thiamine introduced on third day of incubation. Each point represents an average of 3 values, arithmetic plot.

neopyrithiamine/ml from the 2nd to 5th day of incubation. Complete inhibition of growth was produced by 10,000 mμg neopyrithiamine/ml and pronounced inhibition by 1000 and 500 μg/ml (Fig. 9). Demonstration of neopyrithiamine toxicity for *C. albicans* in biotin basal synthetic medium in the absence of thiamine, indicates a blocking action on the utilization of thiamine by the cell. One can infer from this that thiamine biosynthesis is essential to the growth of *C. albicans*. Moreover, the addition of 5 μg thiamine/ml to tubes of neopyrithiamine-inhibited cultures of *C. albicans* on their 3rd

day of incubation on the gyrotory shaker caused prompt reversal of growth inhibition (Fig. 9).

Oxythiamine (pyrimidine modification of thiamine):

This metabolite analogue of thiamine was also inhibitory to the growth of *C. albicans* but less so than neopyrithiamine. The ID_{50} of oxythiamine varied from 850 to 1780 mμg/ml (Fig. 10). Average ID_{50} values of oxythiamine varied from 1100 to 3700 mμg/ml for 5 strains from the 2nd to 4th day of incubation (Fig. 11). As with

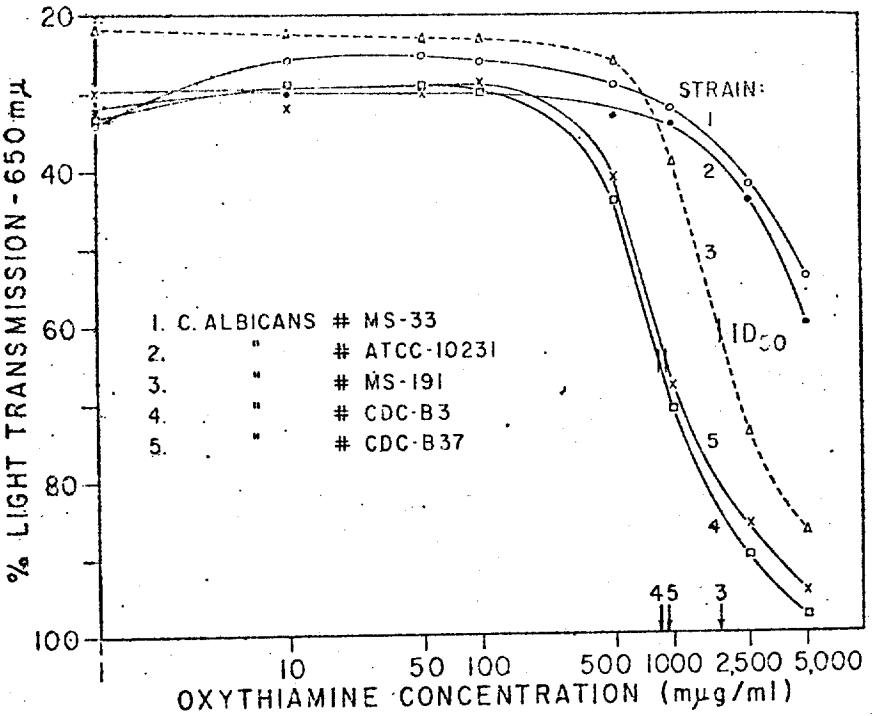


Fig. 10. Growth response of *C. albicans* to oxythiamine in biotin (1mμg/ml) basal synthetic medium, after 3 days incubation at 28° on Gyrotory shaker at 250 cycles/min. Each point represents an average of 3 values, 4-cycle semi-log plot.

neopyrithiamine, the inhibitory effect of 10 mμM oxythiamine/ml on *C. albicans* was partially reversed by the addition of 10 mμM thiamine/ml as well as by 10 mμM/ml of pyrimidine thiamine (2'-methyl-4-amino-5-hydroxymethylpyrimidine-Merck) (Fig. 12), but not by thiazole thiamine. Oxythiamine inhibition was also not reversed by two thiamine thiazoles, 3,4-dimethyl-5-hydroxyethylthiazolium chloride-(Yount) or 3-(—CH_2—CN)-4-methyl-5-β-hydroxyethylthiazolium chloride-(Yount) at 10 mμM/ml. It is significant that the growth inhibitory effect of oxythiamine, a metabolite

analogue with pyrimidine moiety modification, should be reversed by an analogous pyrimidine moiety of thiamine and not by the thiazole moiety. This would imply that oxythiamine selectively blocks utilization of pyrimidine thiamine. As we shall see in subsequent experiments, many pyrimidine and thiazole moieties of thiamine were separately growth stimulatory for *C. albicans*.

It was concluded that all five strains of *C. albicans* were partially dependent on thiamine on the basis of (1) the growth stimulatory

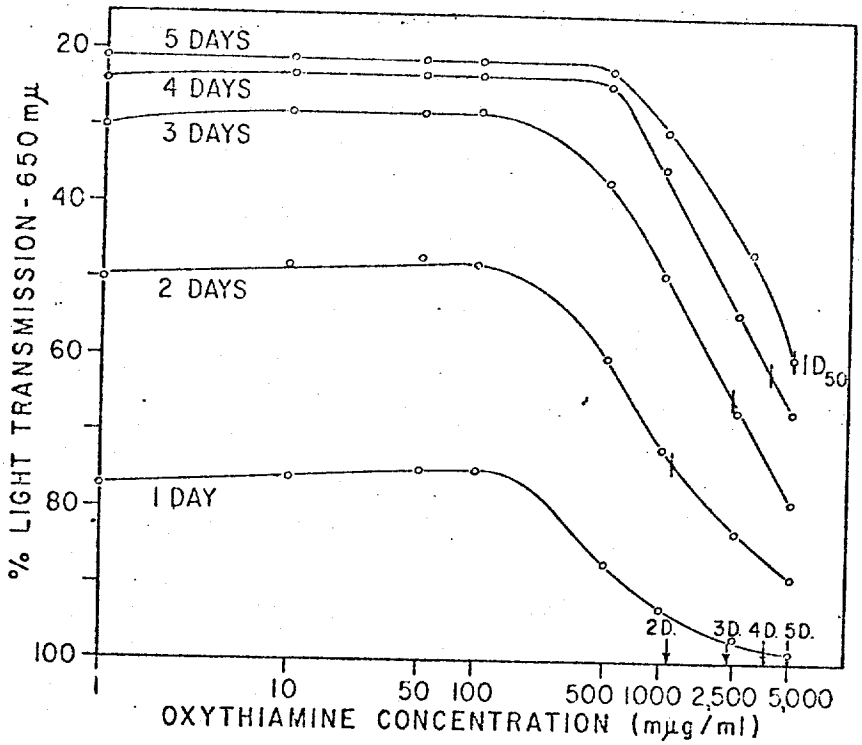


Fig. 11. Growth response of *C. albicans* (average of 5 strains) to oxythiamine in biotin (1 mμg/ml) basal synthetic medium, from 1 to 5 days incubation at 28° on Gyrotory shaker at 250 cycles/min. Each point represents an average of 15 values, 4-cycle semi-log plot.

effect of thiamine on *C. albicans* in biotin basal synthetic medium, (2) the growth inhibitory effect in the absence of thiamine by the thiamine antimetabolites neopyrithiamine and oxythiamine and (3) reversal of growth inhibition of thiamine antimetabolites by thiamine and its moieties.

Effect of pyrimidine thiamine and thiazole thiamine on C. albicans. Oxythiamine differs from thiamine in the substitution of a hydroxyl group for the amino group of the pyrimidine ring, while neopyri-

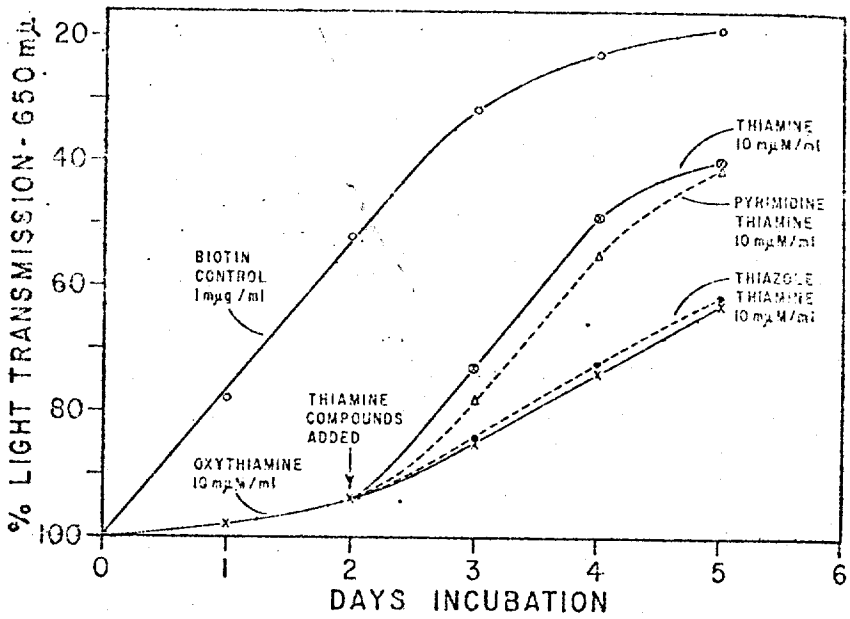


Fig. 12. Inhibitory effect of 10 mμM oxythiamine/ml on *C. albicans* (CDC #B-37) in absence of thiamine, and the reversing effect of thiamine, pyrimidine-thiamine and thiazole-thiamine on the growth inhibition of oxythiamine. Incubated at 28° on Gyrotory shaker at 250 cycles/min. Each point represents an average of 3 values, arithmetic plot.

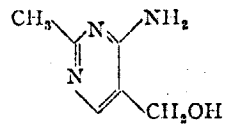
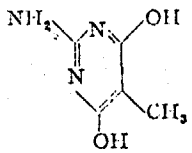
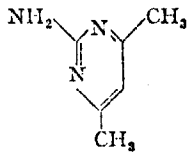
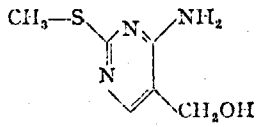
thiamine differs from thiamine in the substitution of a pyridine ring for the thiazole ring (Fig. 13). In determining whether the inhibition caused by neopyrithiamine and oxythiamine was due to the pyrimidine, pyridine or thiazole moieties of these compounds, the reversing effects of related compounds of each of these chemical groups was evaluated with antimetabolite growth-inhibited *C. albicans* shaker cultures.

Results. Neopyrithiamine growth inhibition for *C. albicans* was reversed by thiamine but not by pyrimidine thiamine or thiazole thiamine. Oxythiamine growth inhibition was reversed by intact thiamine and pyrimidine thiamine, but not by thiazole thiamine.

	Reversal of growth inhibition of C. albicans by:		
	Thiamine	Pyrimidine thiamine	Thiazole thiamine
Neopyrithiamine growth inhibition (thiazole analogue)	Yes	No	No
Oxythiamine growth inhibition (pyrimidine analogue)	Yes	Yes	No

TABLE V.

Effect on C. albicans (5 strains) of pyrimidine, thiazole and thiamine analogues in biotin (1 mμg/ml) basal synthetic medium. Incubated at 28° in Gyrotory shaker at 250 cycles/min.

Structure	Chemical name	Effect on growth at 5 μg/ml in biotin basal synthetic medium	
		Stimulation	Inhibition
	2-Methyl-4-amino-5-hydroxymethyl pyrimidine Merck (5H)	+	—
	2-Amino-4,6-dioxy-5-methyl pyrimidine California Foundation for Biochem. Res. (ADMP)	+	—
	2-Amino-4,6-dimethyl pyrimidine Merck (2P)	—	—
	2-Methyl-mercapto-4-amino-5-hydroxymethyl pyrimidine Merck (CP)	+	—

Group I Substituted pyrimidines

1659

558

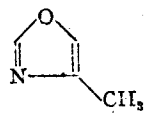
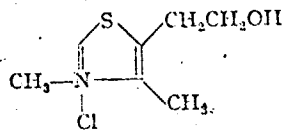
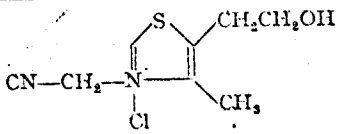
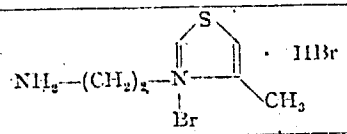
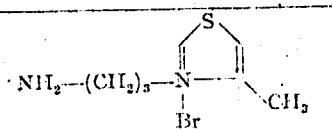
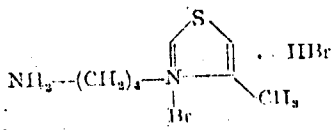
Structure	Chemical name	Effect on growth at 5 μg/ml in biotin basal synthetic medium	
		Stimulation	Inhibition
	α-Methyloxazolium Yount (Y6)	+	—
	3,4-Dimethyl-5, β-hydroxyethyl thiazolium chloride Yount (Y7)	+	—
	3-(-CH_2-CN)-4-methyl-5, β-hydroxyethylthiazolium chloride Yount (Y12)	+	—
	3-(β-aminoethyl)-4-methylthiazolium bromide hydrobromide Merck (M11)	+	—
	3-Aminopropyl-4-methylthiazolium bromide Sealock (Y11)	+	—
	3-Aminobutyl-4-methylthiazolium bromide hydrobromide Sealock (Y10)	+	—

Table V. Group II Substituted thiazoles

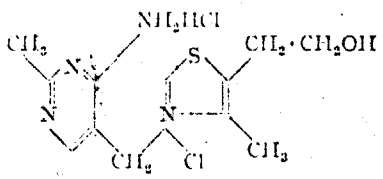
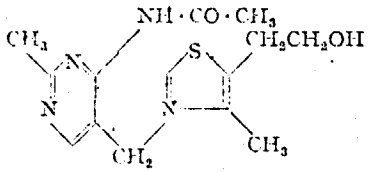
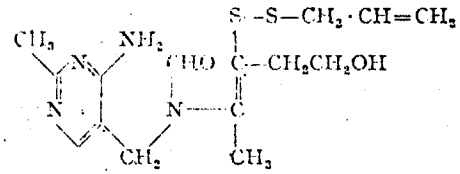
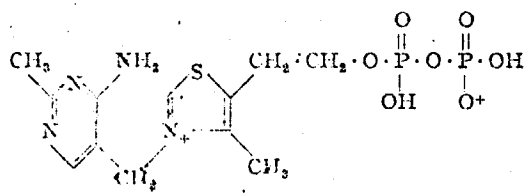
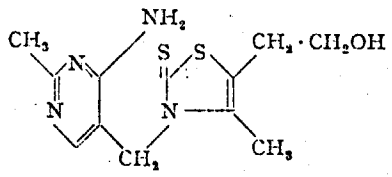
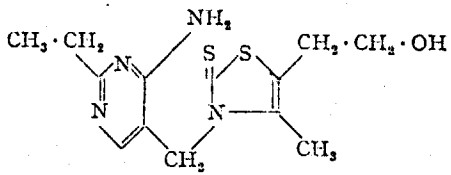
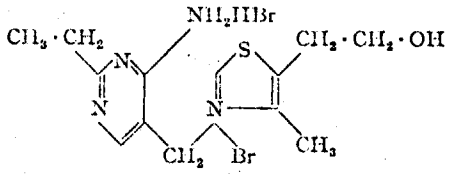
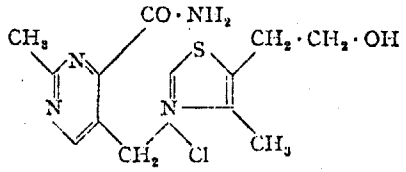
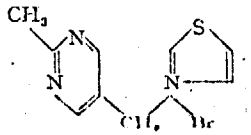
		Stimulation	Inhibition
	3-(2-Methyl-4-amino-5-pyrimidyl-methyl)-4-methyl-5, β-hydroxyethyl thiazolium chloride hydrochloride (U.S.P. Thiamine hydrochloride)	+	—
	3-(2-Methyl-4-acetylamino-5-pyrimidylmethyl)-4-methyl-5, β-hydroxyethylthiazolium Calif. Found. Biochem. Res. (ACT) (Acetylthiamine hydrochloride)	+	—
	2-(2-methyl-4-amino-5-pyrimidyl-methyl)-formamino-5-hydroxyΔ^2 pentenyl-(3)-allyldisulfide Merck (AT) (Allithiamine)	+	—
	Thiamine pyrophosphoric acid ester Nut. Biochem. Corp. (CO) (Cocarboxylase)	+	—
	Dihydrothiamine Yount (Y8)	+	—
	Tetrahydrothiamine dihydrobromide Yount. (Y3)	+	—

Table V. Group III. Thiamine and derivatives

1659

Structure	Chemical name	Effect on growth at 5 μg/ml in biotin basal synthetic medium	
		Stimulation	Inhibition
	3-(2-Methyl-4-amino-5-pyrimidyl-methyl)-4-methyl-5, β-hydroxyethyl -2-thiazolethione Merck (AM)	+	—
	3-(2-Ethyl-4-amino-5-pyrimidyl-methyl)-4-methyl-5, β-hydroxyethyl -2-thiazolethione Merck (ET)	+	—
	3-(2-Ethyl-4-amino-5-pyrimidyl-methyl)-5, β-hydroxyethyl-4-methyl thiazolium bromide hydrobromide Merck (BB)	+	—
	3-(2-Methyl-4-carbamide-5-pyrimidyl-methyl)-5, β-hydroxyethyl-4-methyl-thiazolium chloride Merck (TC)	+	—
	3-(2-Methyl-5-pyrimidylmethyl) thiazolium bromide hydrobromide Merck (MP)	+	—

A 16559

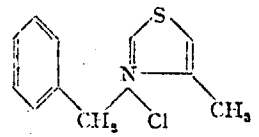
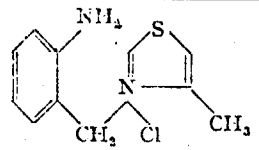
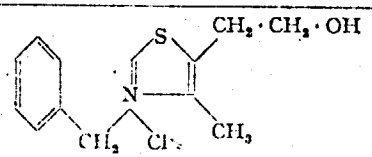
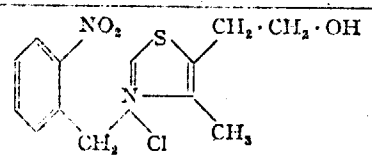
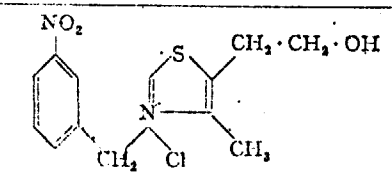
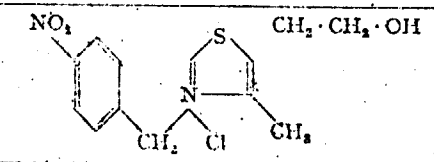
Structure	Chemical name	Medium: Stimulation	Medium: Inhibition
	3-Benzyl-4-methylthiazolium chloride White & Sealock (Y4)	+	—
	3, o-Aminobenzyl-4-methylthiazolium chloride Merck (3M)	+	—
	3-Benzyl-4-methyl-5, β-hydroxyethyl thiazolium chloride Metzler (Y9)	+	—
	3, o-Nitrobenzyl-4-methyl-5, β-hydroxyethylthiazolium chloride Metzler (Y5)	+	—
	3, m-Nitrobenzyl-4-methyl-5, β-hydroxyethylthiazolium chloride Yount (Y1)	+	—
	3, p-Nitrobenzyl-4-methyl-5- β-hydroxyethylthiazolium chloride Yount (Y2)	+	—

Table V. Group III. Thiamine analogues (continued)

1659

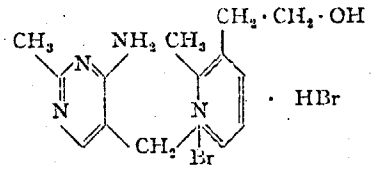
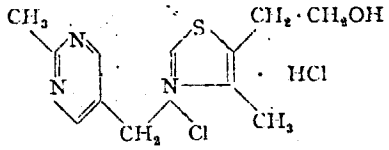
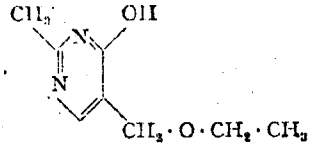
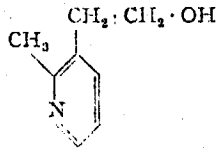
Structure	Chemical name	Effect on growth at 5 μg/ml in biotin basal synthetic medium	
		Stimulation	Inhibition
	1-(2-Methyl-4-amino-5-pyrimidyl-methyl)-2-methyl-3, β-hydroxyethyl pyridinium bromide hydrobromide Merck (NE) (Neopyrithiamine)	—	+
	3-(2-Methyl-4-hydroxy-5-pyrimidyl-methyl)-4-methyl-5, β-hydroxyethyl thiazolium chloride hydrochloride Merck (Oxy) (Oxythiamine)	—	+
	2-Methyl-4-hydroxy-5-ethoxymethyl pyrimidine Merck (PPo) (Pyrimidine half oxythiamine)	—	—
	2-Methyl-3, β-hydroxyethylpyridine Merck (PPN) (Pyridine half neopyrithiamine)	—	—

Table V. Group IV. Thiamine antimetabolites

1659

Furthermore, the halves of neopyrithiamine: 2-methyl-4-amino-5-hydroxymethyl pyrimidine-(Merck) and 2-methyl-3-(β-hydroxyethyl) pyridine-(Merck), were not inhibitory separately at 10 mμM/ml, nor were the two halves of oxythiamine: 2-methyl-4-hydroxy-5-ethoxymethyl pyrimidine-(Merck) and 3,4-dimethyl-5-hydroxyethyl thiazolium chloride-(Yount) at the same concentration. We conclude that the inhibitory activity of neopyrithiamine and oxythiamine on *C. albicans* is due to the characteristic of the entire molecule rather than to its constituent parts.

Effect on growth of C. albicans of substituted pyrimidines (Group I)

In attempting to determine the ability of *C. albicans* to synthesize thiamine when provided with constituent parts of the thiamine molecule, a number of pyrimidines, thiazoles and related compounds were furnished to the organism in the absence of thiamine. As seen in Figure 13, 2-amino-4,6 dimethyl pyrimidine (2p)-Merck, was neither stimulatory nor inhibitory for *C. albicans* at 5 μg/ml in biotin basal synthetic medium. However, the pyrimidines 5H, ADMP and CP (Table V), were all stimulatory at 5 μg/ml after 2 days incubation on the shaker but to a lesser degree than thiamine. These results imply that the organism is able, in each instance, to synthesize the thiazole moiety of thiamine and couple it with either of the preformed pyrimidines (5H, ADMP and CP) to form thiamine.

Effect on C. albicans of substituted thiazoles (Group II)

As seen in Table V, five substituted thiazoles were all stimulatory to the growth of *C. albicans* in biotin basal synthetic medium, but to a lesser extent than intact thiamine. These results imply that the organism is able, in each instance, to synthesize the pyrimidine moiety of thiamine and couple it with a number of preformed thiazoles (Y-6, 7, 10, 11, 12 and MH) to form thiamine. Substitution of sulfur of the thiazole ring with oxygen as in α-methyl-oxazolium, failed to produce an inhibitory compound for *C. albicans.*

Effect on C. albicans of thiamine analogues (Group III)

As seen in Table V, five thiamine compounds at 5 μg/ml concentration (acetylthiamine, allithiamine, cocarboxylase, tetrahydrothiamine and dihydrothiamine) in biotin basal synthetic medium, were equally as stimulatory to *C. albicans* as thiamine at 2 days incubation (Table III and Fig. 6). Other thiamine analogues such as AM, ET, BB, TC and MP at 5 μg/ml concentration in biotin basal synthetic medium were equally as stimulatory as thiamine after 2 days of incubation (Table V). Thiamine analogues in which the pyrimidine ring was replaced by a substituted benzene ring, as in the case of analogues Y-1, 2, 4, 5, 9 and 3M, all at 5 μg/ml, were approximately as stimulatory as 5 μg thiamine/ml at 2 days incubation. These results show *C. albicans* to possess great

1659

versatility with regard to biosynthesis of thiamine, accepting many related compounds in place of thiamine as well as a wide variety of substituted pyrimidines and thiazoles.

Role of thiamine in the growth of C. albicans. The primary function of thiamine in plant metabolism, and presumably in *C. albicans* as well, is as a regulator of carbohydrate metabolism. The metabolically active form of thiamine is cocarboxylase (thiamine pyrophosphate) named because of its coenzyme function in the decarboxylation of pyruvic acid. When thiamine is restricted in the culture medium containing thiamine-deficient fungi, pyruvic acid rapidly accumulates in the culture medium (HAAG, 1940; WIRTH & NORD, 1942; FRIEND & GOODWIN, 1954). Cocarboxylase is also a coenzyme for transketolase and for a variety of enzymatic reactions involving decarboxylation of α-ketoacids (JANSEN, 1954). Previous studies of the thiamine dependency of *C. albicans* have been with low concentrations ranging from 0.1 to 1.0 μg/ml in basal synthetic medium, BURKHOLDER, 1943; BURKHOLDER & MOYER 1943; SCHOFFER, 1944; BURKHOLDER, MCVEIGH & MOYER, 1944; MCVEIGH & BEEL 1951; BONA & HEDRICK, 1952; DROUHET & COUTEAU, 1954; DROUHET & VIEU 1957a; 1957b; MIYASHITA, MIWATANI & FUJINO 1958; KOSER, HODGES, TRIBBY & STUEDELL, 1960). Although most reports agree on the dependency of *C. albicans* for thiamine, there are some exceptions (BONA & HEDRICK, 1952; DROUHET & VIEU, 1957a). Our studies with 5 strains of the organism have shown that the optimal concentration of thiamine in biotin basal synthetic medium is considerably higher than the above range, with optimal values at 30 μg/ml for all 5 strains.

Summary

By employing wide ranges in vitamin concentrations in biotin basal mineral synthetic medium, it was demonstrated that vitamin B_{12} markedly stimulated the growth of *Candida albicans*, the organism showing a partial dependency upon this vitamin. Growth inhibition by 5-fluorouracil was reversed non-competitively by vitamin B_{12}, suggesting that B_{12} has a role in nucleic acid biosynthesis of the organism. Thiamine was growth stimulatory, the organism being partially dependent upon this vitamin as well. Neopyrithiamine and oxythiamine were growth inhibitory in thiamine-free biotin basal mineral synthetic medium although the halves of each inhibitor compound were non-inhibitory. Neopyrithiamine inhibition was reversed by intact thiamine but not by pyrimidine thiamine or thiazole thiamine; while oxythiamine inhibition was reversed by thiamine and pyrimidine thiamine but not by thiazole thiamine, the inference being drawn that oxythiamine selectively blocks utilization of pyrimidine thiamine. Twenty-seven different substituted pyrimidines, thiazoles and related thiamine compounds were all utilizable by *C. albicans* in thiamine-free basal

synthetic mineral medium, the organism presumably synthesizing thiamine when presented with the constituent parts of these thiamine analogues. Substitution of sulfur of the thiazole ring with oxygen, as in α-methyloxazolium, failed to produce an inhibitory compound for *C. albicans*. Acetylthiamine, allithiamine, cocarboxylase, tetrahydrothiamine and dihydrothiamine were equally as growth stimulatory as thiamine.

Acknowledgement

The authors are indebted to Dr. HARRY J. ROBINSON, Merck Institute for Therapeutic Research for contributing numerous thiamine analogues, to Drs. RALPH G. YOUNT and DAVID E. METZLER of Iowa State College for numerous thiamine derivatives, and to Dr. W. E. SCOTT of Hoffman-LaRoche, Inc. for 5-fluorouracil. We also wish to thank Miss YOKO SHIMIZU of Osaka, Japan for technical assistance. This work was supported by Grants G-7072 and G-21044 from the National Science Foundation, U.S.A.

References

ARNSTEIN, H. R. V. (1958). The metabolic function of vitamin B_{12}. Proc. Fourth Int. Congr. Biochem. Vienna, Austria. New York, Pergamon Press. 11, 286.

BONA, J. J. & HEDRICK, L. R. (1952). Vitamin requirements for members of the yeast genus Candida. Bact. Proc. 135.

BURKHOLDER, P. R. (1943). Vitamin deficiencies in yeasts. Amer. J. Bot. 30, 206.

BURKHOLDER, P. R. & MOYER, D. (1943). Vitamin deficiencies of fifty yeasts and molds. Bull. Torrey bot. Cl. 70, 372.

BURKHOLDER, P. R., MCVEIGH, I. & MOYER, D. (1944). Studies on some growth factors of yeasts. J. Bact. 48, 385.

DINNING, J. S. & YOUNG, R. S. (1960). Some effects of folic acid and vitamin B_{12} on nucleic acid metabolism in *Lactobacillus leichmannii*. J. biol. Chem. 235, 3008.

DOWNING, M. & SCHWEIGERT, B. S. (1956). Role of vitamin B_{12} in nucleic acid metabolism. IV. Metabolism of C^{14}-labeled thymidine by *Lactobacillus leichmannii*. J. biol. Chem. 229, 821.

DROUHET, E. & COUTEAU, M. (1954). Facteurs vitaminiques de croissance des *Candida*. C. R. Acad. Sci., Paris 239, 1675.

DROUHET, E. & VIEU, M. (1957a). Facteurs vitaminiques de croissance des *Candida*. Ann. Inst. Pasteur 92, 825.

DROUHET, E. & VIEU, M. (1957b). Biologie des infections à *Candida* I. Diagnostic de laboratoire. Sem. Hôp., Paris 33, 793.

FRIEND, J. & GOODWIN, T. W. (1957). Studies in carotenogenesis. 12. The effect of temperature and thiamine concentration on carotenogenesis by *Phycomyces blakesleeanus*. Biochem. J. 67, 434.

HAAG, E. (1940). Sur le métabolisme du yeast Phycomyces de l'aneurine. C. R. Soc. Phys Hist. nat. Genève. 57, 136.

JANSEN, C. P. (1954). In W. H. STUDELL, JR. and R. S. HARRIS (eds.), The Vitamins, Vol. III., p. 444. New York, Academic Press.

KITAY, E., MCNUTT, W. S. & SNELL, E. E. (1949). The nonspecificity of thymidine as a growth factor for lactic acid bacteria. J. Biol. Chem. 177, 993.

KOSER, S. A., HODGES, E., TRIBEY, I. & STUDELL, J. T. (1960). Growth of Lactobacilli in association with *Candida albicans*. J. infect. Dis. 106, 60.

LASCELLES, J. & CROSS, M. I. (1955). The function of vitamin B_{12} in nitrogen-organisms. Biochem. Soc. Symposia No. 13, 103.

1659

LITTMAN, M. L. & MIWATANI, T. (1961). Reversal of toxicity of 5-fluorouracil and 5-fluorodeoxyuridine for *Candida albicans* by pyridoxine and pyridoxamine. Nature, Lond. **192**, 1155.

LITTMAN, M. L. & MIWATANI, T. (1962). Studies on anticancer compounds with *Candida albicans* test system. I. Toxicity reversing compounds for 5-fluorouracil and 5-fluoro-2'-deoxyuridine growth inhibition. Cancer Chemother. Rep. No. 16, **353**.

LITTMAN, M. L. & MIWATANI, T. (1963). Effect of water soluble vitamins and their analogues on the growth of *Candida albicans*. I. Biotin, pyridoxamine, pyridoxine and fluorinated pyrimidines. Mycopathol. et Mycol. Appl. (In the press, 1963).

MIYASHITA, S., MIWATANI, T. & FUJINO, T. (1958). Studies on the nutrition of *Candida*. I. Vitamin requirements of strains of *Candida*. Biken's J. **1**, 45.

MCVEIGH, I. & BELL, E. (1951). The amino acid and vitamin requirements of *Candida albicans* Y-475 and *Mycoderma vini* Y-939. Bull. Torrey bot. Cl. **78**, 134.

ROBERTS, I. Z., ROBERTS, R. B. & ABELSON, P. H. (1949). Effect of vitamin B_{12} on the phosphorus metabolism of *Lactobacillus leichmannii*. J. Bact. **58**, 709.

SCHOPFER, W. H. (1944). Les besoins en facteurs de croissance de vingt-trois espèces et variétés du genre *Candida*. La production de lactoflavine. C. R. Soc. Phys. Hist. nat. Genève. **61**, 232.

SMITH, E. L. (1958). Biochemical functioning of vitamin B_{12}. Nature, Lond. **181**, 305.

SMITH, E. L. (1960). Vitamin B_{12}. p. 196. New York, John Wiley and Sons.

SPELL, W. H., JR. & DINNING, J. S. (1959). A requirement for vitamin B_{12} in the conversion of ribose to deoxyribose by Lactobacillus leichmannii. J. Amer. chem. Soc. **81**, 3804.

VON WACKER, A., PFAHL, D. & SCHRÖDER, I. (1957). Beziehungen zwischen Vitamin B_{12} und der Desoxyribonucleinsäure. Z. Naturf. **12B**, 510.

WIRTH, J. C. & NORD, F. F. (1942). Essential steps in the enzymatic breakdown of hexoses and pentoses. Interaction between dehydrogenation and fermentation. Arch. Biochem. **1**, 143.

WRIGHT, L. D., SKEGGS, H. R. & HUFF, J. W. (1948). The ability of thymidine to replace vitamin B_{12} as a growth factor for certain lactobacilli. J. biol. Chem. **175**, 475.

1671

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

SU HOON LOOI[4] AND RUTH RENNER
School of Household Economics, University of Alberta, Edmonton, Alberta, Canada T6G 2E2

ABSTRACT Experiments were conducted to study the effect of fat on the chick's requirement for methionine when fat was substituted isocalorically for carbohydrate in a semipurified diet in which protein was supplied by isolated soybean protein. Using either growth or energetic efficiency as the criterion, the chick's requirement for methionine was shown to remain unchanged when hydrogenated fat was substituted isocalorically for glucose, irrespective of the vitamin B-12 status of the chick. Thus, the increased requirement for vitamin B-12 which has been previously observed when fat was substituted isocalorically for glucose cannot be attributed to increased need for methionine, and may reflect a role for vitamin B-12 in fat metabolism. J. Nutr. *104:* 400–404, 1974.

INDEXING KEY WORDS methionine · fat · "carbohydrate-free" nutrient requirement · chick

Previous studies (1) have shown that the isocaloric substitution of fat for carbohydrate in the diet of the chick increased the requirement for vitamin B-12. Since vitamin B-12 has a methionine-sparing action (2) the question arose as to whether the observed increase was actually for vitamin B-12 or whether it merely reflected an increased need for methionine.

Whether source of nonprotein energy affects methionine requirement is unknown. High fat diets might tend to increase methionine requirement if the need for taurine for the synthesis of bile salts is increased, or if the need for one-carbon metabolites increases as fat intake increases. On the other hand, other factors may tend to decrease the requirement for methionine by increasing its utilization. In this regard Pearson and Panzer (3) observed that dietary corn oil tended to lower the urinary excretion of methionine and valine in growing rats. More recently Nakano and Ashida (4) have shown that rats, prefed a diet in which nonprotein energy was supplied by fat, excreted less urinary nitrogen when changed to a high protein diet than did rats prefed a high carbohydrate diet. They found the decreased excretion of urinary nitrogen to be due to retardation of the formation of amino acid-degrading enzymes. These results suggest that fat may have a protein-sparing action.

The following studies were conducted to determine the effect of source of nonprotein energy on the methionine requirement of the chick.

PROCEDURES

In experiment 1, carbohydrate-containing diets with and without vitamin B-12 (100 μg/kg diet) and containing either 0, 0.1, 0.2 or 0.3% supplemental methionine were formulated from basal diet A (table 1) by substituting methionine weight for weight for glucose. Their "carbohydrate-free" counterparts were formulated by substituting hydrogenated fat isocalorically for glucose using the values 7.16 (5) and 3.64 kcal/g (6) for the metabolizable energy

Received for publication July 18, 1973.

[1] Supported in part by a research grant from the National Research Council of Canada.

[2] The data were taken from a thesis submitted by Su Hoon Looi in partial fulfillment for the degree of Doctor of Philosophy at the University of Alberta.

[3] Requests for reprints should be addressed to Ruth Renner, School of Household Economics, Edmonton, Alberta, T6G 2E2.

[4] Present address: Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia.

568

content of hydrogenated fat and glucose, respectively. Thus, all diets have similar energy:protein, energy:mineral and energy:vitamin ratios. Cellulose (6.85 parts) was added to improve the texture of the "carbohydrate-free" diet in an amount to maintain the energy in a given volume equal to that of its high carbohydrate counterpart.

In experiment 2, carbohydrate-containing diets containing 0, 0.075, 0.150, 0.225 and 0.300% methionine were formulated from basal diet B (table 1) by substituting methionine weight for weight for glucose. Their "carbohydrate-free" counterparts were formulated by substituting hydrogenated fat isocalorically for glucose using the value 3.64 kcal/g for glucose and the value determined for hydrogenated fat in experiment 1, that is, 8.59 kcal/g. These diets contained 18.6 kcal metabolizable energy/gram protein. This level is similar to the energy:protein ratio of the "carbohydrate-free" diet used in experiment 1. The diets (expt. 2) were not fortified with vitamin B-12. Since in both experiments 1 and 2 the "carbohydrate-free" diets do not total to 100, their content of methionine will be referred to as the level present in the carbohydrate-containing diets from which they were derived.

In both experiments, each diet was fed to duplicate groups of 10 male day-old crossbred (Dominant White × White Plymouth Rock) chicks to 29 days of age. The chicks were assigned at random at 1 day of age to each experimental group. They were maintained in electrically heated thermostatically controlled battery brooders with raised wire screen floors in a temperature-controlled laboratory. Feed and water were supplied ad libitum. Data on growth and feed consumption were obtained weekly and feed wastage was determined daily. In experiment 1, fecal collections were made from selected groups on days 12, 13 and 14 of life for the determination of metabolizable energy. At 4 weeks

TABLE 1

Composition of basal diets on a dry matter basis

Ingredients	Diet A	Diet B
	g	*g*
Glucose[1]	64.46	69.21
Soybean protein[2]	25.03	20.28
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[3]	1.26	1.26
Vitamin mixture[4]	0.43	0.43
Chromium oxide	0.30	0.30
Antioxidant[5]	0.025	0.025

[1] Cerelose. [2] Promine, Central Soya, Chemurgy Division, Chicago, Ill. 60639. [3] Supplied in milligrams per 367 kcal of ration: KH_2PO_4, 930; $MgSO_4$, 258; KI, 0.29; $FeSO_4 \cdot 7H_2O$, 28; $CuSO_4 \cdot 5H_2O$, 1.56; $ZnCO_3$, 11.50; $CoCl_2 \cdot 6H_2O$, 0.17; $Na_2MoO_4 \cdot 2H_2O$, 0.83; Na_2SeO_3, 0.022; and $MnSO_4 \cdot H_2O$, 33. [4] Supplied per 367 kcal of ration: thiamin, 1.0 mg; riboflavin, 1.0 mg; Ca pantothenate, 4.0 mg; biotin, 0.04 mg; pyridoxine, 2.0 mg; niacin, 8.0 mg; folic acid, 0.3 mg; menadione, 0.3 mg; retinyl, palmitate, 1000 IU; cholecalciferol, 150 ICU; α-tocopheryl acetate, 3.3 IU; chlortetracycline, 1.0 mg; and choline chloride, 148 mg. [5] Contains 25% ethoxyquin, Monsanto Chemical Co., St. Louis, Mo. 63166.

TABLE 2

Effect of level of methionine and vitamin B-12 on weight gain, energy consumption and energetic efficiency of chicks fed carbohydrate-containing and "carbohydrate-free" diets

Supplements		Weight gain		Energy consumption		Energetic efficiency	
		Carbohydrate		Carbohydrate		Carbohydrate	
Vitamin B-12	Methionine	Containing	"Free"	Containing	"Free"	Containing	"Free"
μg/kg	*%*	*g*	*g*	*kcal*	*kcal*	*kcal/g gain*	
0	0	$68^{1,a}$	68^{a}	$628^{1,a}$	594^{a}	$9.19^{1,a}$	8.77^{a}
0	0.1	265^{d}	212^{c}	1748^{d}	1283^{c}	6.54^{c}	$6.20^{c,d}$
0	0.2	$354^{e,f,g}$	$358^{e,f,g}$	$1918^{d,e}$	$1922^{d,e}$	5.40^{e}	5.36^{e}
0	0.3	$398^{h,i}$	$419^{h,i,j}$	$2066^{e,f}$	2157^{f}	5.20^{e}	5.14^{e}
100	0	$94^{a,b}$	114^{b}	$750^{a,b}$	864^{b}	7.95^{b}	7.56^{b}
100	0.1	328^{e}	$341^{e,f}$	1796^{d}	$1850^{d,e}$	5.46^{e}	$5.67^{d,e}$
100	0.2	$375^{f,g}$	448^{j}	$1938^{d,e}$	2250^{f}	5.23^{e}	5.08^{e}
100	0.3	$391^{g,h,i}$	$425^{i,j}$	$2030^{e,f}$	2182^{f}	5.19^{e}	5.11^{e}

[1] Values are averages of duplicate groups each containing 10 chicks. Values without a common letter in their superscript are significantly different ($P < 0.05$).

TABLE 3

Carcass composition, energetic efficiency and protein retention of chicks fed carbohydrate-containing and "carbohydrate-free" diets with graded levels of methionine, with and without vitamin B-12

Supplements		Carcass fat		Carcass protein		Protein retained[1]		Energetic efficiency[2]	
		Carbohydrate		Carbohydrate		Carbohydrate		Carbohydrate	
Vitamin B-12	Methionine	Containing	"Free"	Containing	"Free"	Containing	"Free"	Containing	"Free"
μg/kg	%	% DM[3]	% DM	% DM	% DM	%	%		
0	0	19.7[d,a]	22.6[b]	65.1[h]	62.0[g]	53.1[a]	57.5[a]	4.10[a]	3.60[b]
0	0.1	35.0[d,e]	36.2[d,e]	55.4[e,f]	53.3[c,d,e]	58.3[a]	66.0[b,c]	2.42[d]	2.17[d,e]
0	0.2	34.6[d]	42.2[h,i]	54.6[d,e,f]	48.8[a,b]	65.1[b]	66.9[b,c]	2.18[d,e]	1.88[e,f]
0	0.3	35.9[d,e]	40.8[g,h]	53.9[d,e,f]	49.8[b]	67.5[b,c]	74.4[d,e]	1.98[e,f]	1.78[f]
100	0	24.8[b]	30.3[c]	61.0[g]	56.5[f]	53.7[a]	54.8[a]	3.52[b]	3.06[c]
100	0.1	37.5[e,f]	42.4[h,i]	52.7[c,d]	49.2[b,d]	64.5[b]	67.4[b,c]	2.05[e,f]	1.86[e,f]
100	0.2	36.1[d,e]	44.4[i]	54.9[d,e,f]	47.2[a]	71.7[c,d]	69.0[b,c,d]	1.94[e,f]	1.73[f]
100	0.3	35.9[d,e]	39.2[f,g]	53.8[d,e]	51.1[b,c]	69.3[b,c,d]	75.7[e]	2.02[e,f]	1.91[e,f]

[1] (Gain in carcass protein, g/protein consumed, g) × 100. [2] Kilocalories of metabolizable energy consumed/kilocalorie gained. [3] % of dry matter. [4] Values are averages of duplicate groups each containing 10 chicks. Values without a common letter in their superscript are significantly different ($P < 0.05$).

of age the chicks in experiment 1 were killed and the entire carcasses from each experimental group (minus the livers) were prepared for analysis as described by Hill and Anderson (7).

RESULTS AND DISCUSSION

Summarized in table 2 are data showing weight gains, energy consumption and gross efficiency of feed utilization of chicks fed diets containing graded levels of methionine with and without vitamin B-12 (expt. 1). Analysis of variance and application of Duncan's multiple range test (8) to the data on growth showed that the amount of methionine required to produce optimum growth was reduced by the addition of vitamin B-12 to the diet but was unaffected by the source of nonprotein energy. These results show the requirement of chicks for supplemental methionine when fed these carbohydrate-containing and "carbohydrate-free" diets to be at least 0.3% in the absence of vitamin B-12 and from 0.1 to 0.2% in the presence of vitamin B-12. Calculations assuming that soybean protein contains 1.15 and 0.4% methionine and cystine, respectively, indicated that the basal diet contained 0.63% sulfur-containing amino acids. Thus, results of this experiment indicate that the requirement for sulfur-containing amino acids of chicks fed a diet containing 24% protein was 0.73 to 0.83% when adequately supplemented with vitamin B-12 and at least 0.93% in the absence of vitamin B-12. Since source of nonprotein energy did not affect methionine requirement, it can be concluded that the increased vitamin B-12 requirement of chicks fed "carbohydrate-free" diets observed previously (1) was not due to a methionine-sparing action and may reflect a role for vitamin B-12 in fat metabolism.

Using energetic efficiency as the criterion to assess requirement, the data in table 2 show that the amount of methionine required to produce optimal caloric efficiency was reduced significantly ($P < 0.05$) by the addition of vitamin B-12 to the diet but was unaffected by the source of nonprotein energy ($P > 0.05$). The results indicate that the requirement of chicks for supplemental methionine was not greater than 0.1% and 0.2%, respectively, when diets with and without supplemental vitamin B-12 were fed. Comparison of these results with results obtained using growth as the criterion indicates that the amount of methionine required to promote optimal gain is greater than the amount required for optimal feed efficiency. This finding is in contrast to the results of Almquist (9) and Nelson et al. (10) who found that chicks require more methionine for maximum feed efficiency than for maximum growth.

Data on carcass composition summarized in table 3 show that in this experiment chicks fed diets which contained either 0 or 0.1% supplemental methionine and

TABLE 4

Effect of level of methionine on weight gain, energy consumption and energetic efficiency of chicks fed carbohydrate-containing and "carbohydrate-free" diets without vitamin B-12

Supplemental methionine	Weight gain		Energy consumption		Energetic efficiency[1]	
	Carbohydrate		Carbohydrate		Carbohydrate	
	Containing	"Free"	Containing	"Free"	Containing	"Free"
%	g	g	kcal	kcal	kcal/g gain	
0	74[2,a]	108[b]	956[a]	1008[a]	13.00[a]	9.37[b]
0.075	240[c]	253[c]	2029[c]	1829[b]	8.13[b,c,d]	7.22[c,d,e,f]
0.150	292[d]	360[f]	2389[d,e]	2352[d]	8.20[b,c]	6.54[d,e,f]
0.225	318[e]	394[g]	2346[d],	2482[d,e]	7.36[c,d,e,f]	6.30[e,f]
0.300	329[e]	396[g]	2564[e]	2442[d,e]	7.78[c,d,e]	6.18[f]

[1] Calories of metabolizable energy consumed/g gain. [2] Values are averages of duplicate groups each containing 10 chicks. Values without a common letter in their superscript are significantly different ($P < 0.05$).

which were either severely or moderately deficient in methionine did not increase their fat deposition. This is in contrast to results reported by Carew and Hill (11) and suggests that in this experiment chicks did not overeat to meet their methionine requirement. This experiment, however, differs from those of Carew and Hill (11) in that the basal diet used was severely deficient in methionine but was supplemented with cystine while the basal diet used by Carew and Hill (11) was only moderately deficient in sulfur-containing amino acids and contained no added cystine. Whether these differences contributed to the differences in results obtained is unknown.

Also summarized in table 3 are data showing the proportion of protein retained by chicks fed carbohydrate-containing and "carbohydrate-free" diets containing varying levels of methionine with and without vitamin B-12. Analysis of variance and application of Duncan's multiple range test (8) to the data showed that, in both the absence and presence of vitamin B-12, 0.2% supplemental methionine resulted in maximum nitrogen retention when chicks were fed carbohydrate-containing diets whereas 0.3% supplemental methionine caused a further increase in nitrogen retention when chicks were fed "carbohydrate-free" diets. These results suggest that the isocaloric substitution of fat for carbohydrate increases the methionine requirement for maximum protein retention; however, no firm conclusion can be reached since the diets were not isocaloric. The carbohydrate-containing and "carbohydrate-free" diets contained 15.2 and 18.7 kcal metabolizable energy/gram protein, respectively. This difference was due to the hydrogenated fat having a metabolizable energy value of 8.59 rather than 7.16 kcal/g (5). Thus, whether the increased requirement for methionine for maximum nitrogen retention was due to the isocaloric substitution of fat for carbohydrate or to changes in energy:protein ratio is unknown.

Since the energy:protein ratio of the carbohydrate-containing and "carbohydrate-free" diets fed in experiment 1 differed the question arose as to whether comparable results would be obtained when diets having similar energy:protein ratios were fed. Summarized in table 4 are data showing weight gain, feed consumption and gross efficiency of feed utilization when diets with and without carbohydrate containing graded levels of methionine and having similar energy:protein ratios were fed. Analysis of variance and application of Duncan's multiple range test (8) to the growth data showed that the addition of increasing levels of methionine to both the carbohydrate-containing and "carbohydrate-free" diets caused progressive and significant increases in growth up to a level of 0.225% supplemental methionine. Plots of weight gains versus levels of dietary methionine expressed as $\log_{10}$ (0.240 + % added methionine) and calculation of the two lines which best fit these points using regression analysis showed that, irrespective of source of nonprotein energy, linear ascending and plateau lines intersect at 0.20% supplemental methionine. These results confirmed the results of the preceding

experiment, showing that the methionine requirements of chicks fed carbohydrate-containing and "carbohydrate-free" diets are similar when growth is used as the criterion.

Analysis of variance (8) also showed that source of nonprotein energy affected growth significantly ($P < 0.01$). The data show that, irrespective of the level of supplemental methionine, chicks fed diets in which nonprotein energy was supplied by fat grew significantly faster than chicks fed diets in which nonprotein energy was supplied by carbohydrate even though energy consumption was not increased. That fats, particularly vegetable oils, added to nutritionally complete diets stimulate growth of chicks and improve efficiency of utilization of feed has been observed previously (7, 12–14). The failure of fat to promote more rapid growth in experiment 1 may have been due to the difference in the energy:protein ratio between carbohydrate-containing and "carbohydrate-free" diets. The possibility exists that in experiment 1 chicks fed the "carbohydrate-free" diet containing 18.7 kcal/g protein were able to grow at the same rate as chicks fed the carbohydrate-containing diet having an energy:protein ratio of 15.2 kcal/g protein because of the protein-sparing action of fat.

Statistical treatment of the data on energetic efficiency showed that supplementation with 0.075% methionine caused a significant increase in energetic efficiency in both carbohydrate-containing and "carbohydrate-free" diets with no further significant increase being observed on the addition of higher levels of methionine. These results show that, as in experiment 1, when energetic efficiency was used as the criterion the requirement of chicks for methionine was unaffected by source of nonprotein energy.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Ulla DeBruijn, Joan Davies and Barbara Hohol.

LITERATURE CITED

1. Looi, S. H. & Renner, R. (1974) Effect of feeding "carbohydrate-free" diets on the chick's requirement for vitamin B-12. J. Nutr. *104*, 394–399.
2. Briggs, G. M., Hill, E. G. & Giles, M. J. (1950) Vitamin B_{12} in all-plant rations for chicks and sparing activity of methionine and choline. Poultry Sci. *29*, 723–736.
3. Pearson, P. B. & Panzer, F. (1949) Effect of fat in the diet of rats on their growth and their excretion of amino acids. J. Nutr. *38*, 257–265.
4. 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*, 208–216.
5. Renner, R. & Hill, F. W. (1958) Metabolizable energy values of fats and fatty acids for chickens. Proc. Cornell Nutrition Conference, pp. 95–100.
6. Anderson, D. L., Hill, F. W. & Renner, R. (1958) Studies of the metabolizable and productive energy of glucose for the growing chick. J. Nutr. *65*, 561–574.
7. Hill, F. W. & Anderson, D. L. (1958) Comparison of metabolizable energy and productive energy determinations with chicks. J. Nutr. *64*, 587–603.
8. Steel, R. G. D. & Torrie, J. H. (1960) Principles and Procedures of Statistics with Specific Reference to the Biological Sciences. McGraw-Hill Book Co., New York.
9. Almquist, H. J. (1952) Amino acid requirements of chickens and turkeys—a review. Poultry Sci. *31*, 966–981.
10. Nelson, T. S., Young, R. J., Bradfield, R. B., Anderson, J. B., Norris, L. C., Hill, F. W. & Scott, M. L. (1960) Studies on the sulfur amino acid requirement of the chick. Poultry Sci. *39*, 308–314.
11. Carew, L. B., Jr. & Hill, F. W. (1961) Effect of methionine deficiency on the utilization of energy by the chick. J. Nutr. *74*, 185–190.
12. Dam, R., Leach, R. M., Jr., Nelson, T. S., Norris, L. C. & Hill, F. W. (1959) Studies on the effect of quantity and type of fat on chick growth. J. Nutr. *68*, 615–632.
13. Rand, N. T., Scott, H. M. & Kummerow, F. A. (1958) Dietary fat in the nutrition of the growing chick. Poultry Sci. *37*, 1075–1085.
14. Carew, L. B., Jr., Hopkins, D. T. & Nesheim, M. C. (1964) Influence of amount and type of fat on metabolic efficiency of energy utilization by the chick. J. Nutr. *83*, 300–306.

1706

FEDERATION PROCEEDINGS
Vol. 28, No. 1, January-February, 1969
Printed in U.S.A.
P. 41-5

Vitamin B_{12} absorption and the intestinal cell surface[1]

IAIN L. MACKENZIE AND
ROBERT M. DONALDSON, JR.[2]
Boston University School of Medicine, Boston, Massachusetts

VITAMIN B_{12} CAN BE ABSORBED from the intestine by two different mechanisms. When the amount of the administered vitamin is large, absorption occurs by a diffusion process and is directly proportional to the dose (16). This mechanism is extremely inefficient since only a tiny portion of the administered vitamin is absorbed. Furthermore, absorption by this mechanism is nonspecific in the sense that it occurs not only from the intestine but also from the lungs, oropharynx, nose, and rectum (40). On the other hand, an efficient and extremely specific absorptive mechanism is operative when the dose of the vitamin is in the physiological range of less than 2 μg. Efficient absorption of these more physiological quantities of the vitamin requires gastric intrinsic factor (IF) and occurs only from the distal small intestine. The IF markedly enhances vitamin B_{12} absorption from the distal small intestine, but actually inhibits uptake of the vitamin by proximal small bowel (5). Thus IF-mediated absorption of vitamin B_{12} is dependent not only on IF but also on a specialized attribute of the distal small bowel.

BINDING OF VITAMIN B_{12} BY INTRINSIC FACTOR

Intrinsic factor, secreted in man by the gastric parietal cells (29), is a complex glycoprotein with a molecular weight of approximately 50,000 (24). This glycoprotein tightly binds vitamin B_{12} to form a macromolecular IF-B_{12} complex (22). Gastric-mucosal and gastric-juice preparations containing IF enhance intestinal absorption of vitamin B_{12} in direct proportion to their capacity to bind the vitamin (22). Manipulations that diminish the vitamin B_{12}-binding capacity of IF impair to a similar extent the ability of IF to enhance absorption (22). Furthermore, whenever both free and IF-bound vitamin B_{12} are present in the intestinal lumen, the IF-bound vitamin is preferentially absorbed (3, 12, 39). Thus, binding of the vitamin by IF in a macromolecular complex is essential for efficient vitamin B_{12} absorption.

In preparation for intestinal absorption, dietary vitamin B_{12} is liberated from organic food complexes both by cooking and by gastric pepsin (26). Favored by an extremely low affinity constant (0.38×10^{-10} M) for the interaction of IF and vitamin B_{12} (32), IF presumably competes successfully for this dietary vitamin B_{12} with the non-IF B_{12}-binding proteins present in gastric juice (7). Peristalsis then carries the IF-B_{12} complex to the ileum. During this transit through the small intestine the IF-B_{12} complex is not digested. The IF bound to vitamin B_{12} is less susceptible than IF alone to all denaturing influences so far examined in vitro including peptic (1) and chymotryptic (21) digestion. It seems likely that in vivo vitamin B_{12}-binding similarly protects the IF from gastric and intestinal digestion.

SITE OF VITAMIN B_{12} ABSORPTION

Only during the past 12 years has the distal small intestine been identified as the site of IF-mediated vitamin B_{12} absorption. McIntyre et al. (33) showed that malabsorption and consequent deficiency of vitamin B_{12} occurred in patients with disorders of the terminal ileum, and that the malabsorption of the vitamin was not corrected by antibiotics nor by IF. Subsequently Booth and Mollin (5) demonstrated that resection of the ileum regularly resulted in impaired absorption of the vitamin. In addition, these workers found by surface scanning during surgery that radioactive B_{12} administered to patients before laparotomy was concentrated in the distal ileum. Clearly, the distal small intestine is the site of physiological absorption of vitamin B_{12} in man, and many experimental observations (19) have confirmed in a wide variety of animal species that vitamin B_{12} absorption occurs from distal rather than proximal regions of the small bowel. This ability of the distal small intestine to absorb vitamin B_{12} is not, however, a result of

---

From the Intersociety Symposium on "Gastroenterology: Structure and Function of a Digestive-Absorptive Surface" presented at the 52nd Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April 16, 1968. Sponsored by The American Society for Experimental Pathology.

[1] This study was supported in part by Public Health Service Research Grants AM11867, AM03560, and a Training Grant AM05025.

[2] Address requests for reprints to Dr. Robert M. Donaldson, Jr., University Hospital, 750 Harrison Avenue, Boston, Mass. 02118.

573

the intraluminal environment at this location. When the distal ileum of dogs is interposed in the region of the upper jejunum, this transplanted intestine still retains its ability to take up orally administered vitamin even after several weeks (17).

ATTACHMENT OF THE IF-B_{12} COMPLEX TO THE INTESTINAL SURFACE

Intrinsic factor enhances the uptake of vitamin B_{12} by everted sacs (42) and by mucosal homogenates (18) prepared from distal, but not proximal, small intestine of experimental animals. When IF and vitamin B_{12} are incubated simultaneously with these tissues, uptake of the vitamin is greater than when IF is added to the incubation medium before addition of the vitamin (27, 42). Thus, the distal small intestine appears to possess a receptor mechanism that apparently accepts the entire IF-B_{12} complex. In order to attach to this receptor, precise structural characteristics are required not only for the vitamin B_{12}-binding glycoprotein but also for the cobalamin molecule. Vitamin B_{12}-binding proteins present in serum, saliva, colostrum, and tears are unable to promote tissue uptake of vitamin B_{12} in vitro or to enhance absorption of the vitamin by the intestine (19, 41). In addition, IF action is species specific (14, 48) a fact which further emphasizes the precise structural requirements that must be fulfilled before this macromolecule can promote tissue uptake and absorption of vitamin B_{12}. Moreover, since IF is only partially effective or is completely ineffective in promoting the intestinal absorption of all analogues of cyanocobalamin (7), it appears that precise structural characteristics are also required for the cobalamin molecule.

Considerable evidence has now accumulated to indicate that the IF-B_{12} receptor mechanism is located on the intestinal epithelial cell surface. The IF enhances the attachment of vitamin B_{12} to whole mucosal homogenates, brush borders, and microvillous membranes isolated from distal but not proximal hamster small intestine (14). When comparable quantities of tissue are tested, vitamin B_{12} uptake by microvillous membranes is greater than that by brush borders and greater by brush borders than by whole mucosal homogenates. Thus, the relative uptake of IF-bound vitamin B_{12} increases correspondingly with the proportion of microvillous membranes present in each preparation. These observations provide direct support for an action of IF at the surface of the intestinal cell and indicate that the receptor for the IF-B_{12} complex is located on the microvillous membrane. Since the mucopolysaccharide surface coat is retained in microvillous membrane preparations, it is possible that the IF-B_{12} complex attaches to this surface coat rather than to the underlying unit membrane structure (14).

Studies with antibodies to microvillous membranes may provide an approach to further localization and characterization of the IF-B_{12} receptor (34). Antisera from rabbits injected with purified microvillous membrane preparations isolated from distal but not proximal hamster small intestine inhibit attachment of the IF-B_{12} complex to brush borders of distal small intestine (34). Although antisera from rabbits injected with microvillous membranes isolated both from proximal and distal small intestine contain antibodies which localize to the surface of the small intestine, only antisera to distal microvillous membranes inhibit intestinal uptake of the IF-B_{12} complex. When antibodies to microvillous membranes are conjugated with ferritin and incubated with hamster small bowel, the ferritin-conjugated antibodies can be shown to attach to the mucopolysaccharide surface coat of microvilli. It is possible that the inhibitory antibody may attach similarly and thereby block the IF-B_{12} complex receptor situated in the surface coat. Since the inhibitory antibody containing the ferritin-conjugated antibody mixture has not been separately identified and localized to its combining site, this possibility is as yet untested.

An enzymatic or other energy-requiring process is evidently not involved in the attachment of the IF-B_{12} complex to the cell surface (14, 25). Uptake of the IF-B_{12} complex by brush borders occurs almost immediately, is not affected by changes in temperature from 6 C to 37 C, and is not impaired by the absence of oxygen or glucose from the incubation medium (14). Thus simple physical adsorption of the complex onto the surface of the intestinal cell seems to be the probable mechanism of attachment. Although their role has not been defined, divalent cations are required for this attachment (14, 26). While attachment of the IF-B_{12} complex to the microvillous membrane does not require energy, the process appears to be remarkably specific. Thus, IF from hogs, dogs, rabbits, guinea pigs, and humans does not enhance uptake of the vitamin by hamster brush borders whereas hamster and rat IF preparations are remarkably effective. Moreover, certain macromolecules in serum, saliva, and in extracts of liver, spleen, and kidney bind vitamin B_{12} but are unable to promote uptake of the vitamin by hamster brush borders (14). The affinity of IF-B_{12} complex for the cell surface of everted sacs of guinea pig intestine is striking (affinity constant 0.68×10^{-9} M) (8). A similar affinity of the complex has been shown for the cell surface of hamster (Mackenzie, Donaldson, Kopp and Trier, unpublished observations) and guinea pig (38) brush borders. This degree of affinity provides further evidence that specific binding sites are involved in the interaction. Indeed, this remarkable specificity suggests that the binding forces involved in the attachment of the IF-B_{12} complex to the surface receptor may be similar to those concerned in antigen-antibody reactions. These forces are themselves nonspecific, but because of their short range of action require close intermolecular alignment to be effective (30).

The nature of the IF-B_{12} receptor is unknown, but it is not unreasonable to speculate that it may be a glycoprotein or a mucopolysaccharide. Both glycoproteins and mucopolysaccharides are strongly antigenic as is the

receptor. Furthermore, there is considerable evidence that glycoproteins, or mucopolysaccharides, or both are present at the absorptive surface of the intestinal cell (44). Since IF itself is a glycoprotein and since the affinity of the IF-B_{12} complex for the intestinal receptor is of the same order as the affinity of antibodies for their antigens (30), it is possible that IF-B_{12} complex attachment may resemble the classic interaction between mucopolysaccharide blood group antigens and their gamma globulin antibodies. The species specificity of the interaction between IF and the cell surface further supports this analogy.

Recently, it has been claimed that the IF-B_{12} receptor can be isolated in soluble macromolecular form from brush borders obtained from hamster (13) and guinea pig (38) distal small intestine. Critical to the validity of these claims, however, is evidence that the material exhibiting IF-B_{12} receptor activity is indeed soluble and not particulate in form. In the case of the receptor isolated from hamster brush borders this material was thought to be soluble because it was present in the supernatant fluid after centrifugation at 28,500 $\times$ g (13). Subsequent studies, however, showed that when this supernatant was centrifuged at speeds of 54,500 $\times$ g or greater receptor activity was lost from the supernatant fluid, while the centrifuged pellet containing ribosomes and fragments of microvillous membrane accounted for all the receptor activity (14). Since the centrifugation forces of 30,000 $\times$ g used in studies with guinea pig brush borders (38) may not be sufficient to sediment tiny particles of mechanically fragmented microvillous membrane, further investigations are required to determine whether the IF-B_{12} receptor can be isolated in a nonparticulate, soluble form.

When whole intestinal homogenates are examined, tissue uptake of IF-B_{12} complex exhibits the same characteristics as those observed with isolated preparations of brush borders and microvillous membranes (43). Thus, uptake of the IF-B_{12} complex by whole mucosal homogenates is not temperature dependent, does not require glucose or oxygen, but does need divalent cations. It seems likely, therefore, that the uptake observed with whole mucosal homogenates represents attachment of the IF-B_{12} complex to the surface elements present in these homogenates.

SUBSEQUENT FATE OF THE IF-B_{12} COMPLEX

In contrast to the conditions necessary for attachment of IF-B_{12} complex to the cell surface, IF-mediated uptake of vitamin B_{12} by intact cell systems, such as everted sacs of intestine, is inhibited by lack of oxygen, low temperature, and absence of glucose (42). Loss of integrity of intestinal cells thus appears to be associated with loss of an energy-dependent step required for the absorption of vitamin B_{12} into intact mucosal cells. Absorption of vitamin at the cellular level may therefore be a two-stage process analogous to the uptake of free vitamin B_{12} by *Ochromonas malhamensis* (36). The first stage would be the nonenergy-requiring physical adsorption to the cell surface. The second stage would be the energy-dependent step. No evidence exists at present, however, to indicate the nature of this postulated second step. It may represent digestive activity on the cell surface (9), transport through the membrane, digestion inside the cell, or even accumulation of the absorbed molecule within intracellular organelles (46).

Although little information is available concerning what happens to the IF-B_{12} complex after it attaches to the surface of the ileum, it is convenient to consider three specific questions.

Does the entire IF-B_{12} complex enter the absorptive cell? Ukyo and Cooper (45) and Boass and Wilson (4) have extracted radioactivity from everted sacs of intestine after incubation of these sacs with IF-B_{12} complex. The extracted radioactive vitamin B_{12} was still bound to a substance possessing the biological activity of intrinsic factor. Although these experiments might be interpreted as support for the intact absorption of IF-B_{12} complex into intestinal epithelial cells (4, 45), it is not certain whether the IF-B_{12} complex recovered from intestinal tissue had indeed been absorbed into the cell or had merely been adsorbed onto the cell surface. If it is assumed that the intact IF-B_{12} complex does enter the absorptive cell, how might it do so? The average radius of the postulated water-filled "pores" of intestinal cell membranes has been estimated to be about 4 A (47). Despite its water solubility, IF with an estimated molecular radius of 44 A (47) bound to vitamin B_{12} with a molecular radius of 8 A (47) could not enter through such pores. Studies with rat yolk sacs have led Wilson (47) to conclude that membrane vesiculation and micropinocytosis may be the method of entry. These investigations showed that as rat yolk sacs mature pinocytotic vesiculation diminishes, and at the same time the quantity of vitamin B_{12} taken up by these sacs declines. Furthermore, pinocytosis might explain the generally accepted requirement for calcium in vitamin B_{12} absorption since membrane vesiculation in other systems appears to be calcium dependent (6). Attachment of the IF-B_{12} complex to the cell surface receptor would presumably initiate the process of pinocytosis and hence transport of the complex into the cell.

For several reasons, however, the relevance of these experiments and the importance of pinocytosis in vitamin B_{12} absorption may be questioned. Gamma globulin and other macromolecules (31) are absorbed by intestinal pinocytosis in the fetus and newborn but not in the adult animal; a similar difference may exist with regard to absorption of the IF-B_{12} complex. Certainly an association between pinocytosis and uptake of the IF-B_{12} complex has not been observed in mature intestinal mucosal cells. Furthermore, correlations between the total amount of radioactive vitamin B_{12} present in the yolk sac and the extent of pinocytotic vesiculation cannot be interpreted as evidence that the absorbed vitamin is in fact located in pinocytotic vesicles. Such an interpretation could be made only with radioautography, a technique which

might also distinguish radioactivity adsorbed onto the cell surface from that absorbed into the cell. Thus, the yolk sac observations, while of considerable interest, provide at best only indirect evidence for pinocytosis during vitamin B_{12} absorption.

Carrier-mediated transport is another possible mechanism whereby the IF-B_{12} complex could enter the intestinal cell. Such a mechanism has often been invoked to explain the transport of water-soluble molecules too large to pass readily through intestinal pores (11). Grasbeck (23) has proposed that the intestinal receptor for the IF-B_{12} complex constitutes an incomplete carrier which becomes "complete" with attachment of the complex. The complete carrier then transports the complex into the intestinal cell. Isolation and at least partial characterization of the receptor will be necessary, however, before it can be determined whether attachment of the IF-B_{12} complex to the receptor stimulates pinocytosis, facilitates carrier-mediated active transport, or initiates some other transport mechanism.

Is the IF-B_{12} complex absorbed intact into the circulation? The concept that IF emerges intact from the absorptive cell was originally postulated by Miller in 1957 (35) to explain observations which suggested the existence of a circulating intrinsic factor. However, early reports of the presence of intrinsic factor in serum, bile, and other body fluids (19) have not been confirmed with the more specific analytical techniques currently available (2). Furthermore, when portal blood is examined after subjects are fed radioactive vitamin B_{12} of high specific activity, radioactivity is bound to a macromolecule immunologically distinct from IF (10). Thus, although the possibility has not been excluded that intact IF-B_{12} complex enters the intestinal cell, available evidence argues strongly against the possibility that the IF moiety emerges from the absorptive cell intact and that IF normally circulates through the body.

Is the IF-B_{12} complex digested within or on the surface of the intestinal cell? Destruction of the IF-B_{12} complex with release of the vitamin seems likely since *1*) vitamin B_{12} is removed from the intestine by portal blood rather than by lymphatics (4, 10, 37), *2*) in portal blood the absorbed vitamin is bound to a serum-carrier protein, which is distinct from IF (10), and *3*) there has been no convincing demonstration of the presence of IF in plasma or in tissues other than the stomach (2). Initial support for intestinal splitting of the IF-B_{12} complex came from observations suggesting that a "releasing factor" present in small intestinal homogenates freed radioactive vitamin B_{12} from its complex with rat gastric juice (9). Subsequent studies (28), however, have shown that the "releasing factor" was actually endogenous, nonradioactive vitamin B_{12} which had merely exchanged with the radioactive vitamin bound to IF (12). Thus, although the possibility is attractive, there is no direct evidence of a vitamin B_{12}-releasing mechanism present in the intestinal mucosa.

From in vitro observations (14, 43), it appears that attachment of the IF-B_{12} complex to the cell surface occurs rapidly. Yet IF-mediated absorption of the vitamin in the intact organism is a slow process. When a physiological quantity of radioactive vitamin B_{12} is fed to human volunteers radioactivity does not appear in the plasma for 4 hr, and peak radioactivity occurs only after 6–8 hr (15, 16). Since the same sequence has been observed in a patient with a portacaval shunt (16) the delay in absorption must occur at the surface of or within the intestinal mucosa.

In the complete absence of relevant experimental data, it is tempting to wonder whether this delay involves a complex series of events at the intestinal mucosal cell. Digestion of the IF-B_{12} complex at the digestive-absorptive intestinal cell surface might, for example, yield a small cobalt-containing "active fragment" of the vitamin B_{12} molecule. Such an "active fragment" would pass through the microvillous membrane barrier more readily than the intact vitamin or the IF-B_{12} complex. The complete vitamin B_{12} molecule would then be resynthesized from this absorbed precursor. Such a phenomenon would be analogous to the hydrolysis and resynthesis of triglyceride which occurs during lipid absorption. Although there is no evidence to suggest that this complicated process might occur, there are also no observations that exclude it. Furthermore, surface digestion followed by intracellular resynthesis of vitamin B_{12} might account for the following currently unexplained phenomena: *1*) the way in which the large water-soluble vitamin B_{12} molecule gains access to the intestinal cell, *2*) the long delay in the passage of the vitamin across the intestinal mucosa, and *3*) the energy requirements of the vitamin B_{12} absorptive mechanism. Such speculation, although unwarranted on the basis of available information, may, however, initiate new approaches to the study of vitamin B_{12} absorption.

In summary, then, we can say that the crucial role of vitamin B_{12} binding by IF is well documented as is the specific attachment of the IF-B_{12} complex to a receptor located on the surface of distal small bowel. Completely mysterious, however, is what happens within the intestinal mucosa between the time that the IF-B_{12} complex attaches to the absorptive membrane and vitamin B_{12} bound to serum carrier protein appears in portal blood.

REFERENCES

1. Abels, J., and R. F. Schilling. *J. Lab. Clin. Med.* 64: 375, 1964.
2. Ardeman, S., I. Chanarin and V. Berry. *Brit. J. Haematol.* 11: 11, 1965.
3. Bishop, R. C., M. Toporek, N. A. Nelson and F. H. Bethell. *J. Lab. Clin. Med.* 46: 796, 1955.
4. Boass, A., and T. H. Wilson. *Am. J. Physiol.* 207: 27, 1964.
5. Booth, C. C., and D. L. Mollin. *Lancet* 1: 18, 1959.
6. Brandt, P. W., and A. R. Freeman. *Science* 155: 582, 1967.
7. Castle, W. B. In: *Handbook of Physiology. Alimentary Canal.* Washington, D.C.: Am. Physiol. Soc., 1968, sect. 6, vol. III, chapt. 77, p. 1527.
8. Cooper, B. A. *Medicine* 43: 689, 1964.
9. Cooper, B. A., and W. B. Castle. *J. Clin. Invest.* 39: 199, 1960.

10. Cooper, B. A., and J. J. White. *Brit. J. Haematol.* 14: 73, 1968.
11. Csaky, T. Z. *Ann. Rev. Physiol.* 27: 416, 1965.
12. Donaldson, R. M., Jr., and J. M. Katz. *J. Clin. Invest.* 42: 534, 1963.
13. Donaldson, R. M., Jr., I. L. Mackenzie and J. S. Trier. *J. Clin. Invest.* 45: 1001, 1966.
14. Donaldson, R. M., Jr., I. L. Mackenzie and J. S. Trier. *J. Clin. Invest.* 46: 1215, 1967.
15. Doscherholmen, A., and P. S. Hagen. *Blood* 12: 336, 1957.
16. Doscherholmen, A., P. S. Hagen, M. Liu and L. Olin. *J. Clin. Invest.* 36: 1551, 1957.
17. Drapanas, T., J. S. Williams, J. C. McDonald, W. Heyden, T. Bow and R. F. Spencer. *J. Am. Med. Assoc.* 184: 337, 1963.
18. England, J. M., and K. B. Taylor. *Clin. Res.* 14: 135, 1966.
19. Glass, G. B. J. *Physiol. Rev.* 43: 529, 1963.
20. Glass, G. B. J., and H. Uchino. *Federation Proc.* 18: 53, 1959.
21. Grasbeck, R. *Acta Physiol. Scand.* 45: 88, 1959.
22. Grasbeck, R. *Advan. Clin. Chem.* 3: 299, 1960.
23. Grasbeck, R. *Scand. J. Clin. Lab. Invest.* 19: 7, 1967.
24. Grasbeck, R., K. Simons and I. Sinkkonen. *Biochim. Biophys. Acta* 127: 49, 1966.
25. Herbert, V. *J. Clin. Invest.* 38: 102, 1959.
26. Herbert, V. *Gastroenterology* 54: 110, 1968.
27. Herbert, V., R. R. Streiff and L. W. Sullivan. *Medicine* 43: 679, 1964.
28. Highley, D. R., R. R. Streiff, L. Ellenbogen, V. Herbert and W. B. Castle. *Proc. Soc. Exptl. Biol. Med.* 119: 565, 1965.
29. Hoedemaker, P. J., J. Abels, J. J. Wachters, A. Arends and H. O. Nieweg. *Lab. Invest.* 13: 1394, 1964.
30. Hughes-Jones, N. C. *Brit. Med. Bull.* 19: 171, 1963.
31. Lecce, J. G. *J. Physiol., London* 184: 594, 1966.
32. McGuigan, J. E. *J. Lab. Clin. Med.* 70: 666, 1967.
33. McIntyre, P. A., M. V. Sachs, J. R. Krevans and C. L. Conley. *Arch. Internal Med.* 98: 541, 1956.
34. Mackenzie, I. L., W. L. Kopp, R. M. Donaldson, Jr. and J. S. Trier. *Clin. Res.* 15: 239, 1967.
35. Miller, O. N. *Bull. Tulane Univ. Med. Fac.* 16: 115, 1957.
36. Reeves, R. B., and F. S. Fay. *Am. J. Physiol.* 210: 1273, 1966.
37. Reizenstein, P. G., E. P. Cronkite, L. M. Meyer, E. A. Usenik and D. Driscoll. *Proc. Soc. Exptl. Biol. Med.* 105: 233, 1960.
38. Rothenberg, S. P. *J. Clin. Invest.* 47: 913, 1968.
39. Schilling, R. F., and L. L. Schloesser. *Vitamin B_{12} and Intrinsic Factor*, edited by H. S. Heinrich. Stuttgart: Ferdinand Enke, 1957, p. 194.
40. Shinton, W. K., and A. K. Singh. *Brit. J. Haematol.* 13: 75, 1967.
41. Simons, K. *Soc. Sci. Fennica, Commentationes Biol.* 27: 5, 1964.
42. Strauss, E. W., and T. H. Wilson. *Am. J. Physiol.* 198: 103, 1960.
43. Sullivan, L. W., V. Herbert and W. B. Castle. *J. Clin. Invest.* 42: 1443, 1963.
44. Trier, J. S. In: *Handbook of Physiology. Alimentary Canal.* Washington, D.C.: Am. Physiol. Soc., 1968, sect. 6, vol III, chapt. 63, p. 1125.
45. Ukyo, S., and B. A. Cooper. *Am. J. Physiol.* 207: 27, 1964.
46. Weisberg, H., J. Rohdin and G. B. J. Glass. *Clin. Res.* 16: 294, 1968.
47. Wilson, T. H. *Physiologist* 6: 11, 1963.
48. Wilson, T. H., and E. W. Strauss. *Am. J. Physiol.* 197: 926, 1959.

J. LAB. CLIN. MED. 51, 860-6 1958

SERUM VITAMIN B_{12} CONCENTRATIONS DETERMINED BY L. LEICHMANNII ASSAY IN PATIENTS WITH NEOPLASTIC DISEASE

ROBERT S. MENDELSOHN, M.D.,* AND DONALD M. WATKIN, M.D.
BETHESDA, MD.

THIS paper presents details of a modification of the *U. S. Pharmacopeia XV* *Lactobacillus leichmannii* method[1] of assay developed specifically for determinations of vitamin B_{12} in serum, and reports the results of a survey of serum B_{12} levels measured by this technique in 31 normal controls and 93 patients with neoplastic disease and certain other conditions.

METHODS

The U.S.P. method was followed closely with the exceptions detailed below.

Assay Preparation.—All serum samples were drawn from fasting subjects. To each milliliter of serum, 2 ml. of an aqueous extraction solution was added. Each 100 ml. of extraction solution, prepared just prior to use, contained 1.29 Gm. of disodium phosphate, 1.1 Gm. of anhydrous citric acid, and 0.1 Gm. of sodium metabisulfite. Sodium metabisulfite concentrations of less than 0.001 Gm. per milliliter resulted in incomplete extraction of B_{12} from serum, while greater concentrations (assuming 0.5 ml. of extraction solution present in each final assay tube) resulted in inhibition of growth of the organism. The pH of this extraction solution was approximately 4.6.

The mixture of serum and extraction solution was autoclaved at 121° C. for 10 minutes, centrifuged, and duplicate 0.75 ml. and 0.3 ml. portions of the supernatant (containing 0.25 and 0.1 ml. of serum, respectively) were transferred to the final assay tubes. Sufficient extraction solution was added where necessary to bring its volume in each final assay tube to 0.5 ml. The final volume of each tube (before addition of assay medium) was brought to 5.0 ml. with distilled water.

Assay Medium.—Five milliliters of Bacto B_{12} Assay Medium,† U.S.P., containing 85 Gm. of solid material per liter of distilled water, was added to each final assay tube.

Standard Curve.—A standard B_{12} solution containing 0.050 mμg per milliliter was made up from a B_{12} stock solution prepared monthly from crystalline B_{12} according to U.S.P. instructions. With each assay, 9 dilutions of the working standard, each in duplicate, representing from 0.025 to 0.225 mμg of B_{12} were added to final assay tubes to comprise the standard curve.

Inoculum.—The organism, *Lactobacillus leichmannii*,‡ was transferred daily in Bacto B_{12} Inoculum Broth,† U.S.P. A 20-hour growth was centrifuged and washed 3 times in saline. One drop of material, diluted to give a reading of about 72 per cent transmittance at 600 mμ, in the Coleman Universal spectrophotometer (corresponding to approximately 0.5 mg. cells per 10 ml. of saline), was inoculated into each tube.

From the Nutrition and Metabolism Service, National Cancer Institute, National Institutes of Health, Public Health Service, Department of Health, Education and Welfare, Bethesda 14, Md.

Received for publication Nov. 18, 1957.

*Present address: Cincinnati General Hospital, Cincinnati, Ohio.

†Difco Laboratories, Detroit, Mich.

‡American Type Culture Collection No. 7830.

Procedure.—After either standard B_{12} solution or supernatant from the serum extraction was introduced into the assay tubes, the complementary extraction solution (if required), distilled water, and assay medium were added. The tubes were placed in racks covered with gauze-lined aluminum trays and autoclaved at 121° C. for 5 minutes. They were cooled and, after inoculation as outlined above, were incubated at 37° C. for 20 hours. The per cent transmittance of each tube was then determined in a Coleman Universal spectrophotometer against an uninoculated reagent blank at a wave length between 610 and 623 mμ. The precise wave length for a given assay was ascertained by determining the maximum per cent transmittance of the uninoculated blank for that assay. The per cent transmittance of each tube containing serum extract was converted to B_{12} concentration by reference to the standard curve run with each assay.

TABLE I. RECOVERY EXPERIMENTS

SERUM B_{12} CONCENTRATION MμG B_{12}/ML. OF SERUM	MμG B_{12} ADDED	MμG B_{12} RECOVERED	PER CENT RECOVERY
0.550	0.050	0.041	82
0.550	0.100	0.100	100
0.550	0.025	0.030	120
0.550	0.050	0.051	102
0.380	0.100	0.087	87
0.380	0.050	0.049	98
0.520	0.100	0.090	90
0.520	0.050	0.055	110
0.450	0.100	0.107	107
0.450	0.050	0.045	90
0.530	0.100	0.093	93
0.530	0.050	0.052	104
			Mean = 98.6%
			S.D. = 10.83%
			$S_{\bar{x}}$ = 3.13%

RESULTS

The recoveries from 5 different sera containing various amounts of added crystalline B_{12} are recorded in Table I.

Reproducibility at various B_{12} levels was tested by running the standard curve with 16* replicates at each of 9 B_{12} concentrations. As shown in Table II, the deviations about the mean per cent transmittance at each B_{12} concentration ranged from 2.7 to 10 per cent of the mean. As a further check on reproducibility, 16* replicate assays were performed on each of 9 different quantities of serum from a patient with an elevated B_{12} concentration. The deviations about the mean per cent transmittance for each quantity of serum ranged from 3.4 to 10.5 per cent of the mean. The deviations about the mean quantity of B_{12} in each tube ranged from 5.4 to 22.2 per cent of this mean (See Table II). The per cent values are the quotient times 100 of the standard deviation divided by the mean.

DISCUSSION

The *Euglena gracilis* method of Ross[2] has been used widely from the determination of serum vitamin B_{12} concentrations. It has the advantages of

*See Table II for exceptions.

Thirty-seven patients had serum B_{12} concentrations within 2 standard deviations of the normal mean. These included patients with acute lymphatic leukemia, 5; granuloma fungoides, 4; carcinoma of the mouth and of the testicle, 3 each; carcinoma of the bladder and of the esophagus, 2 each; carcinoma of the prostate, maxillary antrum, thyroid, stomach, endometrium and vulva, 1 each; and malignant carcinoid, lymphosarcoma, chondrosarcoma, multiple myeloma, leukemoid reaction, eosinophilic granuloma of bone, psoriasis with rheumatoid arthritis, myxedema, diabetes with tuberculosis, anemia possibly due to chronic myelocytic leukemia, ileal resection, and neuropathy of unknown etiology, 1 each.

Elevated serum B_{12} concentrations have been reported in patients with myelocytic leukemia,[9] polycythemia vera, carcinomatosis with leukemoid reactions and myelosclerosis.[10] Among the leukemic patients in this study, 4 of 5 with chronic myelocytic leukemia and 6 out of 9 with acute myelocytic leukemia were found to have elevated serum B_{12} levels. Only 1 of 3 patients with chronic lymphocytic leukemia and none of 5 with acute lymphocytic leukemia had elevated levels. The patient with chronic myelocytic leukemia whose level was normal was in the aleukemic phase of her disease. The patient with chronic lymphocytic leukemia whose level was elevated had the nephrotic syndrome apparently antedating his leukemia.

Of the 12 nonleukemic patients with elevated serum B_{12} concentrations, 10 had at least 1 abnormal liver function test. Autopsies on 8 of these patients revealed hepatic involvement, often extensive, in all. In Table V, the findings in these 12 patients are listed. The liver functions tabulated are those measured closest to the date on which blood for B_{12} determinations was drawn.

These findings suggest a relation between hepatic metastases and serum B_{12} elevations. They are in accord with the findings of such elevations in some patients with cirrhosis of the liver[6] and acute hepatitis.[11] Since not all patients with hepatic metastases have elevated serum B_{12} concentrations, if hepatic involvement is casually related to elevated serum B_{12} levels, the particular type of liver injury associated with the elevations remains to be discovered.

SUMMARY

1. A method for serum B_{12} concentration assay using *L. leichmannii* is described in detail. Recovery and reproducibility data are presented.

2. The serum B_{12} concentrations of 31 normals and 93 patients with neoplastic disease and other conditions are reported.

3. Most patients with myelocytic leukemia, acute and chronic, have elevated serum B_{12} levels. Only 1 of 3 patients with chronic lymphocytic leukemia had an elevated level.

4. Most patients with nonleukemic neoplastic disease have normal serum B_{12} concentrations. Those patients with elevated levels display a high incidence of abnormal liver function tests and of gross hepatic metastases.

We are indebted to Miss Helen Noble and Mrs. Virmetta Gilmore for their assistance in developing the assay method and conducting the survey.

being sensitive and specific.[3] It has the disadvantage of requiring 8 to 10 days for incubation of the organism. In addition, although Ross has reported "usually almost complete" recoveries, Pitney and associates[4] have found recoveries in the range of 50 per cent. Harris[5] has reported recoveries of 70 to 90 per cent, although his method since has been modified.[6]

TABLE II. REPRODUCIBILITY EXPERIMENTS

PER CENT TRANSMITTANCE			B_{12} MμG PER TUBE	$S_{\bar{x}}$ OF B_{12} VALUES OBTAINED	ML. OF SERUM PER TUBE
$\bar{x}$	S. D.	$S_{\bar{x}}$			
A. *Standard Curve*					
83.1	2.22	0.79*	0.000		
61.1	2.20	0.56	0.025		
46.9	2.69	0.67	0.050		
34.5	2.22	0.56	0.075		
25.5	1.47	0.37	0.100		
20.5	1.39	0.35	0.125		
17.1	1.36	0.34	0.150		
14.4	1.44	0.36	0.175		
12.9	0.98	0.25	0.200		
10.8	1.03	0.28†	0.225		
B. *Serum*					
81.9	4.03	1.43*	0.002		0
66.7	2.24	0.56	0.018	0.001	0.0016
55.3	1.97	0.49	0.035	0.001	0.0032
44.1	2.82	0.71	0.054	0.001	0.0048
35.3	2.06	0.52	0.073	0.001	0.0064
29.0	2.84	0.73‡	0.092	0.002	0.0080
23.8	2.32	0.58	0.110	0.002	0.0096
20.2	1.78	0.45	0.127	0.003	0.0112
16.8	1.76	0.44	0.152	0.003	0.0128
15.4	1.18	0.30	0.165	0.003	0.0144

*n = 8.
†n = 14.
‡n = 15.

A. Reproducibility tested by running 16 replicate determinations at each of 9 B_{12} concentrations on the standard curve. The mean per cent transmittance is listed in column 1, the standard deviation in column 2, the standard error of the mean in column 3, and the quantity of B_{12} in each tube in column 4.

B. Reproducibility tested by running 16 replicate assays on each of 9 different quantities of serum from a patient with an elevated B_{12} concentration. Columns 1 to 4, as above. The standard error of the mean B_{12} value for each tube is listed in column 5 and the milliliter of serum per tube in column 6.

The principal advantage of the *L. leichmannii* method is the 20-hour incubation time. The method is somewhat less specific than the *E. gracilis* technique. *L. leichmannii* will respond with growth to the presence of desoxyriboses if purine bases are also present in the medium. However, 1,000 times as much desoxyribose as B_{12} is needed for growth.[7] Hence, this factor is largely eliminated from serum by the dilution involved in the method. Although desoxyribose concentrations in serum have not been measured, the B_{12} recovery data presented here together with that of Rosenthal and Sarett[8] indicate that the *L. leichmannii* assay is sufficiently specific and quantitatively accurate for satisfactory determinations. Its convenience and rapidity compared to the *E. gracilis* assay suggest it as the method of choice in assaying B_{12} concentrations in serum.

RESULTS OF SURVEY

Vitamin B_{12} concentrations were measured in the sera of 31 normal individuals. The data are presented in Table III.

TABLE III. LEVELS IN 31 NORMAL SUBJECTS

MEAN (MμG/ML.)	RANGE (MμG/ML.)	S.D. (MμG/ML.)	$S_{\bar{x}}$ (MμG/ML.)
0.533	0.260-0.850	0.166	0.030

Vitamin B_{12} concentrations were measured by 109 determinations in the sera of 93 patients. Elevated levels were defined as those 2 standard deviations or more above the normal mean (i.e., greater than 0.865 mμg/ml.). Of these 109 determinations, 35 were elevated. These 35 elevated levels were found in 23 patients with 12 different diseases. Twenty-four other patients (with 8 of these same 12 diseases) had normal B_{12} levels. In Table IV, the number of elevated and normal B_{12} levels for each disease entity characterized by an elevated serum B_{12} concentration is presented.

TABLE IV. DATA ON PATIENTS WHOSE DISEASES DEMONSTRATED ELEVATED SERUM B_{12} CONCENTRATIONS

DISEASE	TOTAL PATIENTS	NUMBER OF DETERMINATIONS	NUMBER PATIENTS WITH ELEVATED LEVELS	RANGE OF ELEVATED LEVELS (MμG/ML.)	NUMBER PATIENTS WITH NORMAL LEVELS
Chronic myelocytic leukemia	5	11	4	1.2 -12.0	1*
Acute myelocytic leukemia	9	9	6	1.4 - 3.1	3†
Chronic lymphatic leukemia	3	4	1‡	0.92- 1.6	2
Malignant melanoma	8	10	3	1.55- 8.67	5
Carcinoma of cervix	4	4	2	1.1 - 1.57	2
Carcinoma of sigmoid colon	3	3	1	3.10	2
Carcinoma of breast	8	9	1	4.10	7
Hodgkin's disease	3	3	1§	1.90	2
Adenocarcinoma, primary (?)	1	1	1	1.67	
Squamous carcinoma, primary (?)	1	1	1	1.02	
Cirrhosis	1	1	1	1.1	
Myeloid metaplasia	1	5	1	1.07- 2.4	

*In aleukemia phase of disease.
†In 2 of these 3, the marrow was read as acute monocytic or myelocytic leukemia; in the third, as Pelgeroid type of acute myelocytic leukemia.
‡Patient also has nephrotic syndrome.
§Had received "B" vitamin injections.

Of the 93 patients, 9 had serum B_{12} concentrations 2 or more standard deviations below the normal mean (i.e., less than 0.201 mμg/ml.). Of this group, 2 were strict vegetarians and 1 each had mycosis fungoides, malignant carcinoid, and carcinoma of the kidney, lung, mouth, bladder, and tongue. The serum B_{12} concentrations ranged between 0.075 and 0.175 mμg/ml.

TABLE V. DATA OF 12 PATIENTS WITHOUT LEUKEMIA BUT WITH ELEVATED B_{12} CONCENTRATIONS

DISEASE	WHITE BLOOD COUNT	BSP RETENTION (% AT 45 MIN.)	CEPH. CHOL. FLOC.	THYMOL. TURB.	ALKALINE PHOSPHATASE (BESSEY-LOWRY U.)	TOTAL BILIRUBIN (MG. %)	POST-MORTEM FINDINGS
Melanoma	55,000	22	0	4	1.8	0.4	Widespread metastases; diffuse liver metastases
Melanoma	4,100	1	2+	2	3.5	0.4	Widespread metastases; most of liver replaced by tumor
Melanoma	6,200	14	0	1	12.8	0.3	Widespread metastases; extensive large hepatic metastases
Carcinoma of cervix	15,200	4	0	3	1.9	0.4	One small tumor nodule in liver
Carcinoma of cervix	6,700		0	1	1.7	0.3	Still living
Carcinoma of colon	8,000	19	0	3	2.0	0.2	Still living; evidence of hepatic metastases on I^{131} Rose Bengal scanning
Carcinoma of breast	7,500		0	8	10.4	4.2	Extensive hepatic and bone metastases
Hodgkin's disease	14,900	35	0	1	10.0	0.7	Still living
Adenocarcinoma, primary (?)	7,400	10	0	11	3.2	0.3	Still living
Undifferentiated carcinoma, primary (?)	7,200	20	1+	2	4.8		Widespread metastases; extensive hepatic metastases
Myeloid metaplasia	212,000	8	1+	8	7.4	9.5	Hepatosplenomegaly extramedullary hematopoiesis in liver, extensive
Cirrhosis	5,000		4+	4	6.2	7.6	Extensive portal cirrhosis

1812

583

REFERENCES

1. The Pharmacopeia of the United States of America, ed. 15, Easton, Pa., 1955, Mack Printing Company, pp. 885-889.
2. Ross, G. I. M.: Vitamin B_{12} Assay in Body Fluids Using Eugena gracilis, J. Clin. Path. 5: 250-256, 1952.
3. Coates, M. E., and Ford, J. E.: Methods of Measurement of Vitamin B_{12}, Biochem. Soc. Symposium 13: 36-51, 1955.
4. Pitney, W. R., Beard, M. F., and Van Loon, E. J.: Observations on the Bound Form of Vitamin B_{12} in Human Serum, J. Biol. Chem. 207: 143-152, 1954.
5. Harris, J. W.: Vitamin B_{12} Levels in Pernicious Anemia, J. Clin. Invest. (Abst.) 31: 637, 1952.
6. 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: 715-732, 1954.
7. Lascelles, J., and Cross, M. J.: The Function of Vitamin B_{12} in Micro-Organisms, Biochem. Soc. Symposium 13: 109-123, 1955.
8. Rosenthal, H. I., and Sarett, H. P.: The Determination of Vitamin B_{12} Activity in Human Serum, J. Biol. Chem. 199: 433-442, 1952.
9. Beard, M. F., Pitney, W. R., and Sanneman, E. H.: Serum Concentrations of Vitamin B_{12} in Patients Suffering From Leukemia, Blood 9: 789-794, 1954.
10. Mollin, D. L., and Ross, G. I. M.: Serum Vitamin B_{12} Concentrations in Leukemia and in Some Other Hematological Conditions, Brit. J. Haemat. 1: 155-172, 1955.
11. Kristensen, H. P. Ostergaard: B_{12} vitamininhold i plasma ved akut hepatitis og occlusionsicterus, Nord. med. 55: 85-87, 1956.

J. Indian Med. Assoc. 46: 94-95

1820

ACUTE PULMONARY OEDEMA AFTER CYANOCOBALAMIN THERAPY

I. SUDHAKARAN MENON, M.B.

Medical Registrar

West Cumberland Hospital, Whitehaven, U.K.

The first case of pernicious anaemia was reported by Combe (1824) of Edinburgh, but the condition was not brought into prominence until Addison (1855) gave a masterly description of the disease and recognised it as a definite entity. But it is noteworthy that cases of what would now be called pernicious anaemia were recorded apart from Combe and by Andral in 1823, by Marshall Hall in 1837 cited by Cornell (1929). Biermer (1872) was the first to draw attention to the disease in the Continent. His descriptions were apparently independent of those of Addison and his name is usually associated with the disease in European countries (Biermer, 1872).

The isolation of the active anti-pernicious anaemia principle in liver was announced simultaneously by British and American workers in 1948 and was named vitamin B_{12}. Soon after, it was agreed that the name 'cyanocobalamin' should be given to the substance used therapeutically (Dunlop *et al.*, 1964).

No untoward effect after cyanocobalamin therapy is reported in the literature.

The following are three reports of patients who died within 12 to 60 hours of the commencement of cyanocobalamin therapy and in whom the cause of death is presumed to be related to cyanocobalamin therapy.

CASE REPORTS

CASE 1:

A 58-year old publican was admitted on 27-5-64 with 16 years' history of tired feeling when he had to give up his work as a coal miner. His complaints of tired and listless feeling, shortness of breath on exertion and claudication of both calf muscles on walking, gradually got worse through the years and for 5 weeks he was noticing swelling of the ankles and feet. In the past he had rheumatic fever at the age of 13 and 27 and also a perforated duodenal ulcer at the age of 27.

On examination, he was pale with lemon yellowish colouration of skin and was not mentally alert. B.P. was 140/70 mm. Hg. His haemoglobin was 31 per cent with MCHC 31 per cent, ESR 25 mm./1 hour, and WBC count 3,000/c.mm., with no free acid after histamine test meal. A sternal marrow biopsy showed much megaloblastic change and he was commenced on I.M. injection of cyanocobalamin 1000 mcg. on the evening of 29-5-64. He suddenly collapsed and expired on the morning of 30-5-64 at 7-15 A.M.

At *autopsy* he was found to have mild rheumatic mitral stenosis and evidence of marked acute pulmonary oedema.

CASE 2:

A 75-year-old multipara was admitted on 26-8-64 with vague complaints of anorexia, weakness and pallor of face, worsening since six weeks. On examination, she was markedly pale with yellowish tinge of skin and bruises all over. Her tongue was smooth and showed evidence of papillary atrophy. She also had a systolic thrill with systolic murmur in aortic area and left ventricular heave. B.P. was 160/90 mm. Hg. Her haemoglobin was 36 per cent, MCHC 29 per cent and total white cell count 1,800/cmm. Reticulocytes were less than 1 per cent. A sternal marrow biopsy showed evidence of megaloblastic erythropoiesis and she was commenced on 1000 mcg. of cyanocobalamin on the evening of 27-8-64. She could not be aroused on the morning of 28-8-64 and expired the same evening.

At *autopsy* she was found to have atherosclerotic aortic stenosis, left ventricular hypertrophy and acute pulmonary oedema.

CASE 3:

A 72-year-old patient with four months' history of profound anaemia and progressive dementia was admitted on 22-12-64. On examination, he was markedly pale and B.P. was 120/70 mm. Hg. Detailed examination was not possible as the patient was not co-operative. His haemoglobin was 31 per cent, MCHC 33 per cent, ESR 67 mm./1st hour, total white cell count 3,600 c.mm. and reticulocyte count 2 per cent. As peripheral blood contained late megaloblasts, sternal marrow biopsy was not done and he was started on vitamin B_{12} 1000 mcg. daily on the evening of admission. He was found dead in the early hours of the morning of Christmas Day, 1964.

At *autopsy* he was found to have moderately severe atheroma of aorta, and the coronary arteries showed moderately severe atheroma with much calcification and some stenosis. There was also evidence of extensive severe pulmonary oedema.

DISCUSSION

The common feature in all the three cases is low haemoglobin (31, 36, and 31 per cent respectively) and at autopsy the finding of evidence of acute pulmonary oedema. All the three cases died after commencement of cyanocobalamin therapy. When we lost the first case we thought it was just a coincidence that the patient died after vitamin B_{12} therapy, but three cases cannot be brushed off as coincidental, and we thought further probing warranted, though still it may be accidental

585

that they died after the commencement of vitamin B_{12} therapy. It is well known that within a few hours of commencing treatment with vitamin B_{12} the patient usually feels stronger, and some of the characteristic symptoms such as loss of appetite, apathy and digestive symptoms disappear, although the red cell count is as yet unaltered and the anoxia not relieved. Within as short a period as 6-9 hours, and before there is any change in the circulating blood, the bone marrow begins to change its appearance. The earlier megaloblastic cells (i.e. stages I and II) become progressively transformed into the later stages (i.e. stages III and IV) and all the marrow cells revert increasingly to the normoblastic type. In other words, vitamin B_{12} promotes maturation of the erythroid cells and enables them to follow the normal line of development as regards cell size and time of appearance of the haemoglobin (Wright, 1962).

The serum uric acid, which varies greatly during relapse but is usually normal or lower than normal in amount, increases very promptly during remission. This change may occur within twenty-four hours of the administration of an effective therapeutic agent and is soon followed by an increased urinary excretion of uric acid. The maximum increase in serum uric acid characteristically occurs twenty-four hours before the maximal increase in reticulocytes, and subsequent variations in serum and urine uric acid tend to parallel the changes in the percentage of reticulocytes (Riddle, 1929; Wintrobe, 1961).

The total blood volume is reduced, but the reduction is due to the decrease in the number of the cells. The total plasma volume may actually be increased. This was observed in measurements by the Evans' blue dye method (Gibson, 1939) and by the carbon monoxide method (Hallberg, 1955) but, by the Cr-51 method, plasma volume was also low (Massie, 1959). Treatment is followed by a rapid increase in blood volume but, in the first week, a disproportionate increase in plasma volume may mask the rise in red cell volume (Allington and Taylor, 1955, Massey and Rubin, 1954).

In the three cases reported, whether the increase in the total blod volume is the cause for precipitating left ventricular failure or whether the increase in serum uric acid after commencement of vitamin B_{12} therapy has anything to do is anyone's guess and lacks scientific proof. However, the sudden increase in blood volume as the precipitating cause of left ventricular failure sounds logical especially in an anoxic myocardium from severe anaemia. All the three cases reported had a haemoglobin around 35 per cent and two of them had added cardiac pathology, viz., mild rheumatic mitral stenosis in Case 1 and atherosclerotic aortic stenosis in Case 2. In Case 3 coronary arteries showed moderately severe atheroma and some stenosis.

Conclusion

Three cases of pernicious anaemia with haemoglobin around 35 per cent who died within 12 to 60 hours after commencement of vitamin B_{12} therapy have been reported. Though further study is required to correlate the deaths to commencement of vitamin B_{12} therapy, the writer believes that the effect of commencement of vitamin B_{12} therapy in an anaemic patient with haemoglobin of 35 per cent or below has to be observed with caution, especially if they have associated cardiac disease.

Acknowledgment

I am thankful to Dr. J. Simpson and to Dr. C. B. Willey, for allowing me to report on the cases admitted under their care and to Dr. R. Dallachy for the autopsy reports. My thanks are also due to Miss Gillian Morton for typing the script.

References

Addison, T.—On the Constitutional and Local Effects of Disease of the Suprarenal Capsules, 1855, Highley, London.

Allington, M. J. and Taylor, W. H.—*Brit. J. Haemat.*, **1** : 241, 1955.

Biermer, A.—*Korrespbl. Schweiz. Arz.*, **2** : 15, 1872.

Combe, J. S.—*Trans. Med.-Chir. Soc., Edin.*, **1** : 194, 1824.

Cornell, B. S.—Pernicious Anaemia, 1927, Duke University Press, Durham, (Bibliography).

Dunlop, D., Davidson, S. and Alstead, S.—Textbook of Medical Treatment, 1964, p. 462.

Gibson, J. G.—*J. Clin. Invest.*, **18** : 401, 1939.

Hallberg, L.—*Scandinav. J. Clin. Lab. Invest.*, 7 Supp. 16, 1955.

Massey, B. W. and Rubin, C. E.—*Amer. J. Med. Sci.*, **227** : 481, 1954.

Massie, R. W.—*Arch. Intern. Med.*, **103** : 593, 1959.

Riddle, M. C.—*J. Clin. Invest.*, **8** : 69, 1929.

Samson Wright's Applied Physiology, 1962, Oxford, London.

Wintrobe, M. M.—Clinical Haematology, 1961, Lea, Philadelphia, p. 485.

J. Clin. Invest. 37(4): 556-566

#1858

THE *IN VITRO* BINDING OF COBALT^{60} LABELED VITAMIN B_{12} BY NORMAL AND LEUKEMIC SERA

By AARON MILLER with the technical assistance of JOHN F. SULLIVAN

(From the Radioisotope and Medical Services, Boston Veterans Administration Hospital, and The Department of Medicine, Boston University School of Medicine, Boston, Mass.)

(Submitted for publication August 17, 1957; accepted December 26, 1957)

In chronic myelogenous leukemia both the serum concentration of vitamin B_{12} and the capacity of the serum to bind added vitamin B_{12} are markedly increased (1–3), suggesting an abnormality of the serum proteins in this disease. *In vitro* measurements of vitamin B_{12} binding capacity employing a microbiological assay method have not demonstrated a qualitative difference between normal and leukemic sera in respect to the binding of added vitamin B_{12} by the various serum protein fractions (1, 4). In the present study an equilibrium dialysis technique using cobalt^{60} labeled vitamin B_{12} has been employed to measure the binding capacity of normal and leukemic sera for added vitamin B_{12} and to identify the protein fractions which bind the added vitamin B_{12}.

METHODS

A. Experimental plan

Venous blood samples were obtained from patients in the fasting state. After two to three hours at room temperature the serum was separated under sterile conditions and stored at −20° C. until tested. The serum concentration of vitamin B_{12}, the capacity of the serum to bind added cobalt^{60} labeled vitamin B_{12} and the distribution of the added vitamin B_{12} among the serum proteins were determined. Studies were carried out on 13 patients with chronic myelogenous leukemia, 8 patients with other types of leukemias, 8 patients with nonmalignant granulocytic leukocytoses, 2 patients with polycythemia vera and 22 control subjects, the latter group being composed of healthy laboratory personnel or ambulatory hospitalized patients suffering from functional disorders.

B. Analytical procedures

Serum vitamin B_{12} concentration. "Total" vitamin B_{12} concentration of the serum was determined by the modification of Lear, Harris, Castle, and Fleming (5) of the *Euglena gracilis* method of Ross (6). "Free" vitamin B_{12}, measured on occasional serum samples from normal subjects and patients with chronic myelogenous leukemia, constituted 15 per cent or less of the total serum vitamin B_{12}.

Serum vitamin binding capacity. Following the addition of a known excess quantity of radioactive vitamin B_{12} to serum, the degree of binding of the added vitamin B_{12} could be determined by a modification of the equilibrium dialysis method as described by Klotz, Walker, and Pivan (7). To duplicate or triplicate 2 ml. samples of serum was added 2 ml. of a solution containing 0.40 mμg. of Co^{60} vitamin B_{12} and 5 ml. of 1/15 M phosphate buffer (pH 7.3). This mixture was placed in a Visking[1] cellulose dialysis sac at 22° C. A protein-free blank prepared in triplicate with phosphate buffer in place of serum was run simultaneously. Each dialysis bag was placed into 3 by 17 cm. test tubes and the amount of radioactivity in each sample determined by means of a plastic scintillation well counter. After one hour incubation at 22° C., the bags were dialyzed against 250 ml. of 1/15 M phosphate buffer, pH 7.3. Initially, the dialysis was carried on for 24 hours at 22° C. with constant agitation but subsequently this was performed for 48 hours at 4° C. without agitation. Equilibrium was attained in both situations, and similar values for vitamin B_{12} binding were obtained under both experimental conditions. At the conclusion of dialysis each bag was rinsed externally in running water for one-half to one minute after which its radioactivity was determined in the plastic scintillation well counter. The bags were then slit in two, emptied of their contents, washed for one-half to one minute in running water and recounted for the measurement of the residual radioactivity bound to the bag. The unsaturated vitamin B_{12} binding capacity of the serum (UBBC) was determined from the following calculations:

1. Fraction of added $Co^{60}B_{12}$ bound per ml.

$$= \frac{(a)\ Co^{60}B_{12} \text{ in bag postdialysis (cpm)} - Co^{60}B_{12} \text{ bound to bag (cpm)}}{Co^{60}B_{12} \text{ added predialysis (cpm)} \times \text{ml. serum}} - \text{mean } Co^{60}B_{12} \text{ in blank calculated as in } (a)$$

2. UBBC, mμg./ml. = $Co^{60}B_{12}$ added predialysis (mμg.) × calculation *1.*

[1] Secured from Visking Corporation, Chicago, Illinois.

No correction for Donnan's equilibrium was needed as vitamin B_{12} is essentially un-ionized (8). The total vitamin B_{12} binding capacity of the serum (TBBC) was obtained from the sum of the serum vitamin B_{12} concentration and the UBBC.

Technical considerations. Two ml. of a 1:50 dilution of Co^{60} vitamin B_{12} of specific activity 0.97 μc./1.00 μg. were used in each determination of the UBBC unless otherwise stated. This was equivalent to 22 to 23,000 cpm under the counting conditions used. Radioactivity measurements were made in a large plastic scintillation well counter (well, 3.7 cm. in diameter and 10 cm. in depth) with other physical characteristics as previously described (9). A Co^{60} standard was counted each morning to correct for daily variations in the counter. The geometry of counting in pre- and postdialysis samples was constant. Samples were counted sufficiently long to give a counting error of less than 2 per cent except for the counting of empty dialysis bags where less than 5 to 7 per cent counting error was obtained.

The concentration of the stock Co^{60} vitamin B_{12} used for the measurement of the UBBC was determined by the Euglena gracilis assay. Due to the known photolability of the cyano group of the vitamin B_{12} molecule, aliquots of the stock $Co^{60}B_{12}$ solution were exposed to a fluorescent light source at 4° C. for a 12 day period. The binding of the Co^{60} vitamin B_{12} solution exposed to light was 80 per cent greater than a similar aliquot of the vitamin B_{12} solution protected from light. For this reason, the Co^{60} vitamin B_{12} was shielded from any source of light during all phases of the binding measurement. Serum, diluted 1 to 4 with 1/15 M phosphate buffer, pH 7.3, had a similar binding capacity as that of undiluted serum. Increasing or decreasing the amount of Co^{60} vitamin B_{12} over a fourfold range, slightly increased, or did not affect, the amount of vitamin B_{12} bound in both the normal and leukemic sera (Table I).

Experiments designed to evaluate the effect of temperature on vitamin B_{12} binding indicated that similar amounts of Co^{60} vitamin B_{12} were bound by serum at 4° C. and 22° C. No difference in binding was noted between aliquots of serum dialyzed at 4° C. and 22° C. The binding of radioactive material to the dialysis bag was influenced by the dialysis temperature however, 4 to 8 per cent of the Co^{60} vitamin B_{12} becoming bound at 22° C. as compared to 1 to 2 per cent at 4° C. The firmness of this bond is reflected by the fact that only 10 to 20 per cent of the bag radioactivity disappeared after 48 hours of dialysis. Dialysis against distilled water, phosphate buffer, pH 7 to 8, dextran solution, barbital buffer (pH 8.6) and saline had no effect on binding. Multiple successive freezing and thawing of serum did not affect binding capacity. The UBBC of serum stored at −20° C. remained stable over a six month period. In a patient with chronic myelogenous leukemia, no difference in vitamin B_{12} binding was found between serum collected, as described previously, and heparinized plasma separated from the cellular elements at 4° C. immediately after venipuncture. Serum with increased vitamin B_{12} binding showed better agreement between duplicate samples than those with normal binding. The vitamin B_{12} binding values showed a maximum difference in duplicate samples of 15 per cent in serum with binding greater than 4.00 mμg. per ml., 20 per cent with binding from 1.50 to 4.00 mμg. per ml., and 35 per cent in samples with binding less than 1.50 mμg. per ml. If sufficient serum was available, vitamin B_{12} binding was remeasured when the upper limits of precision were approached. The variation in the serum vitamin B_{12} concentration, the UBBC, and the TBBC of a normal subject over a two week interval of time is shown in Table II.

TABLE I

The relationship between the quantity of added vitamin B_{12} and the unsaturated vitamin B_{12} binding capacity of serum aliquots

	Amount of vit. B_{12} added per ml. serum mμg.	Unsaturated vit. B_{12} binding capacity mμg./ml.
Normal serum A	5	1.62
	10	1.86
	20	2.08
Normal serum B	4	1.33
	20	1.56
Myelogenous leukemic serum	10	7.20
	50	7.30

TABLE II

Vitamin B_{12} content and binding capacity of serum and the distribution of serum bound vitamin B_{12} as determined at different times in a normal subject

Subject	Serum B_{12} conc. mμg./ml.	UBBC* mμg./ml.	TBBC† mμg./ml.	Fraction of vitamin B_{12} bound to serum: Albumin %	α-1 globulin %	α-2 globulin %	β-globulin %	γ-globulin %
A. M.								
4/1/56	0.35	1.96	2.31	14	3	28	42	13
4/8/56	0.34	2.20	2.54	14	6	26	37	17
4/15/56	0.33	2.25	2.58	18	0	31	33	18

* UBBC refers to unsaturated vitamin B_{12} binding capacity.
† TBBC refers to total vitamin B_{12} binding capacity.

TABLE III

The vitamin B_{12} content and binding capacity of serum in 22 normal subjects as compared to 10 untreated cases of chronic myelogenous leukemia

	Serum vit. B_{12} concentration mμg./ml.		UBBC* mμg./ml.		TBBC† mμg./ml.	
	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.
Normal	0.27–1.27	0.64 ± 0.10	1.00–2.40	1.51 ± 0.56	1.32–2.81	2.23 ± 0.43
Chronic myelogenous leukemia	4.20–14.30	8.09 ± 4.55	4.75–14.25	8.87 ± 4.12	10.45–26.05	16.86 ± 7.78

* UBBC refers to unsaturated vitamin B_{12} binding capacity.
† TBBC refers to total vitamin B_{12} binding capacity.

Electrophoresis. One ml. of serum and 15 mμg. of Co^{60} vitamin B_{12} (in 0.5 ml.) were allowed to bind for one hour at 22° C. and then dialyzed at 4° C. for 48 hours against frequent changes of phosphate buffer, pH 7.3. Then, 0.12 ml. aliquots of the serum containing bound Co^{60} vitamin B_{12} were electrophoresed for 18 hours on 4 by 47 cm. Schleicher and Schuell paper No. 2043B, using veronal buffer pH 8.6 (ionic strength, 0.08) and a voltage of 110 to 114. At least four individual strips equivalent to 0.48 ml. of the serum-Co^{60} vitamin B_{12} mixture were run on each patient. Strips were then stained by a ninhydrin spray method. No loss of radioactivity occurred after staining the strips. This was in contrast to the bromphenol blue or amido black methods of staining where considerable losses of radioactivity were encountered. Under the conditions used, the albumin fraction usually moved 9 to 12 cm. from the origin while the gamma globulin moved slightly towards the anode (0.05 to 1.5 cm.). "Free" Co^{60} vitamin B_{12} (vitamin in saline solution) remained at the origin or moved slightly towards the anode (0.5 to 1 cm.). In serum which had not been dialyzed containing large amounts of unbound Co^{60} vitamin B_{12}, the free radiovitamin had a mobility similar to that in saline solution, occupying an area just adjacent to or overlapping that occupied by the gamma globulins. Each stained protein fraction was then sepa-

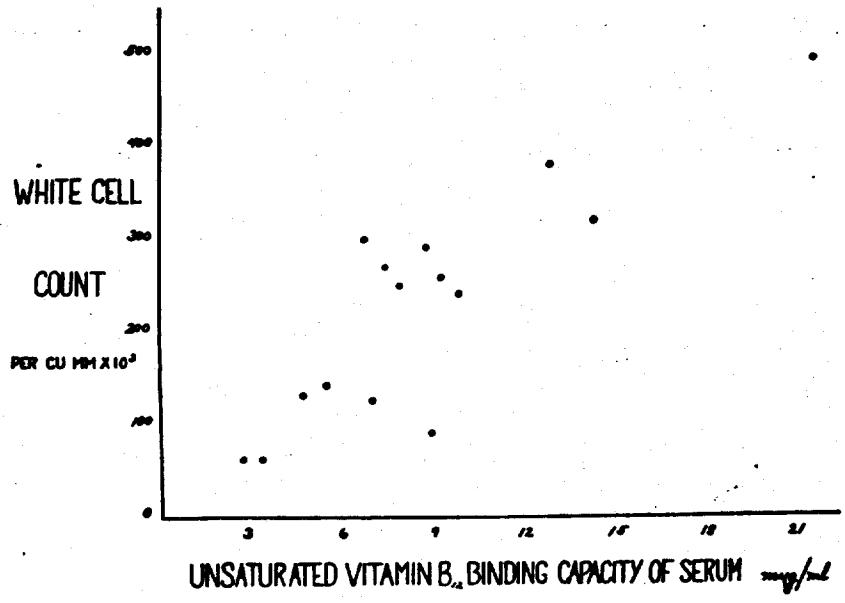


FIG. 1. CORRELATION OF THE WHITE CELL COUNT WITH THE UNSATURATED VITAMIN B_{12} BINDING CAPACITY IN 10 PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA IN RELAPSE

The above points represent the white cell count and the unsaturated vitamin B_{12} binding capacity of a patient with chronic myelogenous leukemia in relapse. Some patients, studied at different times during one or more relapses, are represented by more than one point. The coefficient of correlation between the above two measurements was 0.88.

TABLE IV

Tabular summary of vitamin B_{12} data in treated and untreated cases of chronic myelogenous leukemia

Patient		WBC per cu. mm. $\times 10^3$	Serum B_{12} conc. mμg./ml.	UBBC mμg./ml.	TBBC mμg./ml.	Therapy
F. C.	10/4/56	3,814	14.30	12.75	27.05	X-ray to spleen
	10/29/56	280	11.53	1.70	13.23	
	12/7/56	623	3.30	2.75	6.05	
	4/5/57	5,005	6.50	20.00	26.50	X-ray to spleen
	4/15/57	1,506	5.50	14.40	19.90	
	4/20/57	749	6.20	8.99	15.19	
T. M.	6/13/56	2,109	7.83	9.22	17.05	P-32
	6/28/56	1,942	7.65			
	8/10/56	2,470	7.91	8.72	16.63	X-ray to spleen
	8/23/56	391	7.90	3.79	11.69	
	9/6/56	234	7.68	4.36	12.04	
	10/4/56	107	4.20	1.24	5.44	
	11/15/56	129	2.45	0.90	3.35	
J. C.	10/4/56	2,000	4.35	7.80	12.15	X-ray to spleen
	10/17/56	324	4.05	1.53	5.58	
	11/13/56	286	1.76	1.37	3.13	
	12/13/56	625	3.45	3.40	6.85	
	1/17/57	1,397	5.56	5.50	11.06	
	2/21/57	2,254		6.54		
S. M.	10/23/56	900	7.94	8.96	16.90	Myleran®
	12/13/56	145	3.80	2.90	6.70	
T. E.	9/11/56	766	2.25	5.00	7.25	5 weeks after incomplete response to X-ray, Myleran® begun
	11/15/56	59	2.19	2.18	4.37	
G. L.		185	0.94	1.40	2.34	1 yr. following treatment with Myleran®
S. D.		100	1.70	0.90	2.60	6 months following X-ray to spleen
S. B.		1,201	4.20	7.00	11.20	
A. R.		1,311	5.70	4.75	10.45	
R. L.		2,200	4.65	7.45	12.10	
T. W.		3,000	14.00	6.70	20.70	
T. K.		3,212	8.90	14.25	23.15	
V. P.		1,920	9.00	9.85	18.85	

rated by cutting out each individual protein fraction of the electrophoretic strip. Each of the combined stained protein fractions was then counted in a sodium iodide well counter. As low counting rates were usually found, each fraction was counted for 10 minutes, with the background for each sample determined by 10 minute counting preceding and following the sample count. The chief binding protein fractions in normal sera (beta- and alpha-2 globulins) counted from three-fourths to one and one-half times background, whereas the other fractions counted 10 to 40 per cent above background. In sera with high UBBC's the chief binding protein counted from two to four times background. The binding in each protein fraction was expressed as (*a*) per cent Co^{60} vitamin B_{12} bound calculated by dividing the radioactivity found in each protein fraction by the total radioactivity in all fractions times 100, and as the (*b*) mμg. of Co^{60} vitamin B_{12} bound per ml. calculated from the UBBC and calculation (*a*). The variation in vitamin B_{12} binding by the serum protein fractions of a normal subject measured over a two week interval of time is shown in Table II.

RESULTS

Serum vitamin B_{12} concentration and binding capacity

Normals. In 22 normal subjects the mean serum vitamin B_{12} concentration was equal to 0.64 mμg. per ml. (range, 0.27 to 1.27 mμg per ml.), the mean UBBC equal to 1.51 mμg per ml. (range, 1.00 to 2.40 mμg. per ml.), and the mean TBBC equal to 2.23 mμg. per ml. (range, 1.32 to 2.81 mμg. per ml.) (Table III).

Chronic myelogenous leukemia. The average serum B_{12} concentration, UBBC, and TBBC were greatly increased over normal in 10 patients with active chronic myelogenous leukemia, the first by a factor of 12, the second by a factor of 6, and the third by a factor of 7 (Table III). An excellent correlation was found between the white blood cell count and the UBBC in patients in relapse, the

coefficient of correlation equal to 0.88 (Figure 1). A poorer correlation was found between the white blood cell count and the serum B_{12} concentration (a coefficient of correlation of 0.58). With successful treatment of the disease (X-ray or Myleran®), the UBBC fell rapidly towards normal, paralleling the decline in white blood cell count, this phenomena being noted as soon as five days after the onset of therapy. Restoration of normal UBBC levels occurred within two weeks after the onset of treatment in one patient (Patient J. C., Table IV). By contrast, the concentration of vitamin B_{12} in the serum did not begin to decline for as long as two to three weeks after the onset of treatment. Even in complete remission serum B_{12} levels remained elevated with the sole exception of Patient G. L. (Table IV). With relapse, the serum vitamin B_{12} level and the UBBC rose together.

Other leukemias. The serum vitamin B_{12} content, the UBBC, and TBBC were normal in three patients with chronic lymphocytic leukemia except for a slight increase in TBBC in one of the three (Table V). Two patients with chronic monocytic leukemia had elevated serum B_{12} levels, UBBC's, and TBBC's approaching or equalling that found in chronic myelogenous leukemia. In contrast to chronic myelogenous leukemia, a poor correlation existed between the white blood cell count and the UBBC. After a partial response to X-ray treatment, the serum B_{12} level fell to normal whereas the UBBC and TBBC were elevated (Patient T. D.). One of three patients with acute leukemia had a moderate increase in UBBC and TBBC associated with a normal serum B_{12} level. This patient had a dramatic clinical response to cortisone associated with a decrease in the UBBC and TBBC to normal.

Leukocytosis Eight patients with neutrophilic leukocytoses due to varying causes with or with-

TABLE V

Tabular summary of vitamin B_{12} data in cases other than chronic myelogenous leukemia

Diagnosis and clinical comments	WBC per cu. mm. $\times 10^2$	Serum B_{12} conc. mμg./ml.	UBBC mμg./ml.	TBBC mμg./ml.
Chronic lymphocytic leukemia				
T. B.	4,000	1.06	1.65	2.71
G. M.	610	0.64	1.12	1.76
M. G.	3,866	1.27	1.97	3.24
Chronic monocytic leukemia				
T. D. 9/19/56	486	6.00	9.00	15.00
11/15/56 (Partial response to X-ray treatment)	129	0.50	5.50	6.00
J. M.	32	1.90	5.20	7.10
Acute leukemia				
T. C.	154	0.23	1.74	1.97
B. M.	1,862	0.73	1.26	1.99
S. C. 4/20/57	250	0.26	3.97	4.23
4/29/57 (Good response to cortisone)	41	0.20	1.96	2.16
Leukocytoses				
C. C. Pulmonary tuberculosis and bacterial pneumonia	352	0.98	1.55	2.53
T. D. Polycythemia vera with leukemoid reaction	580	0.85	6.30	7.15
J. F. Epidermoid carcinoma of throat with metastases	322	3.73	5.60	9.33
J. E. Septicemia	484	0.42	3.86	4.28
T. C. Pulmonary tuberculosis with leukemoid reaction	300	0.40	2.79	3.19
J. A. Carcinoma of lung with secondary infection	300	0.50	2.70	3.20
S. H. Polycythemia vera, postoperative after lobectomy for carcinoma of lung	534	0.25	2.94	3.19
T. A. ? Myeloid metaplasia	692	0.74	6.15	6.89
Polycythemia vera				
S. M.	100	0.57	2.40	2.97
T. S.	144	1.96	1.48	3.44
Miscellaneous				
J. R. Chronic pancytopenia of ? etiology	28	0.27	2.75	3.02

TABLE VI

The binding of Co^{60} labeled vitamin B_{12} by the protein fractions of serum from normal subjects

Subject	Albumin		α-1 globulin		α-2 globulin		β-globulin		γ-globulin	
	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.
T. M.	11	0.18	7	0.11	29	0.47	32	0.52	21	0.34
F. M.	5	0.06	8	0.10	32	0.40	38	0.48	17	0.21
J. A.	6	0.11	7	0.12	23	0.41	48	0.85	16	0.28
H. L.	7	0.09	6	0.08	18	0.26	49	0.61	20	0.25
J. C.	4	0.08	1	0.02	16	0.31	66	1.29	13	0.25
A. C.	8	0.09	3	0.03	29	0.31	43	0.46	17	0.18
A. M.	18	0.35	8	0.16	29	0.57	37	0.73	8	0.16
F. M.	8	0.18	0	0.00	31	0.68	44	0.97	17	0.37
S. M.	4	0.10	4	0.10	47	1.14	32	0.78	13	0.31
F. M.	13	0.21	0	0.00	33	0.54	40	0.65	14	0.23
T. D.	11	0.26	2	0.05	34	0.82	56	1.34	17	0.41
T. G.	11		5		19		41		24	
M. S.	3		4		17		57		19	
S. F.	6		2		32		45		15	
M. L.	7		4		31		48		10	
T. M.	8		5		38		33		16	
P. R.	16		6		16		41		21	
T. B.	12		6		24		45		13	
Range	3–18	0.06–0.35	0–9	0–0.12	16–47	0.26–1.14	32–66	0.46–1.34	10–24	0.16–0.41
Mean ± standard deviation	9±4	0.16±0.09	4±3	0.07±0.06	28±7	0.59±0.19	44±10	0.79±0.38	16±4	0.27±0.09

out leukemoid reactions were studied (Table V). The commonest finding was a normal serum vitamin B_{12} concentration and an elevated UBBC and TBBC (six of eight patients). One patient (J. F.) had an elevated serum vitamin B_{12} concentration, UBBC, and TBBC approximating those found in chronic myelogenous leukemia while the last patient had a normal serum B_{12} concentration, UBBC, and TBBC.

Other diseases. One patient with polycythemia vera had a normal serum vitamin B_{12} level and UBBC but a slightly increased TBBC while another patient with this disease had an elevation of vitamin B_{12} content and TBBC. A patient with chronic pancytopenia of unknown etiology had a normal serum vitamin B_{12} level with a slight increase in UBBC and TBBC.

Electrophoresis of serum

Normals. In 16 of 18 normal subjects the beta globulins bound the greatest per cent of the added Co^{60} vitamin B_{12}, average of 44 per cent, range, 32 to 66 per cent (Table VI). The alpha-2 globulins were also an important binding fraction containing an average of 28 per cent of the added Co^{60} vitamin B_{12}, with a range of 16 to 47 per cent, and were the chief binding proteins in the two other subjects. Smaller amounts of radioactivity were bound by the gamma globulin and albumin fractions, whereas binding by the alpha-1 globulins was negligible (average, 4 per cent; range, 0 to 9 per cent). The amount of vitamin B_{12} bound per protein fraction expressed as mμg. per ml. is also shown in Table VI.

Chronic myelogenous leukemia. In contrast to normal subjects the alpha-1 or alpha-2 globulins were the main binding protein fractions of the serum for the added Co^{60} vitamin B_{12} (Table VII). Thus, the alpha-1 globulins contained an average of 38 per cent of the added Co^{60} vitamin B_{12}, approximately nine times that of normal sera, and was the main binding protein in 5 of the 10 patients. The alpha-2 globulins bound an average of 34 per cent of the added Co^{60} vitamin B_{12}, approximately 20 per cent more than that of normal serum, and was the main binding protein in four patients. When expressed in mμg. per ml. the increase in Co^{60} vitamin B_{12} binding by the alpha-1 globulins was approximately 47 times greater than that of normal serum as compared to the sixfold increase over normal by the alpha-2 globulins. The percentage of added Co^{60} vitamin

TABLE VII

The binding of cobalt[60] labeled vitamin B_{12} by the protein fractions of serum from patients with chronic myelogenous leukemia in relapse

Patient	Albumin		α-1 globulin		α-2 globulin		β-globulin		γ-globulin	
	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.
T. M.	3	0.28	56	5.16	16	1.48	10	0.92	15	1.38
S. B.	7	0.49	25	1.75	24	1.68	31	2.17	13	0.91
A. R.	9	0.43	24	1.14	34	1.62	19	0.90	14	0.66
F. C.	3	0.38	56	7.14	29	3.70	10	1.28	2	0.26
S. M.	4	0.36	52	4.66	23	2.06	13	1.17	8	0.72
J. C.	4	0.31	30	2.34	33	2.57	23	1.79	10	0.78
R. L.	1	0.07	21	1.57	55	4.10	18	1.34	5	0.37
T. W.	0	0.00	42	2.81	42	2.81	10	0.67	6	0.40
T. K.	1	0.14	36	5.13	52	7.41	7	1.00	4	0.57
V. P.	4	0.39	39	3.84	33	3.25	20	1.97	4	0.39
Range	0–9	0.00–0.49	21–56	1.14–7.14	16–55	1.62–7.41	7–31	0.67–2.17	4–14	0.26–1.38
Mean ± standard deviation	4±2	0.29±0.18	38±13	3.55±2.29	34±12	3.07±2.04	16±7	1.32±0.66	8±5	0.64±0.32

B_{12} bound by the beta globulins was decreased to about one-third that found for normal serum while slighter decreases were also found for the gamma globulin and albumin fractions. Despite the decrease in the per cent of added Co^{60} vitamin B_{12} bound by these fractions, modest elevations in the mμg. of vitamin B_{12} bound per ml. of serum were found although these values overlapped those found in normal serum. With successful treatment, the serum protein binding pattern reverted towards that found in normal subjects and, with complete remission, a normal pattern of binding was found (Table VIII).

Other leukemias. The binding of Co^{60} vitamin B_{12} by the serum proteins of patients with chronic lymphocytic leukemia was similar to that of normal serum (Table IX). Elevated binding by both the alpha-1 and alpha-2 globulins was found in one patient with chronic monocytic leukemia, whereas in two other patients with chronic mono-

TABLE VIII

The effect of treatment on the binding of cobalt[60] labeled vitamin B_{12} by the protein fractions of serum from patients with chronic myelogenous leukemia

Patient	Albumin		α-1 globulin		α-2 globulin		β-globulin		γ-globulin	
	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.
J. C.										
10/4/56 Rx	4	0.31	30	2.34	33	2.57	23	1.79	10	0.78
10/17/56 (After Rx)	2	0.03	8	0.12	28	0.42	39	0.60	23	0.35
1/17/57 (Relapse)	4	0.22	11	0.61	42	2.31	27	1.49	16	0.88
2/21/57 (Relapse)	2	0.13	22	1.35	42	2.75	22	1.35	12	0.79
F. C.										
10/4/56 Rx	3	0.38	56	7.14	29	3.70	10	1.28	2	0.26
10/29/56 (After Rx)	11	0.19	27	0.46	22	0.37	26	0.44	14	0.24
12/7/56 (Relapse)	2	0.06	20	0.55	55	1.51	18	0.50	5	0.14
4/5/57 Rx	2	0.40	35	7.00	52	10.40	8	1.60	3	0.60
4/15/57 Rx	2	0.29	40	5.76	47	6.77	7	1.01	4	0.58
4/20/57	7	0.63	25	2.25	51	4.59	12	1.08	5	0.45
G. L. Remission	6	0.08	7	0.10	46	0.64	34	0.48	7	0.10

TABLE IX

The binding of cobalt[60] labeled vitamin B_{12} by the protein fractions of serum from patients with varying types of leukemia and leukocytoses

Diagnosis	Albumin		α-1 globulin		α-2 globulin		β-globulin		γ-globulin	
	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.	Vit. B_{12} bound %	Vit. B_{12} bound mμg./ml.
Chronic lymphatic leukemia										
T. B.	8	0.13	3	0.05	30	0.50	47	0.78	12	0.19
G. M.	7	0.14	2	0.04	31	0.61	45	0.89	15	0.30
Chronic monocytic leukemia										
J. M.	4	0.21	3	0.16	40	2.08	41	2.13	12	0.62
T. D. 11/15/56 (After Rx)	1	0.06	18	0.99	54	2.97	22	1.21	5	0.28
Acute leukemia										
T. C.	8	0.14	6	0.11	20	0.35	35	0.61	31	0.54
S. C. 4/20/57	6	0.24	3	0.12	39	1.55	39	1.55	13	0.52
4/29/57 (After Rx)	11	0.22	3	0.06	30	0.59	49	0.96	7	0.14
Leukocytoses										
J. F.	0	0.00	25	1.40	58	3.25	14	0.78	3	0.17
J. A.	7	0.19	5	0.14	18	0.49	45	1.22	25	0.68
S. H.	7	0.20	6	0.18	18	0.53	53	1.56	16	0.47

cytic leukemia and acute leukemia, increased binding by the alpha-2 globulins was found (Table IX).

Leukocytoses. Patient J. F., with the increase in serum vitamin B_{12} concentration, UBBC, and TBBC, also demonstrated increased binding of Co^{60} vitamin B_{12} by the alpha-1 and alpha-2 globulins (Table IX). Two other patients with slight increases in the UBBC and TBBC had normal patterns of Co^{60} vitamin B_{12} binding by the serum proteins.

DISCUSSION

Amounts of vitamin B_{12} bound by the sera of normal subjects as determined in this study differ greatly from those previously obtained using microbiological assay procedures (3, 4, 5). Such differences in results are not surprising in view of the different methods employed. The binding of vitamin B_{12} is defined physicochemically in a dialysis method, *i.e.*, by the ability of the free vitamin to traverse a cellulose membrane. This experimental system is not subject to the variables inherent in microbiological assay procedures.

A relatively constant amount of vitamin B_{12} was bound by normal and leukemic sera when increasing concentrations of Co^{60} vitamin B_{12} were allowed to bind with normal and leukemic sera before dialysis. A similar limit in the vitamin B_{12} binding has been reported using a dialysis system (10). Recently, others have reported different results using a dialysis method differing slightly from that used in this study (11). They found no limitation in the amount of vitamin B_{12} bound by normal serum when the concentration of Co^{60} vitamin B_{12} was progressively increased. No reason for these differences in results is apparent at present.

In normal sera, using microbiological methods, the alpha globulins have been found to be the chief binding proteins for added vitamin B_{12} (4). However, the beta globulins bound the greatest amount of added Co^{60} vitamin B_{12} in the present study. The alpha-2 globulins were next in vitamin B_{12} binding ability but only negligible amounts were bound by the alpha-1 globulins. In previous investigations, excess vitamin B_{12} was added to serum, the serum proteins fractionated by paper electrophoresis, and the vitamin B_{12} content of the isolated protein fractions then determined. The considerable vitamin B_{12} found in the beta globulin fraction was ascribed to "free" vitamin B_{12},

i.e., that available to *Euglena gracilis* without preliminary heating of the serum. However, we have found that a large fraction of the Co^{60} vitamin B_{12} "bound" by normal serum as determined by dialysis is measured as "free" by the *Euglena gracilis* assay (12). The vitamin B_{12} in human liver homogenates has been found to be "free" as measured by *Euglena gracilis* even though the vitamin B_{12} was associated with a protein which had a mobility similar to the beta globulins on electrophoresis (13). Actually, on electrophoresis "free" vitamin B_{12} (the vitamin in saline solution) moves with the endosmotic flow and not as a beta globulin. It seems apparent that the differences in our findings and those previously reported are due to the different methods employed for the determination of vitamin B_{12} binding. The interpretation of the *Euglena gracilis* method of determining serum vitamin B_{12} binding is difficult if the vitamin B_{12} moving with a protein on electrophoresis can be measured as "free."

It is of interest that the vitamin B_{12} content of normal serum is primarily bound to the alpha-1 and alpha-2 globulins (4), whereas added vitamin B_{12} was principally bound by the beta and alpha-2 globulins. The interpretation of these findings is not certain. It may be that the alpha-1 globulins are already saturated with vitamin B_{12} accounting for their inability to bind additional vitamin B_{12}, whereas the alpha-2 globulins are only partially saturated and still capable of binding added vitamin B_{12}. The ready binding of added vitamin B_{12} by the beta globulins could be due to an *in vitro* avidity of the protein for the vitamin having no significance *in vivo*. However, *in vivo* binding of vitamin B_{12} by the beta globulins cannot be ruled out. Small amounts of the serum vitamin B_{12} content were found bound to the beta globulins in some normal sera (4). A protein in human liver homogenates resembling a beta globulin on electrophoresis is also capable of binding vitamin B_{12} (13). The small amount of vitamin B_{12} bound to the beta globulins *in vivo* could result if its rate of serum turnover was rapid as compared to the vitamin bound to the alpha globulins.

The vitamin B_{12} concentration, UBBC, and TBBC were all greatly elevated in chronic myelogenous leukemia as has been noted by other workers (1–3). The increase in UBBC was a labile abnormality decreasing rapidly to normal with the onset of treatment in contrast to the gradual decline in serum vitamin B_{12} levels following treatment. Of the other leukemias studied, only the patients with chronic monocytic leukemia had elevations in vitamin B_{12} concentration, UBBC, and TBBC approaching that found in chronic myelogenous leukemia. Elevated vitamin B_{12} levels have also been reported in monocytic leukemia (3, 14), although no increase in binding capacity was found using the *Euglena gracilis* binding method (3). Similarities between the monocytic and myelogenous leukemic processes are suggested by these findings. Patients with granulocytic leukocytoses of varying etiologies usually had normal serum vitamin B_{12} levels with increased UBBC's and TBBC's although the increase in UBBC and TBBC was usually not in the range found in chronic myelogenous leukemia. Thus, serum vitamin B_{12} levels were more helpful in distinguishing these patients from those with chronic myelogenous leukemia. However, this was not uniformly so, and Mollin and Ross have reported increased serum vitamin B_{12} levels in two patients with chronic leukocytoses (2).

In contrast to the findings in normal sera, the alpha-1 and alpha-2 globulins were the proteins responsible for tremendous increase in the UBBC of chronic myelogenous leukemic sera. The increase in binding by the alpha-1 globulins was most striking as negligible amounts of vitamin B_{12} were bound by this protein in normal serum. Similar increased binding of added vitamin B_{12} by the alpha-1 and alpha-2 globulins of myelogenous leukemic sera had been found, using microbiological assay techniques (1). However, the difference between normal and myelogenous leukemic sera was not described by the above workers since they found the alpha-1 and alpha-2 globulins of normal serum to be the chief binding proteins for added vitamin B_{12}. The vitamin B_{12} content of myelogenous leukemic serum is also bound to the alpha globulins (1). It is of interest that, following treatment, the alpha globulins containing the vitamin B_{12} content of the serum were cleared slowly from the circulation, whereas the alpha globulins responsible for the increased binding of added vitamin B_{12} disappeared rapidly from the serum. Patients with an increased UBBC due to diseases other than chronic myelogenous leukemia always had increased binding of vitamin

B_{12} by the alpha-2 globulins but only occasionally had elevated alpha-1 globulin binding. This would suggest that the increase in alpha-1 globulin binding of vitamin B_{12} is the more specific change in chronic myelogenous leukemia. The percentage Co^{60} vitamin B_{12} bound by the other three protein fractions of myelogenous leukemic sera was decreased, whereas the Co^{60} vitamin B_{12} bound in mμg. per ml. was slightly increased as compared with the increase in alpha-1 or alpha-2 globulin binding.

The cause of the increased concentration of vitamin B_{12} in chronic myelogenous leukemia serum is unknown. The rise in serum vitamin B_{12} concentration to levels greater than the TBBC of normal serum and the invariable increase in UBBC both suggest an etiological relationship between the increased binding capacity and the hypervitaminemia. If vitamin B_{12} is bound more firmly than normal by the binding substances in myelogenous leukemic sera, a decreased serum turnover of the vitamin with a resultant rise in its serum concentration would ensue. Rates of serum turnover of bound vitamin B_{12} have been determined in chronic myelogenous leukemia following the intravenous administration of Co^{60} vitamin B_{12} (15). However, the estimation of turnover rates for normal subjects was not possible due to the small amounts of bound Co^{60} vitamin B_{12} remaining in the serum after 24 hours. The absence of hypervitaminemia despite the increase in UBBC in diseases other than chronic myelogenous leukemia is somewhat against such a hypothesis. Hypervitaminemia could also result from an excess amount of vitamin B_{12} entering the serum without any change in the serum turnover rate of the vitamin. Myelogenous leukemic leukocytes dying intravascularly could be the source of the added vitamin (2, 16). Against such a hypothesis is the absence of an increased concentration of vitamin B_{12} in myelogenous leukemic leukocytes (2, 16). If the survival time of chronic myelogenous leukemic leukocytes was shorter than that found in the other leukemias, an increased amount of vitamin B_{12} could also be presented to the serum. However, studies of white cell survival have demonstrated that the leukocytes in chronic myelogenous leukemia have a longer survival time than those of the acute leukemias (17).

The chemical nature of the vitamin B_{12} binding substances in myelogenous leukemic sera moving electrophoretically with the alpha globulins is also obscure. Whether these substances are proteins found normally in serum, but increased in chronic myelogenous leukemia, proteins of the serum that have been altered by the disease process, or abnormal tissue proteins foreign to normal serum, cannot be answered by this study.

SUMMARY

1. An equilibrium dialysis method for the determination of the vitamin B_{12} binding capacity of serum (UBBC) has been described. The serum concentration of vitamin B_{12}, the UBBC, and the total vitamin B_{12} binding capacity (TBBC) of sera from normal subjects and patients with chronic myelogenous leukemia, other leukemias and leukocytoses have been determined.

2. In normal subjects, the average serum vitamin B_{12} concentration was equal to 0.64 mμg. per ml., the UBBC to 1.51 mμg. per ml., and the TBBC to 2.23 mμg. per ml.

3. Patients with chronic myelogenous leukemia had an average serum vitamin B_{12} concentration of 8.09 mμg. per ml., a UBBC of 8.87 mμg. per ml., and a TBBC of 16.86 mμg. per ml. Following treatment, the UBBC fell rapidly to normal, while the decline in serum vitamin B_{12} concentration was a delayed and more gradual one.

4. Two patients with chronic monocytic leukemia, and one patient with leukocytosis had an increase in serum vitamin B_{12} concentration and UBBC. Six of eight patients with leukocytosis and one of three patients with acute leukemia had normal serum vitamin B_{12} levels but elevated UBBC's. Patients with chronic lymphocytic leukemia had a concentration of vitamin B_{12} and UBBC within the normal range.

5. The beta globulins bound an average of 44 per cent of the added Co^{60} vitamin B_{12} (average, 0.79 mμg. per ml.) and was the chief binding protein in 16 of 18 normal subjects. The alpha-2 globulin was next in vitamin B_{12} binding ability (an average of 28 per cent of the added Co^{60} vitamin B_{12} and 0.59 mμg. per ml.). The alpha-1 globulins bound the least Co^{60} vitamin B_{12} of any of the five protein fractions.

6. The alpha-1 globulins were the chief binding protein in 5 of 10 patients with chronic myeloge-

nous leukemia, binding an average of 38 per cent of the added Co^{60} vitamin B_{12} (3.55 mμg. per ml.). The alpha-2 globulins were the chief binding protein in 4 of the 10 patients, binding an average of 34 per cent of the added Co^{60} vitamin B_{12} (3.07 mμg. per ml.). With treatment of the disease, the pattern of vitamin B_{12} binding by the serum proteins returned towards normal.

7. An increase in the binding of Co^{60} vitamin B_{12} by the alpha-1 and alpha-2 globulins was noted in one patient with chronic monocytic leukemia, and one other patient with leukocytosis. Four other patients with elevated UBBC's of varying degree, due to diseases other than chronic myelogenous leukemia, had an increase in vitamin B_{12} binding by the alpha-2 globulins but no increase in alpha-1 globulin binding.

8. In addition to an increased serum concentration of, and binding capacity for, vitamin B_{12} in chronic myelogenous leukemia, there is an abnormal distribution of added vitamin B_{12} among the serum protein fractions in this disease.

ACKNOWLEDGMENTS

We are indebted to Merck and Company who kindly supplied the cobalt[60] labeled vitamin B_{12} used in this study.

REFERENCES

1. Beard, M. F., Pitney, W. R., and Sanneman, E. H. Serum concentrations of vitamin B_{12} in patients suffering from leukemia. Blood 1954, 9, 789.
2. Mollin, D. L., and Ross, G. I. M. Serum vitamin B_{12} concentrations in leukaemia and in some other haematological conditions. Brit. J. Haemat. 1955, 1, 155.
3. Raccuglia, G., and Sacks, M. S. Vitamin B_{12} binding capacity of normal and leukemic sera. J. Lab. clin. Med. 1957, 50, 69.
4. Pitney, W. R., Beard, M. F., and Van Loon, E. J. Observations on the bound form of vitamin B_{12} in human serum. J. biol. Chem. 1954, 207, 143.
5. 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. 1954, 44, 715.
6. Ross, G. I. M. Vitamin B_{12} assay in body fluids using Euglena gracilis. J. clin. Path. 1952, 5, 250.
7. Klotz, I. M., Walker, F. M., and Pivan, R. B. The binding of organic ions by proteins. J. Amer. chem. Soc. 1946, 68, 1486.
8. Smith, E. L., Ball, S., and Ireland, D. M. B_{12} vitamins (cobalamins). 2. Neutral, basic and acidic cobalamins. Biochem. J. 1952, 52, 395.
9. Hine, G. J., and Miller, A. Large plastic well makes efficient gamma counter. Nucleonics Oct., 1956, 14, 78.
10. Rosenthal, H. L., and Sarett, H. P. The determination of vitamin B_{12} activity in human serum. J. biol. Chem. 1952, 199, 433.
11. Bertcher, R. W., and Meyer, L. M. Co^{60} vitamin B_{12} binding capacity of normal human serum. Proc. Soc. exp. Biol. (N. Y.) 1957, 94, 169.
12. Miller, A. Unpublished observations.
13. Pitney, W. R., Beard, M. F., and Van Loon, E. J. The vitamin B_{12} content of electrophoretic fractions of liver homogenates. J. biol. Chem. 1955, 212, 117.
14. Beard, M. F., Pitney, W. R., Sanneman, E. H., Sakol, M. J., and Moorhead, H. H. Serum concentrations of vitamin B_{12} in acute leukemia. Ann. intern. Med. 1954, 41, 323.
15. Miller, A., Corbus, H. F., and Sullivan, J. F. 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. 1957, 36, 18.
16. Thomas, J. W., and Anderson, B. B. Vitamin B_{12} content of normal and leukaemic leukocytes. Brit. J. Haemat. 1956, 2, 41.
17. Osgood, E. E., Tivey, H., Davison, K. B., Seaman, A. J., and Li, J. G. The relative rates of formation of new leukocytes in patients with acute and chronic leukemias. Cancer (Philad.) 1952, 5, 331.

Amer. J. Med. Sci. 225:113-119

1902

THE AMERICAN JOURNAL OF THE MEDICAL SCIENCES

FEBRUARY, 1953

ORIGINAL ARTICLES

CRYSTALLINE B_{12} INHALATION THERAPY IN PERNICIOUS ANEMIA*

By RAYMOND W. MONTO, M.D.**

JOHN W. REBUCK, M.D., PH.D.***

AND

MICHAEL J. BRENNAN, M.D.**

(From the Division of Hematology** and Department of Laboratories***, the Henry Ford Hospital, Detroit 2, Michigan.)

OF the previously suggested routes for B_{12} administration in the treatment of pernicious anemia, parenteral injection alone has thus far proved satisfactory. Oral[1,2] as well as sublingual administration has resulted in unsatisfactory clinical and hematologic responses, except when given in massive doses or in conjunction with intrinsic factor[4].

The success of parenteral injection is predicated upon the ready access of the injected material to capillaries and lymphatics. One of the largest capillary beds in the body which contacts the external environment is that of the pulmonary circulation. Inhalation therapy seemed to be a logical route for attempted administration of B_{12}. Reports of the effectiveness of aerosol therapy in other fields such as the antibiotics, hormones and vasoconstrictors lend support to the validity of this concept[2]. B_{12} inhalation therapy has resulted in adequate clinical and hematologic responses in 3 patients, 2 presenting severe and one mild manifestations of pernicious anemia.

Materials and Methods. Crystalline B_{12} in physiological saline solutions without a preservative was utilized in the first 2 cases. The material was administered initially by means of pressure tank and a vaponephrin nebulizer and contained 15 μgm. of the vitamin per 1.0 cc. The dosage was that usually employed in parenteral administration of this agent. In one patient after 9 days of administration utilizing the pressure tank, self-administration was effected by means of a hand atomizer.

In the third case, 1000 μgm. of crystalline B_{12} in 0.1 cc. by volume of lactose powder was utilized for administration as a dust. This was an attempt to further simplify self-administration. The B_{12} lactose powder was administered by means of a dust inhaler (aerohalor-Abbott). The patient was directed to take 10 deep inhalations per administration at the times corresponding to usual parenteral dosage schedules.

Later experience with this method has shown that larger amounts of active material are deposited in the lungs if a forced expiration immediately precedes a slow, deep inspiration. Immediately following this inspira-

* Presented at the American Clinical and Climatological Association, Elmira, N. Y., October 16, 1952.

598

FIG. 1. CASE 1.—Representative megaloblasts and a giant neutrophilic metamyelocyte in the marrow aspirate prior to treatment. X1200.

FIG. 2. CASE 1.—Normoblasts and unaffected members of the neutrophilic series in the marrow aspirate after crystalline B_{12} aerosol treatment. X1200.

FIG. 3. CASE 1.—Megalocytes and poikilocytes in the peripheral blood prior to treatment.

FIG. 4. CASE 1.—Normocytes with only an occasional macrocyte in the peripheral blood after crystalline B_{12} aerosol treatment. X1200.

tion the breath should be held for a few moments then exhaled through the nose[6].

Case Reports. CASE 1. F. Z., HFH Case No. 674802, a white male aged 56, was first seen April 14, 1952, complaining of weakness, anorexia and exertional dyspnea of 8 weeks' duration. For several weeks he had noted numbness in the lower extremities. The past history was non-contributory.

Physical examination revealed a well-developed, well-nourished male with a lemon yellow skin color. There was moderate atrophy of the tongue and marked pallor of the mucous membranes. Neurological examination was normal except for mild hypesthesia of the lower extremities.

Pertinent laboratory data on admission included a gastrointestinal survey, including liver function tests and other biochemical blood determinations, that was normal excepting a histamine-fast achlorhydria; hemoglobin, 5.9 gm. per 100 cc.; erythrocytes 1,360,000 per c.mm.; hematocrit 17.1%; mean corpuscular volume 125.7 c.μ.; mean corpuscular hemoglobin 43.3 mμgm.; mean corpuscular hemoglobin concentration 34.5%; reticulocytes 2.2%; leukocytes 6,300 per c.mm.

The blood smear (Fig. 3) revealed a macrocytic erythrocytic picture characterized by the presence of megalocytes, poikilocytosis, anisocytosis and polychromasia. The neutrophilic leukocytes presented hypersegmentation of their nuclei.

The sternal aspirate revealed paradoxical hyperplasia and the presence of 44.2% megaloblasts and 12% giant neutrophils of the pernicious anemia type indicative of marked deficiency of anti-pernicious anemia factors (Fig. 1, Table 1).

The patient was given daily inhalations of 15 μgm. of crystalline B_{12} in 1 cc. of physiological saline. No other anti-anemic preparations were given.

The hematologic response is shown in Chart 1. On the 7th day of therapy the reticulocyte response was 21% and on the 8th day of therapy 44.2%. The patient continued on daily self-administered inhalations

TABLE 1.—DIFFERENTIAL COUNTS (1000 CELLS) OF STERNAL MARROW ASPIRATES OF PERNICIOUS ANEMIA PATIENTS BEFORE AND AFTER TREATMENT WITH CRYSTALLINE B_{12} AEROSOL AND B_{12}-LACTOSE DUST INHALATION

	Before	*After**	*Before*	*After**	*Before*	*After*
Myeloid: erythroid ratio	1.1:1	2.4:1	5:1	5.5:1	0.9:1	5.6:1
Reticulum cells (occ.)	0.4	0.5	1.0		0.6	0.4
Myeloblasts (1.)	0.2	0.4	0.4		0.1	0.4
Progranulocytes (2.)	1.4	2.8	4.2	0.6	1.3	3.7
Neutrophilic myelocytes early (4.)	4.2	5.4	4.0	2.8	4.4	5.3
Neutrophilic myelocytes late (13.)	5.6	5.4	5.4	3.8	5.2	10.0
Neutrophilic metamyelocytes (15.)	3.2	7.0	5.4	4.6	6.6	8.3
Band cells (16.)	7.2	10.6	10.4	5.0	6.7	13.9
Neutrophils segmented (15.)	12.6	26.0	29.2	35.8	5.9	24.1
Immature neutrophils, P.A. Type (0)	9.0	2.4	3.4	0.6	5.6	2.3
Neutrophils segmented, P.A. type (0)	3.0	2.0	2.6	1.2	1.3	0.1
Eosinophilic myelocytes early		0.3		0.2	0.1	0.2
Eosinophilic myelocytes late (2.)	0.4	0.8	0.8	0.2	0.4	1.6
Eosinophilic metamyelocytes	0.4	0.4	0.6		0.4	0.6
Eosinophils (1.)	1.0	0.5	2.8	0.8	0.2	1.0
Basophils, immature (occ.)		0.2	0.4			0.2
Basophils (0.5)			0.4		0.5	
Prolymphocytes (occ.)		0.1			0.3	0.6
Lymphocytes (10.)	5.2	6.5	11.2	31.4	7.2	10.6
Leukocytoid lymphocytes (0)	0.2		0.2			
Monocytes (1.)		1.7		7.2	0.1	0.9
Proplasmocyte (occ.)						0.1
Plasmocyte (1.)		0.2	1.8	0.2	0.5	0.3
Pronormoblasts (0.5)		0.7	0.8	0.2	1.3	
Basophilic normoblasts (2.)		1.8	1.4	0.6	2.7	0.8
Polychromic normoblasts (12.)		20.9	6.2	4.0	5.5	7.9
Orthochromic normoblasts (4.)	0.2	2.1	4.2	0.4	2.4	3.7
Promegaloblasts (0)	7.2	0.2			2.8	0.1
Basophilic megaloblasts (0)	17.0	0.2	0.2		21.8	0.1
Polychromic megaloblasts (0)	16.6	1.1	1.8		12.8	1.5
Orthochromic megaloblasts (0)	3.4	0.6	0.4		2.2	0.7
Phagocytic histiocytes (occ.)	0.2					0.1
Lipid histiocytes (0)						0.1
Fat cells (occ.)			Predominate			
Mitoses, white	0.2	0.1			0.5	0.1
Mitoses, red	1.2	0.9		0.4	1.4	0.3
Cellularity	Packed	Normal	Hypo-plastic	Hypo-plastic	Packed	Normal

* Three weeks after therapy

of B_{12} at home without symptoms or signs of pulmonary irritation. A continued rise in hemoglobin and erythrocyte count has been noted on return visits to the outpatient department. By the 20th day the erythrocyte count had more than doubled with an increase of over 500,000 erythrocytes per week.

Examination of the marrow aspirate (Table 1) revealed a drop in megaloblasts to only 2.1% and of pernicious anemia type neutrophils to 4.4% (Fig. 2). At this time the patient presented marked symptomatic improvement. Therapy was continued by inhalation of 15 μgm. three times a week for another month followed by inhalation of 100 micrograms in 1 cc. saline once a week by hand atomizer. By July 1st, the erythrocyte count reached 4,680,000 per c.mm., and the hemoglobin 14.5 gm.

CASE 2. L. H., HFH Case No. 425149, a white female aged 71 years, was first seen on April 20, 1952. Complaints were weakness, weight loss and paresthesias of all extremities. The past history was non-contributory.

Physical examination revealed a poorly nourished, pale, elderly female. There was mild atrophy of the tongue and pallor of the mucous membranes. Neurological examination demonstrated mild peripheral neu-

ritis in both legs, considered by the neurological consultant as consistent with the early stage of subacute combined degeneration of the cord.

A gastrointestinal survey, including liver function tests, and biochemical studies of the blood were normal except for the presence of histamine-fast achlorhydria.

Pertinent hematological data on admission were as follows: Hemoglobin 12.1 gm. per 100 cc.; red cells 3,480,000 per c.mm.; hematocrit 37.8%; mean corpuscular volume 108.5 c.μ., mean corpuscular hemoglobin 31.8 mμgm.; mean corpuscular hemoglobin concentration 32.0%; reticulocytes 1.7; leukocytes 6,200 per c.mm. Examination of the blood smear revealed a macrocytic erythrocytic picture with anisocytosis and polychromasia as additional features. An occasional neutrophilic leukocyte presented nuclear hypersegmentation.

Examination of the sternal aspirate revealed hypoplasia and the presence of 2.4% megaloblasts and 6% giant neutrophils of the pernicious anemia type indicative of definite but moderate deficiency of anti-pernicious anemia factors.

The patient was given daily inhalations of 15 μgm. of crystalline B_{12} for 6 days, then, 3 times a week for 2 weeks. The hematologic response is shown in Chart 2. Three weeks after the institution of aerosol therapy the red blood cell count increased from 3,480,000 to 4,400,000 per c.mm. and the hemoglobin rose from 12.1 to 14.2 gm. There was symptomatic improvement of the peripheral neuritis and a weight gain of 10 pounds.

Case 3. S. G., HFH Case No. 686563, a white female aged 51 years, was first seen August 4, 1952, complaining of weakness and sore tongue as well as numbness and tingling of all extremities. The past history was noncontributory.

Physical examination revealed a pale, slightly obese female. A distinct yellow tint to the skin and sclerae were evident. The tongue presented a mild degree of atrophy of the lateral margins and a reddened tip. Neurological examination showed moderate hyperesthesia of the extremities.

Pertinent laboratory data on admission were as follows: a gastrointestinal survey, including liver function tests and other biochemical blood determinations, revealed cholelithiasis and a histamine-fast achlorhydria; hemoglobin, 7.5 gm. erythrocytes 1,640,000 per c.mm.; hematocrit 23.6%; mean corpuscular volume 143.8 cμ.; mean corpuscular hemoglobin 45.7 mμgm.; mean corpuscular hemoglobin concentration 31.7%; reticulocytes 1.4%; thrombocytes 100,000

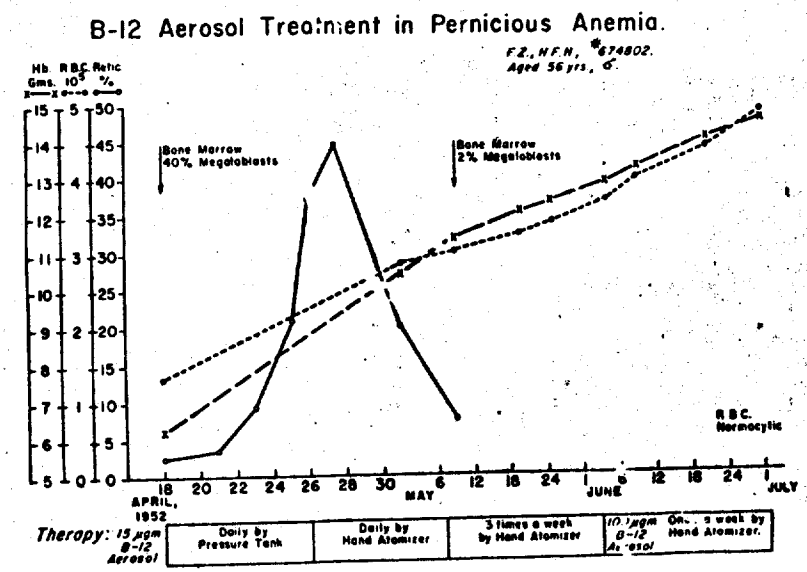


Chart 1.—Hematologic response to crystalline B_{12} aerosol treatment in pernicious anemia, Case 1.

per c.mm.; leukocytes 5,200 per c.mm. Examination of the blood smear revealed a macrocytic erythrocytic picture characterized by the presence of megalocytes, poikilocytosis, anisocytosis, polychromasia, basophilic stippling and an occasional normoblast. The neutrophilic leukocytes presented hypersegmentation of their nuclei and a slight "shift to the left."

Examination of the sternal aspirate revealed paradoxical hyperplasia and the presence of 39.6% megaloblasts and 6.9% giant neutrophils of the pernicious anemia type indicative of marked deficiency of anti-pernicious anemia factors (Table 1).

The patient was given daily inhalations of crystalline B_{12} in lactose powder. Ten deep inspirations per day were self-administered for 8 days. No other anti-anemic preparations were given. The hematologic response is shown in Chart 3. On the 8th day of therapy the reticulocyte response was 22.0%. Study of Chart 3 reveals a persistence of reticulocytosis above 10% for over 15 days. In the first 3 weeks of therapy the erythrocyte count increased from 1,640,000 to 2,960,000 per c.mm., or an average of over 400,000 erythrocytes per c.mm. per week. The hemoglobin rose from 7.5 to 10.4 gm. The glossitis and hyperesthesias disappeared and color and strength improved.

The patient was discharged to her home after 8 days of hospitalization with instructions to take 5 deep inspirations of B_{12} three times a week for the first week and twice a week for the second week. In the succeeding 3 week period she took only 5 inspirations of B_{12} lactose dust on one occasion. On this reduced dosage schedule the erythrocyte count dropped from 2,960,000 to 2,520,000 per c.mm. Upon reinstitution of 10 inspirations of B_{12} lactose dust three times a week, the patient again demonstrated an increase in her erythrocyte count at the rate of 500,000 cells per c.mm. per week (Chart 3). Examination of the marrow aspirate (Table 1) at the end of this period revealed a drop in megaloblasts to 2.4%. The thrombocytes had risen from the original level of 100,000 to 375,000 per c.mm.

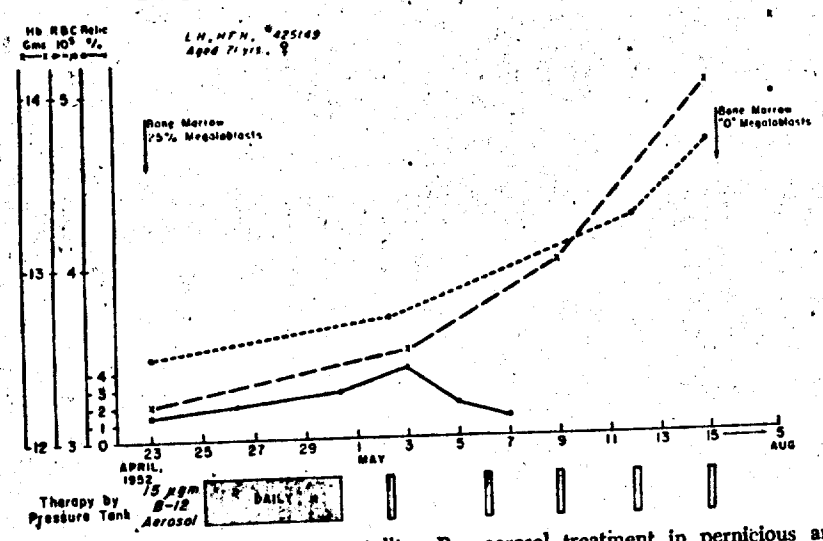


CHART 2.—Hematologic response to crystalline B_{12} aerosol treatment in pernicious anemia, Case 2.

Discussion. The suitability of B_{12} inhalation therapy requires some amplification. Few drugs now being used in inhalation therapy meet the exacting requirements of lack of toxicity and need for closely controlled dosage. Inhalation of crystalline B_{12} in physiological saline or lactose powder has, to date, produced no objective or sub-

jective evidence of toxicity or sensitivity at the pulmonary site.

There is no question but that B_{12} inhalation therapy involves additionally some degree of oral therapy including sublingual and gastrointestinal routes. However, satisfactory responses to inhalation of the vitamin were obtained at dosage levels far below those needed for effective oral therapy[4,5].

per cc.[1]. After inhalation of only 200 μgm of B_{12} in saline, B_{12} activity in the urine reached the level of 0.26 mμgm per cc., and following inhalation of approximately 500 μgm of B_{12} in lactose powder B_{12} activity in the urine rose to 0.134 mμgm per cc. Direct pulmonary instillation of 100 μgm. of crystalline vitamin B_{12} by means of the bronchoscope, resulted in a urinary excre-

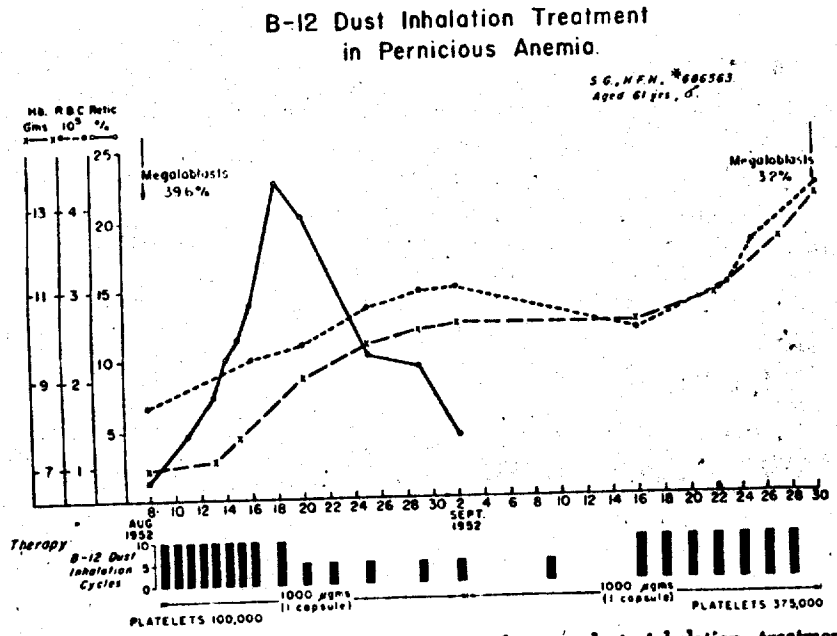


CHART 3.—Hematologic response to crystalline B_{12}-lactose dust inhalation treatment in pernicious anemia, Case 3.

To evaluate further the efficacy of pulmonary absorption of the inhaled B_{12} over and above the evidence afforded by the clinical and hematological response previously described, urinary excretion studies were performed. These latter consisted of measurement in the urine of B_{12}-like growth stimulating substance, using the test organism, *Lactobacillus leichmanii*. Upon oral administration of B_{12} at dosages as high as 10,000 μgm., B_{12} activity in urine has been shown to be less than 0.05 mμgm

tion (9.76 mμgm. per cc.) pattern equivalent to the intramuscular injection of 60 to 80 μgm. of B_{12}. These results indicate a definite increase in urinary B_{12}-like growth substance in the urine of those subjects receiving B_{12} inhalation administration.

Intranasal B_{12} drops and dust, as routes of administration, are being investigated.

Although these results are uniformly encouraging, they, of course, constitute only preliminary observations as to the

efficiency of inhalation crystalline B_{12} treatment of pernicious anemia. Additional investigations will concern the problem of long-term maintenance of the pernicious anemia patient with these newer methods of specific anti-pernicious anemia therapy.

Summary. Inhalation therapy with crystalline B_{12} in physiological saline and lactose dust has resulted in adequate clinical and hematologic responses in 3 patients, 2 presenting severe and one mild grades of pernicious anemia. Detectable amounts of B_{12} activity in the urine were found after pulmonary administration of this substance.

ADDENDUM: Subsequent to the submission of this manuscript, 3 additional pernicious anemia patients in relapse have been adequately treated by inhalation crystalline B_{12}.

ACKNOWLEDGMENT: The authors wish to express appreciation to H. W. Schoenlein, Director of Bacteriological Laboratories, Difco Laboratories Incorporated, for supplying the crystalline B_{12}-lactose powder and the urinary B_{12} determinations.

REFERENCES

1. Conley, C. L., Krevans, J. R., Chow, B. F., Barrows, C., and Long, C. A.: J. Lab. & Clin. Med., 38, 84, 1951.
2. Dautrebande, L.: Physiol. Rev., 32, 214, 1952.
3. Meyer, L. M., Sawitsky, A., Chen, B. S., Krim, M., and Fadem, R.: Am. J. Med. Sci., 220, 604, 1950.
4. Ungley, C. C.: Brit. Med. J., 2, 905, 1950.
5. Idem: Nutrit. Abst. Rev., 21, 11, 1951.
6. Vermilye, H. N.: J. Am. Med. Assn., 129, 251, 1945.

J. Amer. Diet. Assoc. 39: 476-480

Vitamin B_{12} and Ascorbic Acid in Protein Utilization[1]

ELLEN H. MORSE, Ph.D.,[2]
ELNA E. DANIELS,
FLORENCE A. HANLON, and
GEORGIANNA R. WALKER[3]

School of Home Economics and Storrs Agricultural Experiment Station, University of Connecticut, Storrs

UTILIZATION OF dietary protein is influenced in part by the action of certain vitamins. To promote growth in many species, vitamin B_{12}, in particular, is needed to supplement a vegetable-protein ration. In studying vitamin B_{12} as a growth stimulant in human beings, growth might be affected by other variables such as differences in thyroid activity, a chance cold or infection, or even psychologic disturbances which affect appetite. However, a vitamin B_{12} deficiency may occur in areas of the world where the diet is deficient in animal protein (1).

There are indications of interrelationships between B_{12}, ascorbic acid, and protein. Vitamin B_{12} appears to be involved in protein synthesis through its relationship to two amino acids, serine and glycine, in methyl group synthesis (2). The relationship of ascorbic acid to protein metabolism occurs in the hydroxylation of tyrosine (3).

Mueller and Will observed (4) that in disease states, such as pernicious anemia, with low blood levels of ascorbic acid, vitamin B_{12} appeared to return these low levels to normal before body stores of ascorbic acid were restored from dietary sources. They postulated that vitamin B_{12}, by its ability to stabilize SH groups and thus protect other reducing substances, such as glutathione and cysteine, might prevent rapid oxidation of ascorbic acid. The present study was designed to show a possible interrelationship of vitamin B_{12} and ascorbic acid to the utilization of protein, as well as the independent effects of these two vitamins.

Criteria selected to investigate these relationships, using young women as subjects, were: nitrogen balance, erythrocyte counts, hemoglobin, non-protein nitrogen and amino acid nitrogen in blood, and total serum cholesterol.

If supplementation of the diet with vitamin B_{12} and ascorbic acid increased the utilization of protein, an increase in nitrogen retention might be expected. However, changes in nitrogen balance following vitamin supplementation were slight and without statistical significance. Hence the results will not be discussed here.

Changes in hemoglobin values also are not reported. The responses to the diet, either with or without supplementation, were negative in this study, although slight rises in hemoglobin values following ascorbic acid supplementation of the diets of college women have been reported by Leverton and Marsh (5).

Determinations of non-protein nitrogen and amino acid nitrogen in blood were carried out to explore the possibility that they might reflect the effect on

---

[1]This study was part of a Northeast Regional Project NE-37, Relationships between Protein and Other Selected Nutrients and Their Metabolism and Utilization, a cooperative study involving agricultural experiment stations in the Northeast Region, and supported in part by regional funds of the U.S. Department of Agriculture. Presented in part at the 24th annual meeting of the American Institute of Nutrition in Chicago, April 11, 1960 (Fed. Proc. 19: 418, 1960). Received for publication December 1, 1960.

[2]Present address: Department of Home Economics, University of Vermont, Burlington.

[3]The authors wish to acknowledge the services of John B. LeRoy, M.D., University physician; and the statistical advice of D. G. Gosslee, Ph.D., and the suggestions of H. D. Eaton, Ph.D., Mary L. Greenwood, Ph.D., and A. A. Spielman, Ph.D., in carrying out the experiment and in the preparation of the manuscript. The technical assistance of the following is also acknowledged: Mary Breese, Mary Callender, Ko-Cheng Chao, Julianne Keegan, Jeanne Fortier, Nancy M. Kline, Virginia A. Overley, and Grace deVries. Gratitude is expressed to the twenty students who served cheerfully as subjects.

605

protein metabolism of the vitamin supplementation. The results of these analyses, as well as the effects of supplementation on erythrocyte counts, are discussed. The average change in total serum cholesterol as a result of using a low-fat, largely vegetable-protein diet for six weeks is also reported.

Experimental Procedure

Twenty women students of the University of Connecticut were selected to serve as subjects from a group of volunteers following a physical examination by the University physician. They ranged from eighteen to twenty-seven years in age and from 46 to 78 kg. in weight. At the time of the physical examination, hemoglobin values ranged from 10.6 to 13.6 gm. per 100 ml. blood. Subjects with medium to low values were chosen in order to elicit possible hematologic response to vitamin B_{12} and ascorbic acid supplementation.

A controlled dietary experiment was conducted for six weeks in a campus dining room under the supervision of the School of Home Economics, with meals served seven days a week. The first two weeks served as a standardizing period during which all subjects ate the same basal diet. During the next four weeks the twenty subjects were divided according to a completely randomized 2^2 factorial design (6) into groups receiving the four following dietary treatments. Group I ate the basal diet and served as controls. Group II ate the basal diet plus a supplement of 1000 mcg. vitamin B_{12}. Group III received the basal diet plus a supplement of 200 mg. ascorbic acid. Group IV were given both 1000 mcg. vitamin B_{12} and 200 mg. ascorbic acid in addition to the basal diet.

The dietary specifications called for 40 to 45 gm. protein a day with approximately 85 per cent in the form of plant protein to keep the intake of vitamin B_{12} at a minimum. The chief sources were cereals, bread, macaroni, rice, peas, kidney, and Lima beans. The sources of animal protein consisted of 120 gm. whole milk daily for breakfast cereal, the protein in 30 gm. cream for Postum, the 4 per cent milk solids in the commercial enriched white bread, and the small amount of milk solids and powdered eggs in a few commercial baked products. Thus, with milk as the chief source of vitamin B_{12}, the dietary intake of this vitamin was estimated by calculation to be less than 1.0 mcg. per day (7).

Ascorbic acid intake was held at less than 30 mg. per day, by selecting foods low in ascorbic acid, by cooking all vegetables in a large amount of water, and by mashing the potatoes.

The Recommended Dietary Allowances (8) were met for most of the nutrients except the three under study, either from the daily food intake or by supplementation with one capsule daily of B-complex[1] without vitamin B_{12} and with two, 500 mg. tablets of dicalcium phosphate[2] containing 120 mg. calcium. The one exception was the allowance for iron; food intake alone provided 80 per cent of this standard.

Extra calories in the form of margarine, French dressing, sugar, and ginger ale were allowed *ad libitum* to satisfy individual preferences; intake of these was recorded daily. The average intake was 2154 calories per day, with a range of 1794 to 2641. The average fat intake was low, averaging only 28 per cent of total calories. Approximately 12 per cent of the fat calories were from cream, 12 per cent from salad oil, and the remainder from margarine and hydrogenated shortening.

After the two-week standardizing period, supplementation of the diets with vitamin B_{12} and or ascorbic acid was administered by a staff member at the evening meal. Crystalline vitamin B_{12} in 1000-mcg. tablets[6] was given to Groups II and IV. Groups I and III received a placebo tablet[7]. Capsules containing 200 mg. crystalline ascorbic acid[8] were given to Groups III and IV, while Groups I and II received placebos[9].

A venous blood sample was taken from each subject between 10 and 12 a.m. in the week before the study was started, at the end of the standardizing period, and at the end of the fourth, fifth, and sixth weeks. Part of the sample was prepared for frozen storage as protein-free filtrate by the method of Folin and Wu (9) for the determination of non-protein nitrogen and amino acid nitrogen. Non-protein nitrogen was determined by a blood micro-Kjeldahl method adapted by the authors from the method of Kind (10). Amino acid nitrogen was determined by the method of Frame *et al.* (11). A small portion of each blood sample was centrifuged, the serum removed and stored frozen for determination of total serum cholesterol using the method of Clayton *et al.* (12). Finger tip blood samples from each subject were taken between 10 and 12 a.m. on the day preceding the venous blood samples.

Results and Discussion

Table 1 presents the treatment means for erythrocyte counts, non-protein nitrogen, and amino acid nitrogen in blood for each group obtained after the two-week standardizing period and at the end of the four-week comparison period, as well as the means at the end of the comparison period adjusted by regression methods for the means at the end

[1]Supplied by Upjohn Company, Kalamazoo, Michigan.

[2]Supplied by Abbott Laboratories, North Chicago, Illinois.

[6]Supplied by Merck and Company, Rahway, New Jersey.

[7]Supplied by Merck and Company.

[8]U.S.P. grade, supplied by Merck and Company.

[9]Capsules prepared by School of Pharmacy, University of Connecticut.

TABLE 1 Means for erythrocytes, non-protein nitrogen, and amino acid nitrogen obtained with control and three experimental groups

PERIOD	GROUP I	GROUP II	GROUP III	GROUP IV	STANDARD DEVIATION*
			Erythrocyte Counts		
			millions/mm.³		
Standardizing period —unadjusted mean	4.58±0.06†	4.43±0.48	4.50±0.23	4.26±0.24	±0.31
Comparison period					
Unadjusted mean	4.24±0.25	4.63±0.44	4.52±0.17	4.60±0.32	±0.32
Adjusted mean	4.15	4.62	4.49	4.72	±0.26
			Non-Protein Nitrogen		
			mg./100 ml. blood		
Standardizing period —unadjusted mean	25.9 ±2.3	24.8 ±3.8	26.1 ±1.9	27.1 ±1.8	±2.6
Comparison period					
Unadjusted mean	27.1 ±3.3	25.4 ±2.8	24.8 ±1.1	27.6 ±1.7	±2.4
Adjusted mean	27.2	26.1	24.7	26.9	±1.8
			Amino Acid Nitrogen		
Standardizing period —unadjusted mean	8.68±0.35	8.80±0.36	8.59±0.84	8.38±0.82	±0.64
Comparison period					
Unadjusted mean	9.18±0.30	8.62±0.34	8.64±0.41	8.65±0.82	±0.51
Adjusted mean	9.14	8.52	8.65	8.78	±0.40

*20 values.
†Based on 5 values per group.

of the standardizing period. The adjustment of means and analysis of covariance were calculated according to the Snedecor's procedures (6). The use of covariance to adjust for the uncontrollable genetic and environmental conditions by application of regression methods to analysis of variance significantly increased the relative efficiency of the experiment. The relative efficiencies based on the ratio of the unadjusted error mean square to the effective adjusted error mean square were 144 per cent for the erythrocyte counts, 169 per cent for non-protein nitrogen, and 161 per cent for amino acid nitrogen in blood.

The mean responses and differential responses based on the adjusted means according to the procedure of Cochran and Cox (13) are presented in Table 2. The mean response represents the difference between the mean values for the ten subjects with and the ten without one of the two supplements. The differential responses represent differences between the adjusted means for the five subjects in each of the four treatment groups.

ERYTHROCYTE COUNTS

Positive responses to supplementation were obtained during the four-week period (Table 2). The mean response of the ten subjects receiving vitamin B_{12} was greater than that of the ten subjects receiving no vitamin B_{12}, with a difference of 0.355 million per cubic millimeter. This was shown by covariance to be significant at the 2.5 per cent level ($P < 0.025$). The mean response for the subjects receiving ascorbic acid was greater than that for subjects receiving no ascorbic acid, with a difference of 0.215 which just missed being significant at the 5 per cent level. The interaction between the two vitamins was negligible and without statistical significance ($P > 0.25$).

All but one girl receiving vitamin B_{12} supplementation had an increase in red cell count. This subject had a menstrual period of longer duration than the others. The fact that the means of erythrocyte counts for Group I receiving no vitamin B_{12} declined markedly during the six weeks suggests that a vegetable-protein diet might eventually lead to anemia as reported by Wokes *et al.* (14).

The response to the 1000-mcg. supplement of vitamin B_{12} showed that the level chosen was high enough to produce significant results. These normal young women, with a normal supply of intrinsic factor, were able to utilize the crystalline vitamin when orally administered in large dosage.

NON-PROTEIN NITROGEN AND AMINO ACID NITROGEN IN BLOOD

As seen in Table 1, the means for non-protein nitrogen showed very slight changes. The effects of vitamin B_{12} and of ascorbic acid administered alone were negative (Table 2). However, when given jointly, there was a positive response. The analysis of covariance showed that the interaction of the two vitamins was very close to the 5 per cent level of significance.

As with the non-protein nitrogen, the response of amino acid nitrogen to vitamin B_{12} and to ascorbic acid alone were negative, while the two vitamins

TABLE 2 **Mean and differential responses based on adjusted means reported in Table 1**

VITAMIN	MEAN RESPONSE*	DIFFERENTIAL RESPONSE: Ascorbic acid, Absent	Ascorbic acid, Present	Vitamin B_{12}, Absent	Vitamin B_{12}, Present	STANDARD ERROR†
		Erythrocyte Counts				
	← *millions mm.* →					
Ascorbic acid	+0.215	—		+0.31	+0.09	
Vitamin B_{12}	+0.355	+0.48	+0.23	—		±0.16
Standard error†	+0.12					
		Non-Protein Nitrogen				
	← *mg. 100 ml. blood* →					
Ascorbic acid	−0.85	—	—	−2.5	+0.8	±1.1
Vitamin B_{12}	+0.55	−1.1	+2.2	—	—	
		Amino Acid Nitrogen				
Ascorbic acid	−0.115	—	—	−0.49	+0.26	±0.25
Vitamin B_{12}	−0.245	−0.62	+0.13	—	—	

*For differential responses.
†For mean response.

combined produced a positive response, which, by covariance analysis, was just short of the 5 per cent level of significance. For both non-protein and amino acid nitrogen, the interaction was so close to the 5 per cent level of significance that a few more subjects might have produced significant results.

TOTAL SERUM CHOLESTEROL

Although there have been indications that a vitamin B_{12} deficiency induced hypercholesteremia in chicks (15), no significant effects of treatment with this vitamin or with ascorbic acid were observed for serum cholesterol. However, the average levels for the twenty subjects considered as one group proved of interest. The average serum level for samples taken in the week before the study started was 190 mg. per 100 ml. At the end of the first two weeks on the plant-protein, low-fat diet, it had dropped to 174 mg. per 100 ml. This drop was highly significant ($P<0.001$). From the end of two weeks to the end of the sixth week, the decline to a level of 166 mg. per 100 ml. was slight but, by "t" test, was significant at the 5 per cent level ($P<0.05$). The change to a fat intake averaging only 28 per cent of calories probably caused this decrease in total serum cholesterol, although the use of vegetable protein may have had some effect (16).

Summary

A six-week dietary experiment, using twenty young women subjects, was designed to determine the effect of vitamin B_{12} and ascorbic acid on protein utilization. All subjects ate a vegetable-protein, low-fat basal diet (40 to 45 gm. protein) for a two-week standardizing period. The next four weeks served as a comparison period during which the subjects received one of four dietary treatments. Group I served as controls, continuing on the basal diet. Group II ate the basal diet plus a supplement of 1000 mcg. vitamin B_{12} daily. Group III were given the basal diet plus a supplement of 200 mg. ascorbic acid daily. Group IV received both 1000 mcg. vitamin B_{12} and 200 mg. ascorbic acid in addition to the basal diet.

At the end of the study, the erythrocyte counts were significantly higher for the group receiving vitamin B_{12} than for those not receiving this vitamin. A positive response to ascorbic acid supplementation approached significance. The interaction between the two vitamins was negligible and was without statistical significance.

While the effect of either vitamin alone on non-protein nitrogen and amino acid nitrogen in blood was not significant, the effect of both vitamins together approached the 5 per cent level of significance for both criteria.

The average serum cholesterol level of the twenty subjects dropped significantly.

References

(1) Howe, E. E.: Effect of vitamin B_{12} on growth-retarded children: A review. Amer. J. Clin. Nutr. 6: 18, 1958.

(2) Johnson, B. C.: Studies on the mechanism of action of vitamin B_{12} in animal nutrition. Amer. J. Clin. Nutr. 6: 34, 1958.

(3) Udenfriend, S., Clark, C. T., Axelrod, J., and Brodie, B. B.: Ascorbic acid in aromatic hydroxylation. 1. A model system for aromatic hydroxylation. J. Biol. Chem. 208: 731, 1954.

(4) Mueller, J. F., and Will, J. J.: Interrelationship of folic acid, vitamin B_{12} and ascorbic acid in patients with megaloblastic anemia. Amer. J. Clin. Nutr. 3: 30, 1955.

(5) Leverton, R. M., and Marsh, A. G.: The comparative effect of iron, protein, ascorbic acid and the vitamin B complex on hemoglobin formation in humans. J. Nutr. 21: (abstr.) 8, 1941.

(6) Snedecor, G. W.: Statistical Methods. 5th ed. Ames, Iowa: Iowa State College Press, 1956.

(7) JUKES, T. H., AND WILLIAMS, W. L.: Vitamin B_{12}. Occurrence in foods. *In* The Vitamins. Chemistry, Physiology, Pathology. Vol. I. (Harris, R. S., and W. H. Sebrell, Jr., eds.) N. Y.: Academic Press, Inc., 1954, p. 476.

(8) FOOD & NUTRITION BD.: Recommended Dietary Allowances, Revised, 1958. Natl. Acad. Sci.–Natl. Research Council Pub. No. 589, 1958.

(9) FOLIN, O., AND WU, H.: A system of blood analysis. J. Biol. Chem. 38: 81, 1919.

(10) KIND, C. A.: Personal communication, 1957.

(11) FRAME, E. G., RUSSELL, J. A., AND WILHELMI, A. E.: The colorimetric estimation of amino nitrogen in blood. J. Biol. Chem. 149: 255, 1943.

(12) CLAYTON, M. M., ADAMS, P. A., MAHONEY, G. B., RANDALL, S. W., AND SCHWARTZ, E. T.: Micro methods for the determination of chylomicron counts, fatty esters, lipid phosphorus, and cholesterol in blood serum. Clin. Chem. 5: 426, 1959.

(13) COCHRAN, W. G., AND COX, G. M.: Experimental Designs. 2nd ed. N. Y.: John Wiley & Sons, Inc., 1957, p. 192.

(14) WOKES, F., BADENOCH, J., AND SINCLAIR, H. M.: Human dietary deficiency of vitamin B_{12}. Amer. J. Clin. Nutr. 3: 375, 1955.

(15) HSU, J. M., AND CHOW, B. F.: Vitamin B_{12} deficiency and hypercholesterolemia. Fed. Proc. 16: 63, 1957.

(16) OLSON, R. E., VESTER, J. W., GURSEY D., DAVIS, N., AND LONGMAN, D.: The effect of low-protein diets upon serum cholesterol in man. Amer. J. Clin. Nutr. 6: 310, 1958.

Biochem. Biophys. Res. Commun. 35:121-126

Derangement in B_{12} Metabolism Leading to Homocystinemia, Cystathioninemia and Methylmalonic Aciduria

S. Harvey Mudd, Harvey L. Levy and Robert H. Abeles
National Institute of Mental Health, Bethesda, Maryland, Joseph P. Kennedy, Jr. Laboratories, Massachusetts General Hospital and Graduate Department of Biochemistry, Brandeis University

Received March 6, 1969

In mammals, vitamin B_{12} derivatives are known to function as coenzymes for two reactions: (a) methionine formation from 5-methylfolate-H_4 and homocysteine and (b) isomerization of methylmalonyl-CoA to succinyl-CoA (Weissbach and Dickerman, 1967; Hogenkamp, 1968). We have recently investigated an infant with abnormalities of both the sulfur amino acids and methylmalonic acid. This paper summarizes the results of our investigation which indicate the occurrence of a hitherto unrecognized metabolic abnormality, a defective ability to accumulate the coenzymatically active derivatives of B_{12}. As far as we are aware, this is the first proven instance of deranged vitamin metabolism in a human.

Experimental: The child was a patient at the Massachusetts General Hospital from 4 weeks of age until his death at 7-1/2 weeks. Tissues were obtained at postmortem examination and stored frozen. Quantitative analysis of amino acids was performed by ion-exchange chromatography (Efron, 1966). Methylmalonic acid in urine was assayed colorimetrically (Giorgio and Plaut, 1965). Serum folate and serum B_{12} bioassays were kindly performed by Dr. Roy Misliuk and William Beck using respectively L. casei and L. leishmanii. Total tissue B_{12} was measured by Euglena gracilis bioassay (Lear, et al., 1954) and by a radioisotope method (Lau, et al., 1965). The two methods agreed within 5 percent. Deoxyadenosyl-B_{12} was measured as described (Abeles, et al., 1966). Betaine-homocysteine methyltransferase (betaine: L-homocysteine S-methyltransferase, EC 2.1.1.5) and methionine-activating enzyme (ATP-L-methionine-S-adenosyltransferase, EC 2.5.1.6 were assayed according to Finkelstein and Mudd (1967), cystathionine synthase and cystathionase (EC 4.2.1.15) according to Mudd, et al. (1965), and methylfolate-H_4 methyltransferase by a modification of the method of Weissbach, et al. (1963).

Results: Abnormalities of sulfur amino acids. The urine and plasma of the patient consistently contained abnormal elevations of homocystine, cystathionine, and the mixed disulfide of homocysteine and cysteine (Table I). Methionine was extremely low in plasma and not detected in urine. Other amino acids were not correspondingly decreased.

The data of Table I show also that methionine concentrations of frontal and occipital lobes of the brain were 10 percent or less of values for control tissues. The methionine of liver was low, but within the control range.

Cystathionine concentrations were high in both brain and liver.

Table I

Sulfur-containing amino acids in plasma, urine, and tissues

	Methionine	Homocystine	Cystathionine	Homocysteine-cysteine mixed disulfide
Plasma (day 12)	0.07 (0.4-0.6)	1.92 (0)	0.20 (0)	1.61 (<0.5)
Urine (days 11-12)	0 (<1.0)	5.10 (<1.0)	2.04 (<0.5)	0.83 (0)
Urine (days 16-17)	0	2.89	3.73	0.21
Brain, frontal lobe	0.1 (1.0-2.3)	0 (0)	34.4 (1.7-12.3)	1.6 (0)
Brain, occipital lobe	0.2 (2.1-2.6)	0 (0)	32.0 (1.8-9.1)	0 (0)
Liver	1.9 (1.0-8.0)	0 (0)	3.9 (0.7-2.9)	0 (0)

Plasma values are expressed as mg/100 ml; urine, as mg/24 hours; and tissue, as mg/100 g wet tissue. The values for the patient are shown, followed by control values in parentheses. The homocystine and cystathionine were isolated from the urine by column chromatography and further identified by paper chromatography, high voltage electrophoresis, and characteristic staining reactions. Ranges for free amino acids of liver and brain were obtained by analysis of tissues from four control infants. The infants from whom tissues were obtained postmortem for use as controls in the studies reported in this paper ranged in age from 7 days to 17 months. They had died of diseases without known vitamin or amino acid abnormalities.

The following enzymes were assayed in extracts prepared from liver and kidney: betaine-homocysteine methyltransferase, methylfolate-H_4 methyltransferase, cystathionine synthase, cystathionase, and methionine-activating enzyme. Of these enzymes, only methylfolate-H_4 methyltransferase was consistently decreased (Table II). In both liver and kidney a slight activity of methylfolate-H_4 methyltransferase was present, amounting to 4 percent and 23 percent of the mean control value in kidney and liver respectively. In each case the decrease was statistically significant ($p < 0.05$ by the "one-tailed" probability test (Fisher, 1948)). Betaine methyltransferase was not decreased in liver, but was slightly lower in kidney from the patient than in kidney from any control subject. The value was 39 percent of the mean control value. Although this decrease is statistically significant, the decrease is unlikely to have been sufficient to contribute markedly to the patient's difficulties, especially in view of the normal concentration of this enzyme in his liver.

Table II

Enzyme specific activities in extracts of tissues from the patient and control subjects.

Enzyme and source	Patient	Control subjects Mean ± SE*	Range
Methylfolate-H_4 methyltransferase			
liver	0.25	1.10 ± 0.14	0.52-1.62
kidney	0.10	2.34 ± 0.46	0.80-4.56
Betaine methyltransferase			
liver	4.66	6.22 ± 0.98	2.1 -12.5
kidney	4.05	10.4 ± 1.1	7.33-15.4

*Mean ± standard error.

For the control series, each enzyme was assayed in an extract of liver from each of 10 control subjects. Similar studies were performed in extracts of kidneys from 8 control subjects (not necessarily the same subjects that were used for the studies of liver). Specific activities, all per mg protein, are given in the following units: methylfolate-H_4 methyltransferase, mμmoles methionine/60 minutes; betaine-homocysteine methyltransferase, mμmoles methionine/45 minutes.

The possibility that a lack of 5-methylfolate-H_4 contributed to the deficient methionine synthesis was eliminated by investigation of the folate derivatives in a specimen of serum obtained from the patient on hospital day 20 five days after the administration of folic acid. The total concentration, measured by the growth response of L. casei, was 20 mμg/ml (calculated as 5-methylfolate-H_4), a high value. No growth response of either P. cerevisiae or E. faecalis could be demonstrated. These findings, while short of absolute chemical identification, strongly suggest that the dominant folate derivative in the patient's plasma was 5-methylfolate-H_4 (Stokstad and Koch, 1967) and thus are in accord with the evidence that there was a deficient activity of the enzyme which utilizes this compound.

Methylmalonic aciduria: Elevated amounts of methylmalonic acid were found in the urine of our patient. The compound was identified by thin layer and paper chromatography. For unequivocal identification the methylmalonic acid was isolated from urine (days 20-21) by chromatography on Dowex 2. The eluate was extracted into ether and crystallized from toluene-acetic acid. The crystalline material had properties typical of authentic methylmalonic acid during IR spectroscopy and gas-liquid chromatography. Quantitatively, the patient

excreted 17.6-19.3 mg/24 hours before the administration of cyano-B_{12}, 22.0 mg on hospital day 20, five days after receiving 50 µg cyano-B_{12} parenterally and 16.4 mg on hospital day 23, two days after receiving an additional 25 µg cyano-B_{12}.

B_{12} derivatives in serum and tissues (Table III): A serum sample, obtained on day 20, contained a high concentration of B_{12}. Total B_{12} in liver of the patient was within the control range. In contrast, deoxyadenosyl-B_{12} in the patient's liver was reduced to less than ten percent of the mean control value. This decrease was highly significant statistically ($p<0.01$). No deoxyadenosyl-B_{12} was detected in the patient's kidney.

Table III

B_{12} derivatives in serum and liver of the patient and control subjects

	Patient	Control subjects	
		Mean ± SE	Range
Serum, total B_{12} (day 20), mµg/ml	1.75	—	0.2-0.5
Liver, mµg/g wet tissue			
total B_{12}	56	104 ± 22	52-150
deoxyadenosyl-B_{12}	13	160 ± 22	67-240
Kidney, mµg/g wet tissue			
deoxyadenosyl-B_{12}	<2	—	9-14

Total B_{12} was measured in livers from four control subjects; deoxyadenosyl-B_{12}, in liver from seven and kidney from two control subjects. The patient's tissues were frozen within one hour of death and assayed for deoxyadenosyl-B_{12} six months later. Control tissues were obtained more than one hour after death and assayed after storage from three months to three years. The values for liver are discordant in that the content of deoxyadenosyl-B_{12} is apparently higher than total B_{12}. That this discrepancy results from a systematic difference in the two assay systems employed, rather than random error, is indicated by the fact that for the control patients the two sets of values correlate well ($r = 0.96$, $p<0.05$).

Discussion: The combination of homocystinemia (-uria), cystathioninemia (-uria) and a specific decrease of methionine in a desperately ill infant led us to postulate a difficulty in remethylation of homocysteine to methionine. This postulate was confirmed postmortem by demonstration of a specific deficiency in methylfolate-H_4 methyltransferase activity. Betaine methyltransferase activity was not commensurately decreased. The normal activities of cystathionine synthase and cystathionase rule out the enzyme deficiences which are known alternative causes of homocystinuria (Mudd, et al., 1964; Mudd, et al., 1966) and

cystathioninuria (Frimpter, 1965; Finkelstein, et al., 1966). Together, the findings indicate the physiological importance of methylfolate-H_4 methyltransferase.

The methylmalonic aciduria (discovered postmortem) indicated a second area of abnormality, most probably a deficient activity of methylmalonyl-CoA isomerase. Since methylfolate-H_4 methyltransferase and methylmalonyl-CoA isomerase are the only two mammalian enzymes known to be dependent upon B_{12} derivatives as coenzymes, an inability to accumulate coenzymatically active derivatives of B_{12} was postulated. This postulate was confirmed by demonstration that the tissues contained very little deoxyadenosyl-B_{12}, although total B_{12} was within the control range. The normal concentration of total B_{12} in the patient's liver suggests that his defect lay not in failure to transport B_{12} into cells, but rather in an abnormal metabolism of B_{12}. The abnormality could have been a failure to transform vitamin B_{12} into coenzymatically active derivatives. A less likely alternative is excessive breakdown or loss of such derivatives. The precise location of the metabolic block and the relationship of this patient to others recently described who share one or more abnormalities with him are currently under investigation.

Future cases of the disease described here might benefit from methionine treatment and/or large doses of betaine or choline. Possibly, massive doses of vitamin B_{12} (Rosenberg, et al., 1968) or hydroxy-B_{12} will be useful. Attempts should certainly be made to circumvent the presumptive metabolic block by administration of methyl-B_{12}, deoxyadenosyl-B_{12} or another modified form of B_{12}.

Acknowledgments: We wish to thank Dr. Herbert Weissbach for several helpful discussions and for aid in setting up the methylfolate-H_4 methyltransferase assay, Dr. Hugo Moser for advice and encouragement, Drs. Robert Scheuplein and Christa Mueckenhausen, respectively, for the IR spectroscopic and gas-liquid chromatographic studies, and Mrs. P. Auld for technical assistance. This work was supported in part by a grant from the American Cancer Society and NIH Grant NB-05096. This is publication number 631 from the Graduate Department of Biochemistry, Brandeis University.

References

Abeles, R. H., Myers, C., and Smith, T. A., Analyt. Biochem. 15, 192 (1966).
Efron, M. L., in Automation in Analytical Chemistry, Skeggs, L. T., Jr., Ed. (Mediad Inc., New York, 1966) p. 637.
Finkelstein, J. D., Mudd, S. H., Irreverre, F., and Laster, L., Proc. Natl. Acad. Sci. 55, 865 (1966).
Finkelstein, J. D., and Mudd, S. H., J. Biol. Chem. 242, 873 (1967).
Fisher, R. A., Statistical Methods for Research Workers, Oliver and Boyd, Edinburgh, 1948.
Frimpter, G. W., Science 149, 1095 (1965).
Giorgio, A. J., and Plaut, G. W. E., J. Lab. and Clin. Med. 66, 667 (1965).

Hogenkamp, H. P. C., Ann. Rev. Biochem. 37, 225 (1968).
Lau, K.-S., Gottlieb, C., Wasserman, L. R., and Herbert, V., Blood 26, 202 (1965).
Lear, A. A., Harris, G. W., Castle, W. B., and Fleming, H., J. Lab. and Clin. Med. 44, 715 (1954).
Mudd, S. H., Finkelstein, J. D., Irreverre, F., and Laster, L., Science 143, 1443 (1964).
Mudd, S. H., Finkelstein, J. D., Irreverre, F., and Laster, L., J. Biol. Chem. 240, 4382 (1965).
Mudd, S. H., Laster, L., Finkelstein, J. D., and Irreverre, F., in Amines and Schizophrenia, Himwich, H. E., Kety, S. S., and Smythies, J. R., Ed. (Pergamon Press, Oxford and New York, 1966) p. 247.
Rosenberg, L. E., Lilljeqvist, A.-C., and Hsia, Y. E., Science 162, 805 (1968).
Stokstad, E. L. R., and Koch, J., Physiol. Revs. 47, 83 (1967).
Weissbach, H., and Dickerman, H., Physiol. Revs. 45, 80 (1965).
Weissbach, H., Peterkofsky, A., Redfield, B. G., and Dickerman, H., J. Biol. Chem. 238, 3318 (1963).

Proc. Soc. Exp. Biol. Med. 71: 226-227

17143. Effect of Pteroylglutamic Acid and Vitamin B_{12} on Growth of Rous Tumor Implants.

J. J. Oleson and Paul A. Little. (Introduced by Guy W. Clark.)

From the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

Earlier work from this laboratory showed that pteroylglutamic acid was essential for the growth of implants of Rous sarcoma in young chicks.[1] The effect of other dietary factors was much less pronounced.[2] A search was then made for additional dietary factors which might affect the growth of this sarcoma in chicks.

Day-old chicks in groups of 10 were fed the synthetic pteroylglutamic acid deficient diet and water *ad libitum* and were injected with tumor suspension as previously described.[2] Supplements of pteroylglutamic acid and the preparations reported here were injected intramuscularly daily at the levels indicated in the table.

It was found that 15 unit liver extract* would apparently replace pteroylglutamic acid at levels as low as 0.01 ml per day in stimulating the growth of Rous sarcoma in chicks fed this synthetic diet. It is known that parenteral liver extracts contain very little pteroylglutamic acid[3] and therefore cannot substitute for this factor in the nutrition of chicks. A preliminary study was then made of the properties of the factor in liver which was responsible for tumor stimulation.

Since 15 unit liver extract (beef) is a rich source of vitamin B_{12} and the properties of the liver factor were found to be similar to this vitamin, highly purified concentrates of vitamin B_{12} were also tested. These were found to give the same results as the liver extract itself. The preparations were previously assayed microbiologically† and by a chick growth method[4] for their vitamin B_{12} content.

Some typical results are presented in Table 1. Pteroylglutamic acid alone gives a high incidence of growing tumors of good size. The liver extract alone gives quite similar results. The effect of the liver is duplicated by dosages of vitamin B_{12} as low as 0.05 γ per day. The higher levels of this factor give larger sized tumors than the lowest levels in the 14-day test period. When pteroylglutamic acid and vitamin B_{12} are given together, the tumors come up earlier and attain greater size than with corresponding levels of either factor alone.

Thus it appears that vitamin B_{12} does not entirely replace pteroylglutamic acid in its growth promoting effect on the Rous tumor but is required in addition to give maximum tumor growth under these particular dietary conditions.

Summary. Vitamin B_{12} has been demonstrated to have a role in the growth of Rous sarcoma in chickens comparable to that previously shown for pteroylglutamic acid.

Received May 3, 1949. P.S.E.B.M., 1949, 71.

[1] Little, P. A., Sampath, A., Paganelli, V., Locke, E., and SubbaRow, Y., *Tr. N. Y. Acad. Sci.*, Series II, 1948, **10**, 91.

[2] Little, P. A., Oleson, J. J., and SubbaRow, Y., *J. Lab. and Clin. Med.*, 1948, **33**, 1139.

[3] Clark, G. W., *Am. J. Med. Sci.*, 1945, **209**, 520.

* 15 Unit liver-Lederle (prepared from beef liver).

† We thank Mr. A. C. Dornbush for these assays.

[4] Whitehill, A. R., Wieland, O., and Oleson, J. J., unpublished data.

TABLE 1.
Comparative Effects of Pteroylglutamic Acid, Liver Extract and Vitamin B_{12} on the Growth of Rous Sarcoma.

Supplements	Amt inj. daily	No. of tests	Total No. of chicks	% chicks w. tumors	Avg tumor size
None	—	6	60	3	+
Pteroylglutamic Acid	5 γ	3	23	61	+
,, ,,	10 γ	5	50	78	++
,, ,,	50 γ	6	57	88	+++
15 Unit Liver	.01 cc	1	10	70	+
15 ,, ,,	.025 cc	2	20	60	+
15 ,, ,,	.05 cc	2	20	60	++
15 ,, ,,	.10 cc	2	20	85	+++
Vit. B_{12} concentrate	≅ .05 γ	2	20	55	+
,, ,, ,,	≅ 0.1 γ	1	10	60	++
,, ,, ,,	≅ 0.5 γ	2	20	75	+++
,, ,, ,,	≅ 1.0 γ	2	19	68	+++
,, ,, ,,	≅ 2.0 γ	2	20	85	+++
Vit. B_{12} + Pteroylglutamic Acid	≅ 0.5 γ + 10 γ	1	10	90	++++
Vit. B_{12} + Pteroylglutamic Acid	≅ 2.0 γ + 10 γ	1	10	100	++++

Lab. Anim. 4:1-12

2092

Laboratory Animals (1970) 4, 1-12.

VITAMIN B_{12} DEFICIENCY IN CAPTIVE MONKEYS AND ITS EFFECT ON THE NERVOUS SYSTEM AND THE BLOOD

by

C. E. OXNARD*

W. THOMAS SMITH

and

I. TORRES†

Departments of Anatomy and Pathology,
The Medical School, University of Birmingham,
Birmingham, 15

SUMMARY

Monkeys fed vegetarian diets develop neurological and haematological abnormalities; paralysis can occur though the neural lesions (cerebral degeneration, posterior and lateral column degeneration of the spinal cord, segmental and wallerian degeneration of the peripheral nerves) are usually not evident during life. It is clearly important that the diet given to captive animals should contain an adequate amount of vitamin B_{12}, and that newly captive monkeys should not be used for research purposes unless shown to have normal serum levels of the vitamin.

The use of animals in experimental situations, particularly those involving prolonged captivity, necessitates maintenance of normal biological parameters. When animals used for any experiment are abnormal, conclusions drawn from the data obtained may be invalid. Recent work has shown that in 40 specimens of 8 genera of Old World primates fed adequate diets, the serum vitamin B_{12} levels were of the same order as is found in man (Huser & Beard, 1969); also that various biological changes, not manifest in obvious illness, may be due to a deficiency of this same vitamin in some primates maintained on vegetarian diets (reviewed by Oxnard, 1967).

Rhesus monkeys when recently captive showed amounts of vitamin B_{12} in the serum that resembled those found in man (150-600 pg/ml—Krohn, Oxnard

*Present address: Department of Anatomy, The University of Chicago, Chicago, Illinois 60637, U.S.A.

†Present address: Departamento de Morfologia, Universidad de Oriente, Ciudad Bolivar, Venezuela.

617

& Chalmers, 1963). When kept in captivity under the conditions previously existing in the primate colony at the University of Birmingham, the amount of the vitamin in the serum gradually fell to levels as low as 20 pg/ml after 2 years (Oxnard, 1964). The evidence of Wilson & Pitney (1955) in Northwestern University and of Das Gupta, Chatterjea, Ghosh & Banerjee (1955) in India, when viewed in retrospect, suggests that this phenomenon was not confined to the Birmingham colony. It seems to be associated with the vegetarian diet (supplemented with iron and with vitamins other than B_{12}) that was, until recently, commonly given to monkeys in captivity. Hypovitaminosis B_{12} probably does not occur when monkeys are given animal-food products, vitamin B_{12} supplements, or commercial pelleted diets which contain food of animal origin or vitamin B_{12} additives.

When allowed to become deficient, rhesus monkeys do not often show obvious changes; for instance, the macrocytic anaemias that occur in man do not appear, individual animals sometimes have greater amounts of haemoglobin, higher red cell counts and higher serum iron levels than are found in recently captive animals, and obvious macrocytosis has not been noted (Oxnard & Smith, 1966). Anaemias that have been found from time to time in isolated animals are microcytic and hypochromic. Macrocytic anaemias reported in rhesus monkeys have been shown to respond to folic acid (and sometimes ascorbic acid) rather than to treatment with vitamin B_{12} (reviewed by Smith, 1965).

Neuropathies are also well known in man with vitamin B_{12} deficiency associated with pernicious anaemia, in malabsorption syndromes (Cooke & Smith, 1966), and in true vegetarians; subacute combined degeneration of the cord may be the sole manifestation of the deficiency.

Similarly, in a few monkeys that have been maintained on vegetarian diets and have been found to have low levels of the vitamin in the serum, paralysis of the hindlimbs and tail can occur. A continued finding over many years has been the association of some cases of 'cage paralysis' with demyelination of the posterior (mainly) and lateral columns of the cord (reviewed by Innes & Saunders, 1962). Recent evidence indicates that hypovitaminosis B_{12} is a causative factor in certain cases of cage paralysis, and that widespread occult histological lesions also occur in a high percentage of non-paralysed animals (Oxnard & Smith, 1966). These occult lesions include: spongiform degeneration of the posterior and lateral columns of the spinal cord; patchy demyelination of peripheral nerves of the hindlimb; and, occasionally, spongiform degeneration of the cerebral white matter and demyelination of the optic nerves. In some monkeys, lesions were found after less than 8 months in captivity on a vegetarian diet; the earliest onset of paralysis was after 14 months.

Lesions of the spinal cord and brain accompanied by loss of axons are

presumably irreversible. Recent evidence suggests, however, that when the lesions involve the peripheral nerves treatment can result in functional improvement, paralyses in affected monkeys becoming less severe (Oxnard & Smith, 1966). In non-paralysed monkeys segmental remyelination of peripheral nerves has been observed following administration of vitamin B_{12} (Oxnard, Smith & Torres, 1967), though in the present paper we shall show that such remyelination is also found in animals with untreated vitamin B_{12} deficiency.

The purpose of this present paper is to assess in some further detail the extent and nature of the neurological lesions in a large number of animals with levels of vitamin B_{12} that are low compared with those of treated and recently captive animals, and also to assess whether or not clinical neurological and haematological examination can reveal prior signs of the condition.

MATERIALS AND METHODS

Neuropathological investigation

Some 43 monkeys in the Birmingham colony were studied: there were 40 rhesus monkeys (*Macaca mulatta*), 2 patas monkeys (*Erythrocebus patas*) and 1 baboon (*Papio anubis*). Of these, 12 (including the baboon and 1 patas monkey) had been maintained on wholly vegetarian diets for periods varying from 11 months to 10 years, (Group I); 14 monkeys (including the other patas monkey) kept in captivity from 11 months to more than 18 years, and originally given vegetarian diets, were later given a series of injections of vitamin B_{12} followed by a normal diet (Group II); and 17 recently captive monkeys had been given a normal diet since arriving in the colony (Group III). Of the Group III monkeys, 14 had been in captivity for less than 21 days, and the other 3 for 6, 7 and 14 months. In Group I, 10 animals had low serum vitamin B_{12} levels (<100 pg/ml); in Group II, 13 animals had high serum levels of the vitamin (500 pg/ml); and in Group III the serum levels were normal except in 2 estimations made in pregnancy, which is frequently associated with reduced levels (Oxnard, 1964). 5 animals in Groups I and II were paralysed; all of the others were clinically normal.

The brains, spinal cords and peripheral nerves were examined in all cases by routine neuropathological techniques. In 29 animals (10 from Group I, 10 from Group II and 9 from Group III) single nerve fibres from the proximal sciatic, proximal tibial and common peroneal, and distal tibial nerves were studied after fixing individual nerves in 4 per cent formaldehyde in saline and staining with 1 per cent osmium tetroxide: the fibres were then teased apart in glycerine under a dissecting microscope.

Clinical examination of the nervous system

Neurological examination presented considerable difficulties and could not be carried out on the full range of animals. Full clinical status was assessed in 9 animals of Group I, 7 animals of Group II, and 5 animals of Group III, and this included observations of the behaviour of the animals while in their cages, and examinations out of the cage. In 22 animals it was only possible to observe the animals from outside their cages. During observation of the monkeys, special attention was paid to the coordination, precision and strength of their spontaneous movements and alterations in their normal behaviour. The examination included a search for trophic changes and the testing of passive movements, muscle power and tendon reflexes. Tests for more subtle reflexes, such as the placing reaction, together with electrophysiological examination could not be carried out. The recording of objective phenomena was somewhat problematical and it is likely that only the most gross signs were recognised.

Haematological investigation

Detailed haematological examinations were carried out on 2 further groups of monkeys. The first consisted of 25 recently captive animals that had normal levels of vitamin B_{12} in the serum (Group IV); the second consisted of 15 rhesus monkeys that had been maintained in the colony on a vegetarian diet for longer than 2 years, and of which all save 1 had levels of the vitamin below 60 pg/ml (Group V). The specimen of serum in the exceptional case was heavily haemolysed so that the level of the vitamin was not known.

The 15 deficient rhesus monkeys (Group V) were given, at intervals of about 10 days, intramuscular injections of 1 000 μg of vitamin B_{12}. Serum determinations after the injections showed that the animals did indeed receive the vitamin. Repetition of treatment was carried out because previous experience showed that in severely deficient animals serum levels fall rapidly after single injections of the vitamin. Repeated assays confirmed that such a regimen was sufficient to maintain high serum levels in all animals.

Blood was drawn from the short saphenous vein with minimal stasis and, for haematological examination, anticoagulated with EDTA.

The following haematological values were estimated using the methods given by Dacie & Lewis (1963).

Haemoglobin (Hb): the oxy-haemoglobin method was used with a colorimeter (Evans Electroselenium Ltd, Harlow, Essex) which was standardised daily with a neutral gray screen of 0.475 density (Ilford Ltd, 4 New Oxford Street, London, W.C.1.).

Haematocrit (PCV): the Hawksley micro-haematocrit was used with the usual precautions. The capillary tubes were centrifuged for 5 minutes and no allowance was made for 'trapped plasma'.

Red cell count (RBC): erythrocytes were counted on an electronic cell counter (Model A, Coulter Electronics Ltd, High Street South, Dunstable, Bedfordshire) using the 'threshold value' for the machine estimated from rhesus monkey red cells suspended in an albumin-saline solution. As many thousands of cells are counted in a few minutes by electronic counters, the accuracy achieved is greater than with manual methods. Thus, the derived values (e.g. MCH) are of greater significance.

Derived values: mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), and mean corpuscular haemoglobin (MCH) were calculated from the usual formulae.

All the above values were estimated at least twice as a check on the accuracy of the techniques.

Details of diets

Vegetarian diet

This consisted of steamed potatoes, carrots and other raw vegetables, fresh fruit, plus a slice or two of white bread covered with 'Bemax' (Bemax Ltd, 23 Upper Mall, London, W.6)—which contains vitamins B and E but not B_{12}—as well as supplements of vitamins A, C and D. The diet for adults is, therefore, exclusively vegetable; small infants received additional milk. The total daily consumption was about 500-600 g (700-800 cal, 2 900-3 300 J) and the various items per adult animal per day on this regime were estimated to be as follows (Eckstein & Kelly, 1966):

carbohydrate—30 per cent of diet—170 g (680 cal, 2 850 J)

protein: total—3.5 per cent of diet—20 g (80 cal, 330 J)
animal—0 per cent of diet
vegetable—100 per cent of total protein

fat—trace

vitamins: B complex (added as 'Bemax')
B_{12} ? trace
E—2 mg (128 mg/lb 'Bemax')

iron—occasional supplement

ash—2 per cent

The actual diet consumed probably varied somewhat. On days when root vegetables were not adequately cleaned, vitamin B_{12} may have been imbibed from that in adherent soil or root hairs.

Normal diet

The adult animals received pelletted diet ('Oxoid' 41B, 'Oxoid' Ltd, Southwark Bridge Road, London, S.E.1), together with fresh occasional fruit

and vegetables, about 200 g per day (500 cal, 2100J); the various items per adult animal per day were estimated to be as follows:

carbohydrate—49 per cent of diet—90 g (350 cal, 1 470 J)

protein: total—13.5 per cent of diet—25 g (100 cal, 420 J)

animal—40 per cent of of total protein

vegetable—60 per cent of total protein

fat—3.5 per cent of diet—6.5 g (55 cal, 230 J)

vitamins: B complex present

B_{12} about 0.5 μg

E about 0.15 mg

iron—occasional supplements

ash—3 per cent

This diet thus contains less vitamin B complex and less vitamin E than vitamin B_{12}-deficient diet. Again it must be emphasised that the quantity consumed was variable, so that the estimations can only be regarded as approximate.

RESULTS

Neuropathological investigation

Histological sections

Groups I and II (26 animals): 23 animals (including all 5 paralysed animals) showed degeneration of the posterior and lateral columns of the spinal cord similar to that previously described by Oxnard & Smith (1966), that closely resembled human subacute combined degeneration resulting from vitamin B_{12} deficiency. Such degeneration was exceptionally severe in 3 of the 5 paralysed animals, in contrast to the mild to moderate severity found in all 18 of the 21 non-paralysed animals showing cord changes. The posterior nerve roots showed demyelination, or the posterior root ganglia showed secondary neuronal degeneration (chromatolysis, neuronophagia), or both, in 9 of 24 animals in which they were examined. The peripheral nerves showed demyelination in frozen sections in 21 of the 26 animals, being most marked in 3 paralysed and 3 non-paralysed animals. The cerebral white matter showed spongiform degeneration in 3 of the 5 paralysed animals and in 2 of the 21 non-paralysed animals (Table 1). In Group II, 3 non-paralysed animals did not show spinal cord degeneration; the posterior nerve roots showed degeneration in 1 of these, the peripheral nerves showed degeneration in another, and the posterior roots and peripheral nerves were normal in the 3rd.

2092

Group III (17 animals): 1 animal showed slight degeneration of the cord, 2 showed slight degeneration of the peripheral nerves and 1 showed slight degeneration of the cord and posterior roots and ganglia, and moderate peripheral nerve degeneration (Table 1).

Table 1. Incidence of neurohistological lesions in the monkeys studied.

Group	*Number of animals*	*Number paralysed*	*Normal*	*Abnormal*							
				peripheral nerve lesions		*spinal cord lesions*		*posterior root &/or ganglion lesions*		*cerebral lesions*	
				*NP**	*P*	*NP*	*P*	*NP*	*P*	*NP*	*P*
I deficient	12	1	0	9	1	11	1	2	1	2	1
II deficient, then treated	14	4	2	7	4	7	4	3	3	2	0
III recently captive controls	17	0	13	3	0	2	0	1	0	0	0

*NP** non-paralysed; *P* paralysed; the posterior roots and/or ganglia were not examined in 2 cases in Group I, neither was the brain-stem in 1 case in Group II.

Teased preparations of peripheral nerves

These results were mentioned briefly and illustrated by Torres, Smith & Oxnard (1969).

Groups I and II—teased preparations were examined from 20 of the 26 animals: 15 animals showed lesions. Segmental demyelination was seen in every affected case, in 13 cases it was the most pronounced abnormality and was always associated with clear evidence of segmental remyelination. Wallerian (axonal) degeneration was present in only 4 affected animals, in 3 of which there was evidence of axonal regeneration and remyelination. In 2 animals the wallerian degeneration was more pronounced than the segmental demyelination.

Group III—nerve fibres from 9 of the 17 animals were teased: 3 animals showed segmental demyelination and remyelination, and in 1 of these there was limited evidence of wallerian degeneration. The 3 animals with segmental demyelination were the same 3 animals that showed degeneration in routine histological sections of peripheral nerves.

There was no evidence in any of the 3 groups that the lesions were more pronounced in the distal parts of the nerves; the distribution pattern suggested random involvement.

623

Examination in life

The animals of Group III appeared healthy, with brisk, easily elicited reflexes. Those in Groups I and II, when not paralysed, did not show trophic lesions and also appeared healthy. But in these groups, though one must interpret such a finding with caution, it did seem that reflexes in the hindlimbs were reduced, difficult to elicit, or even absent. It was also an impression that these captive rhesus monkeys were more aggressive and morose; however this remains to be tested by some objective method.

The 5 animals with chronic paralysis showed, in general, considerable flexion deformity, trophic changes on the heels and toes, and loss of hair and skin on the tail. These lesions appeared to be painless; however, hyperaesthesia seemed to be caused in some animals by stroking or rubbing the digits or sole, and by pressure on the muscles and tendons of the leg. The worst of these cases presented complete paralysis; the less affected showed impaired mobility so that they could not climb, or climbed with difficulty. A typical posture for these animals was to sit with the toes curled and with only the lateral aspect of the foot resting lightly on the bars or wires of the cages. There seemed to be considerable resistance to bearing the body weight on the soles of the feet. The reflexes were reduced or absent.

The 2 patas monkeys both developed their first attack of paralysis within a few months of captivity, and were consequently examined frequently during the course of the disease. As there were 4 other patas monkeys also available in the colony, there was good material for comparative observations.

First the affected patas monkeys merely appeared unwell and with reduced mobility. Gradually over subsequent days they showed a marked disinclination or inability to jump up to their perches, and sat for considerable periods of time with the limbs held inverted so the plantar surfaces did not touch the substrate. Touching or stroking the feet elicited withdrawal and signs of discomfort from the animals—this was worse on one side. In 1 animal the hand showed a tremor when holding a nut or when held in a sustained posture against gravity. Clinical examination of the 2 animals showed extreme hyperreflexia in the hindlimbs, greater on the more affected side. The forelimbs appeared to show normally brisk reflexes. 1 of the animals became very much worse and was killed. The other was treated and showed definite improvement in motor activities. Over the succeeding days the reflexes gradually became normally brisk. Treatment with parenteral cyanocobalamin was not maintained, though the animal was put on a pellet diet which included animal products and presumably, therefore, vitamin B_{12}. Certainly the serum level of the vitamin was maintained. 7 months later the animal again became unsteady; at this time it showed a picture more like that of the other paralysed animals.

Haematological investigation

The comparison of 25 recently captive monkeys (Group IV) with 15 that had been maintained on deficient diets for more than 2 years (Group V), confirmed that for haemoglobin and for haematocrit there were no significant differences at the probability level of 1 in 20 (Table 2). But the RBC was significantly lower in the deficient animals as compared with the recently captive animals (P=0.05-0.02). Though the MCH and MCHC did not differ significantly between the 2 groups, the MCV was significantly lower in the recently captive (P=0.05-0.02; the means differed by 5.4 μm^3).

Table 2. Haematological findings on recently captive monkeys (Group IV), compared with those maintained for more than 2 years on a vegetarian diet both before (Group V) and after (Group V+) treatment with vitamin B_{12}.

Means ± S.E.

	Group IV	*Group V*	*Group V+*	*Significant differences* between:*
Hb (per cent)	85.1±7.9	82.8±5.8	89.2±7.6	V and V+
PCV (per cent)	42.7±3.4	41.0±2.4	43.0±3.1	V and V+
RBC (millions/mm^3)	4.93±0.16	4.65±0.5	5.1±0.5	IV and V; V and V+
MCHC (per cent)	29.0±3.2	29.1±2.1	30.1±1.6	...
MCV (pm)	86.7±9.4	92.1±10.4	86.1±5.9	IV and V; V and V+
MCH (pg)	25.3±2.9	26.3±2.9	26.1±2.8	...

*Differences significant at the 5 per cent level or less. Differences between IV and V+ not significant.

Table 2 also shows measurements made on the 15 Group V monkeys approximately 6 weeks after treatment with B_{12}. Here all parameters save the MCH and MCHC were significantly different. The haemoglobin was raised by 6.4 per cent (P= 0.01) and the PCV by 2 per cent (P 0.05-0.02). The RBC was raised by nearly half a million cells per mm^3 (P 0.01-0.001) and the MCV was correspondingly reduced by 6 μm^3 from 92 to 86 (P=0.05-0.02).

DISCUSSION

Groups I and II were considered together because both consisted of long-stay animals that had been fed vegetarian diets for long enough to have induced neurological lesions; Oxnard & Smith (1966) detected early histological changes after 8 months and clinical paralysis after 14 months. Group II

was originally distinguished from Group I because all animals in Group II had been given vitamin B_{12} injections followed by a normal diet for periods varying from 6 months to 4 years before death, in an attempt to assess the effect of replacement therapy. We have not yet found histological evidence of reparative changes attributable to such therapy in either central or peripheral nervous systems. The evidence of functional improvement previously noted (Oxnard & Smith, 1966) probably resulted from recovery of temporarily suppressed nerve function, after therapy had arrested the degeneration due to deficiency. In the present series, active sudanophilic degeneration of myelin was still seen in the cord of an animal that had received a normal diet continuously for 35 months after 43 months on a deficient diet; and in another animal paralysis recurred after 6 months of treatment.

The minor changes found in 4 of the animals in Group III can be explained in several ways. 2 of these animals were pregnant and had very low levels, and 1 non-pregnant animal had a low-normal serum-vitamin B_{12} level, after only 10, 13 and 19 days respectively on a non-vegetarian diet (the vitamin B_{12} level was not estimated in the 4th animal). This suggests that low levels due to pregnancy or a previously inadequate diet may have already affected the nervous system before the animals arrived in the Birmingham colony. Advancing age and peripheral nerve trauma can also result in minor peripheral nerve lesions similar to those shown in 3 of 4 of these animals, but unfortunately both the age and the previous history of the animals prior to their arrival in the colony was unknown. These findings should be borne in mind when recently captive monkeys are used for neurological research, particularly when the peripheral nerves are under investigation.

Unless the animals developed overt paralysis, there was no evidence during life of the lesions found on histological examination. The hyporeflexia that was found in most of the animals in Groups I and II was only evident when recently captive animals were available for comparison, and even then the finding must be treated with caution. It seems likely that tests for more subtle reflexes such as the placing reaction, or investigation of motor nerve conduction velocities, might provide prior evidence of the disease process.

Routine examination of blood films, Hb, PCV, MCHC and MCV is probably of little value in the diagnosis of simian vitamin B_{12} deficiency. Oxnard & Smith (1966) found no significant differences between deficient animals and recently captive animals. The results reported here seem to be a little at variance in that the RBC and MCV now differed significantly. However, this can perhaps be attributed in part to somewhat larger samples and perhaps also to the increased sensitivity of the RBC as determined with the Coulter counter (previous determinations were done by hand). In fact, in the previous work the RBC and MCV were just not significantly different at the 1 in 20 level, and in the present study they just attained that level of significance.

The results obtained from the longitudinal haematological investigation of the same animals before and after treatment show that although in most parameters a statistically significant improvement did occur, the changes were small enough to pass undetected in the routine examination of the animals. Certainly the somewhat larger size of the red blood cells in the animals before treatment (i.e. when deficient) could not be labelled 'macrocytosis'. As in the previous studies qualitative examination of blood films did not reveal a single case that was consistent with a macrocytic anaemia. Nevertheless the improvement on treatment was sufficient to produce mean values resembling those of the more normal recently captive animals.

Recently Huser & Beard (1969) in a small series of 5 rhesus monkeys, which had received diets adequate in vitamin B_{12} for at least 2 years, found haematological parameters which showed even better figures than were found here after treatment. Thus, for instance, their mean RBC was 5.5 millions (range 5.2-5.9) and their mean MCV was as low as 77 μm^3. This suggests the possibility that even more marked improvements might have been found in the animals in this study had the experiments been carried further. It also suggests that the animals accepted as normal in this study (either because they had been recently imported and therefore had not had time to develop the vitamin B_{12} deficiency on the vegetarian diet, or because they were receiving supplements) were still not in an optimal nutritional state.

It is clear, then, that unless captive monkeys are fed diets containing natural vitamin B_{12} or are given supplements of the vitamin, many of them will develop neural lesions. The progression of such lesions may be arrested by treatment, though the restoration of disordered function that ensues is likely to be due to cessation of degeneration and release of inhibition, rather than to remyelination and regeneration. Evidence of remyelination after segmental demyelination, and regeneration after wallerian (axonal) degeneration, was found in the peripheral nerves in animals with untreated vitamin B_{12} deficiency, a state of affairs not unlike that in experimental lead poisoning (Fullerton, 1966); treatment did not appear to influence the degree of segmental remyelination and axonal regeneration.

Little is known about the long-term effects of vitamin B_{12} deficiency on the growth and weight of monkeys. Flinn & Oxnard (1966) found there were significant increases in the growth of infant monkeys studied for about 2 years after post-weaning treatment with vitamin B_{12}, as compared with the growth patterns in untreated monkeys. But this was a retrospective study based on body weights previously recorded, and could not be correlated with other desirable data such as radiological studies.

It is possible that other nutritional factors were also deficient in the monkeys studied, though the similarity of the lesions to those of human 'subacute combined degeneration', and the functional improvement previously reported after

treatment with vitamin B_{12} (Oxnard & Smith, 1966) suggest that deficiency of this vitamin was the most important factor. The practical point that emerges is that vegetarian-fed monkeys should not be used for neurological or psychological research, in view of the high incidence of neural lesions found in this investigation. In addition, recently captive animals may be suspect unless serum assays confirm their normal nutritional status for the vitamin.

ACKNOWLEDGEMENTS

We are indebted to the Agricultural Research Council of Great Britain (grant no. 40319 to C. E. Oxnard during the tenure of his appointment in the Department of Anatomy, University of Birmingham) and to the Ministerio de Sanidad y Asistencia Social through the Universidad de Oriente, Venezuela (Research Fellowship to I. Torres) for generous financial support.

REFERENCES

Cooke, W. T. & Smith, W. T. (1966). Neurological disorders associated with adult coeliac diseases. *Brain* **89**, 683.

Dacie, J. V. & Lewis, S. M. (1963). *Practical haematology*. London: Churchill.

Das Gupta, C. R., Chatterjea, J. B., Ghosh, S. K. & Banerjee, D. K. (1955). Vitamin B_{12} concentration of serum in monkeys. *Bulletin of the Calcutta School of Tropical Medicine* **3**, 148.

Eckstein, P. & Kelly, A. W. (1966). A survey of the breeding performance of rhesus monkeys in the laboratory. In *Some recent developments in comparative medicine. Symposia of the Zoological Society of London* **17**.

Flinn, R. M. & Oxnard, C. E. (1966). The relationship between growth and the administration of cyanocobalamin in the rhesus monkey. *Folia Primatologica* **4**, 432.

Fullerton, P. M. (1966). Chronic peripheral neuropathy produced by lead poisoning in guinea-pigs. *Journal of Neuropathology and Experimental Neurology* **25**, 214.

Huser, H. J. & Beard, M. E. J. (1969). Studies on folate and vitamin B_{12} metabolism in primates. I. Blood and bone marrow morphology, folate and vitamin B_{12} levels. *Folia Primatologica* **10**, 172.

Innes, J. R. M. & Saunders, L. Z. (1962). *Comparative neuropathology*. New York & London: Academic Press.

Krohn, P. L., Oxnard, C. E. & Chalmers, J. N. M. (1963). Vitamin B_{12} in the serum of the rhesus monkey. *Nature, London* **197**, 186.

Oxnard, C. E. (1964). Some variations in the amount of vitamin B_{12} in the serum of the rhesus monkey. *Nature, London* **201**, 1188.

Oxnard, C. E. (1967). Some occult lesions in captive primates. *American Journal of Physical Anthropology* **26**, 93.

Oxnard, C. E. & Smith, W. T. (1966). Neurological degeneration and reduced serum vitamin B_{12} levels in captive monkeys. *Nature, London* **210**, 507.

Oxnard, C. E., Smith, W. T. & Torres N., I. (1967). Observaciones recientes acerca de la deficiencia de vitamina B_{12} degeneracion neurologica en primates cautivos. *Acta Neurologica Latinoamericana* **13**, 1.

Smith, E. L. (1965). *Vitamin* B_{12}. London: Methuen.

Torres N., I., Smith, W. T. & Oxnard, C. E. (1969). Degeneration of the peripheral and central nervous system in vitamin-B_{12}-deficient monkeys. *Experientia* **25**, 273.

Wilson, H. E. & Pitney, W. R. (1955). Serum concentrations of vitamin B_{12} in normal and nutritionally deficient monkeys. *Journal of Laboratory and Clinical Medicine* **45**, 590.

Acta. Med. Scand. 180(5): 561-564

2093

Acta Medica Scandinavica. Vol. 180, fasc. 5, 1966

From the Central Laboratory (Head: P. Paaby, M. D.), Aalborg County Hospital, Aalborg, Danmark, and the Skørping Sanatorium (Head: E. Strandgaard, M. D.), Skørping, Danmark

The Absorption of Vitamin B_{12} During Treatment with Para-aminosalicylic Acid

By

P. Paaby and E. Norvin

It has been reported, that treatment with Para-aminosalicylic acid (PAS) can be the cause of various haematological complications including megaloblastic anaemia, and in 1964 Heinivaara and Palva (3) published a report on the effect of PAS-treatment on the Schilling test.

They reported a significant fall in the Schilling-test values, with or without intrinsic factor, within a few weeks after the institution of PAS-treatment; the values returning to pretreatment levels a few weeks after discontinuation of the medication. The most striking feature of this publication was, that all of 10 cases responded so definitely to the PAS-treatment with rapid falls in Schilling-values.

We have tried to evaluate the influence of PAS-treatment upon the Schilling-test and various other parameters in a series of 26 patients.

Methods

Schilling test (7). Normal values 11—35 % (1). Each test dose of vitamin-B_{12}-CO^{58} contained 1 μg vitamin-B_{12}, and the flushing dose was 1,000 μg. The amount of intrinsic factor was 100 mg freeze dried hog pyloric mucosa (Biofac). Analysis of the concentration of vitamin-B_{12} in serum (serum-B_{12}) was carried out by the Central Laboratory, Central Hospital, Randers, by the method of E. Hoff-Jørgensen (4). Normal values 150—800 pg/ml. (All other analyses were performed in the Central Laboratory, Aalborg County Hospital.) Concentration of iron and transferrin in serum by Ramsay's (6) method. Normal values: serum iron: 46—154 μg%, serum-transferrin 245—405 μg% (5). Haemoglobin was determined as cyanmethaemoglobin (2). Normal values: ♂ 14—17 g%, ♀ 12—15 g% (5). Haematocrit value was determined by Wintrobe's method and erythrocytes counted mechanically on the Celloscope.

Mean cell volume (MCV) was calculated as

$$MCV = \frac{\text{Haematocrit} \times 10}{\text{red cell count mill}/\mu l}$$

Normal range: 86—107 mpl (5).

Mean cell haemoglobin concentration (MCHC) was calculated as

$$MCH = \frac{\text{Haemoglobin g\%} \times 100}{\text{Haematocrit}}$$

Normal range: 32—36 g/100 ml (5).

Submitted for publication April 13, 1966.

629

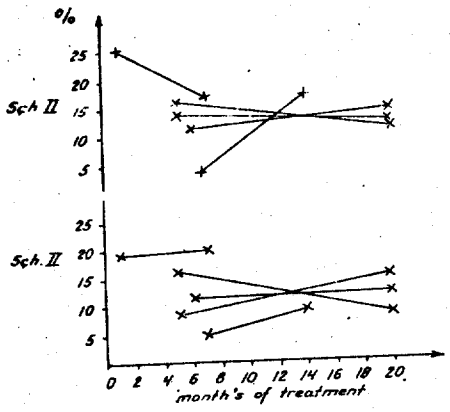


Fig. 1. Schilling values without (Sch. I) and with (Sch. II) intrinsic factor in five patients examined twice during treatment with para-aminosalicylic acid (PAS).

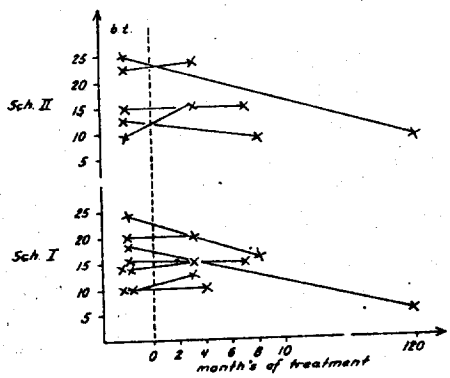


Fig. 2. Schilling values before (b.t.) and during treatment with PAS.

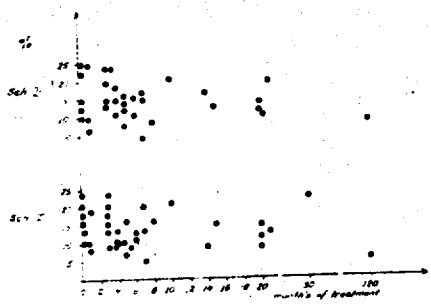


Fig. 3. Schilling values in 26 patients during treatment with PAS.

Patient material and treatment

Twenty-six patients. Fourteen were ♂, 25—71 years of age. Twenty-two were ♀, 28—78 years of age. Five ♂ and 7 ♀ were investigated twice. Two ♂ and 0 ♀ received 3 g phenyl-PAS + 1 g PAS 3 times daily. No ♂ and 4 ♀ received 4 g phenyl-PAS 3 times daily. Twelve ♂ and 8 ♀ received 9 g Ca-PAS once daily.

Results

No differences could be found between patients treated with Ca-PAS and those treated with Phenyl-PAS or Phenyl-PAS + PAS. Statistically they are therefore treated as belonging to the same group.

Fig. 1 shows the relationship between duration of treatment and Schilling-test without intrinsic factor (Sch. I) and with intrinsic factor (Sch. II) in five patients examined twice. No statistical difference could be shown between the groups of patients examined 1st and 2nd time.

Fig. 2 indicates changes in Sch. I (7 patients) and II (5 patients) before and after commencement of treatment. (One patient, who had been treated during 120 months was first examined immediately before cessation of treatment and then again after 5 months without PAS.)

No statistical difference in Schilling values could be shown between the groups of untreated and treated patients, but it may be noted that the two patients whose Schilling values dropped were those who had had the longest periods of treatment (8 and 120 months respectively).

Fig. 3 shows Sch. I and II in all the patients investigated. Regression anal-

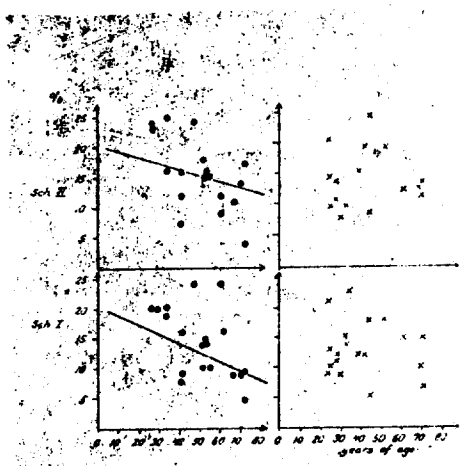


Fig. 4. Relationship between age and Schilling values. Females = ●, males = ×.

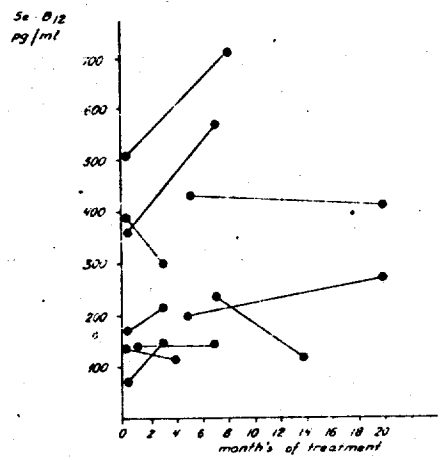


Fig. 5. Serum concentration of vitamin-B_{12} (Se-B_{12}, pg/ml) in 10 patients examined twice during treatment with PAS.

ysis was carried out. (When a patient had been examined twice, only the last set of results was used in the calculation.)

No relationship between Schilling values and duration of treatment could be shown. Many results were below the normal range, and there was a feeling that older women predominated among patients with low results.

Fig. 4 shows the regression lines calculated for relationships between age and Schilling-values in female patients.

The regression coefficients were not significant. There was no relation between Schilling values and age of the male patients, but fig. 4 indicates the critical point of the Schilling-test in routine work; the collection of 24-hour urine specimens.

Fig. 5 shows serum-B_{12} in 10 patients examined twice during treatment, and fig. 6 shows serum-B_{12} values in all the

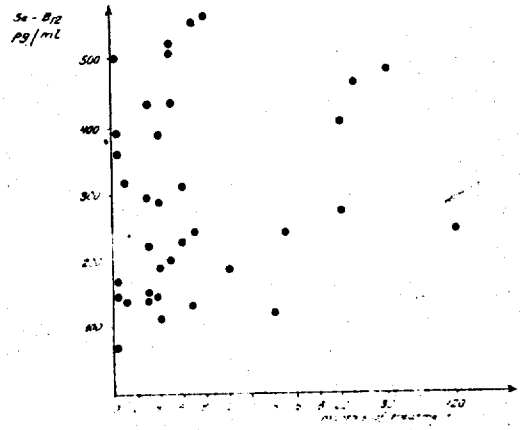


Fig. 6. Vitamin B_{12} in serum (pg/ml) versus duration of treatment with PAS.

patients. There was no relationship (regression analysis) between serum-B_{12} values and the duration of treatment.

The statistician also tested the possibility of relationship between serum-B_{12} and Sch. I and II, duration of treatment and Hb, MCV, MCHC, serum iron, transferrin, but none of them were significant.

He found, though, a strongly significant relationship between Sch. I and Sch. II. This in itself is quite trivial, but from an analytical point of view it is satisfactory. When, as in this material, the administration of intrinsic factor is unnecessary, Sch. I and Sch. II should give identical results.

If not, the technical work is bad.

Since the patient material consists of seriously ill persons (TB) it is not surprising that so many of the results fall outside the normal ranges.

Summary

In 26 patients treated with paraaminosalicylic acid the possibility of relationship between duration of treatment and Schillingtest values, concentration of vitamin-B_{12} in serum, concentration of iron and transferrin in serum, concentration of haemoglobin in whole blood, mean cell volume, and mean cell haemoglobin concentration were tested.

No such relationships could be proved, and the results of Heinivaara and Palva (3) could not be corroborated.

References

1. Bierring, E. & Ebbesen, I.: Ugeskr. Laeg. *122:* 277, 1960.
2. Crosby, W. H., Munn, J. I. & Furth, F. W.: U.S. Armed Forces M. J. *5:* 693, 1954.
3. Heinivaara, O. & Palva, I. P.: Acta med. scand. *175:* 469, 1964.
4. Hoff-Jørgensen, E. & Glick, D.: Meth. Biochem. Anal. *1:* 105, 1954.
5. Paaby, P.: Personal investigation.
6. Ramsay, V. N. M.: Clin. chim. Acta *2:* 214, 1957.
7. Schilling, R. F.: J. Lab. Clin. Med. *42:* 860, 1953.
8. Wintrobe, M. M.: Clinical haematology. 4th. ed. p. 366. Harry Kimpton, London 1957.

Brit. J. Haematol. 20(3): 299-305

2134

British Journal of Haematology, 1971, 20, 299.

Absorption of Vitamin B_{12} in the Guinea-Pig

III. THE FORMS OF VITAMIN B_{12} IN ILEAL MUCOSA AND PORTAL PLASMA IN THE FASTING STATE AND DURING ABSORPTION OF CYANOCOBALAMIN

T. J. Peters, J. C. Linnell, D. M. Matthews and A. V. Hoffbrand

Medical Research Council Malabsorption Research Group, Department of Medicine and Department of Haematology, Royal Postgraduate Medical School, and Department of Experimental Chemical Pathology, Westminster Medical School

(*Received* 20 *August* 1970; *accepted for publication* 10 *September* 1970)

Summary. The forms of vitamin B_{12} in guinea-pig ileum, liver and portal plasma were studied using a chromatographic-bioautographic technique. 5′ Deoxyadenosylcobalamin was the principal cobalamin in each tissue although significant amounts of methylcobalamin were detected in plasma. An unidentified cobalamin was also found in all three tissues.

During the absorption of labelled cyanocobalamin a proportion of this analogue was converted to 5′ deoxyadenosylcobalamin in the ileum; preliminary experiments suggest that the site of inter-conversion was the mitochondria of the ileal epithelial cells. A significant proportion of the cyanocobalamin was transferred to the portal plasma unchanged however, indicating that the mitochondrial localization of vitamin B_{12} during its absorption, shown in previous studies, is not solely related to the conversion of vitamin B_{12} analogues from one form to another.

Previous studies suggest that the mitochondria of the ileal epithelial cells play a fundamental role in the absorption of vitamin B_{12} by the guinea-pig (Peters & Hoffbrand, 1970). These studies showed that cyanocobalamin enters the mitochondria during its transport through the cell and that it remains in these organelles for approximately 1 hr before the absorbed vitamin B_{12} enters portal plasma. Two physiological vitamin B_{12} analogues, methylcobalamin and 5′ deoxyadenosylcobalamin (adenosylcobalamin), have also been shown to localize to the ileal mitochondria during absorption (Peters *et al*, 1971). The explanation for this localization is unknown but one possibility is that the ileal mitochondria are the site of interconversion of different vitamin B_{12} analogues during their absorption.

The present study was undertaken to determine whether cyanocobalamin is converted to other vitamin B_{12} analogues during absorption and, if so, the intracellular site of this conversion. In addition data is presented on the endogenous forms of vitamin B_{12} in guinea-pig plasma, small intestine and liver. A preliminary communication of this work has appeared (Hoffbrand *et al*, 1970).

METHODS

Analytic procedures. The total vitamin B_{12} content of the different tissues was assayed

633

microbiologically using the 'z' strain of *Euglena gracilis* (Anderson, 1965). The individual cobalamins were separated and quantitated by chromatography and bioautography (Linnell *et al*, 1969b, c). The cobalamins were extracted from the tissue samples with hot aqueous ethanol (80% v/v) for 20 min at 80°C, separated by thin-layer chromatography and detected bioautographically using a vitamin B_{12}-dependent strain of *Escherichia coli* with a growth indicator dye. The crimson stained zones on the bioautogram were quantitated by densitometric scanning. In experiments using labelled vitamin B_{12}, the crimson zones were excised from the bioautogram and transferred, together with the underlying areas of chromatographic absorbent, to a series of small, stoppered tubes. Radioactivity was measured in a gamma scintillation counter and the radioactivity of each analogue expressed as cpm/mg protein or in the case of plasma samples, cpm/ml plasma.

Protein was estimated by the method of Lowry *et al* (1951) using bovine serum albumin (Armour) as standard.

Experimental procedure. Fasted male guinea-pigs (Hartley strain) weighing between 350 and 450 g were fed approximately 10 ng of high specific activity ^{57}Co-labelled cyanocobalamin (80–120 μCi/μg, Radio Chemical Centre, Amersham). The animals were killed by a blow on the head at ½, 1, 2, 3, 4 and 24 hr post-feeding. The ileal mucosa was homogenized in 10 vols ice-cold 0.15 M NaCl. Ileal mitochondria were also prepared by the method of Parsons *et al* (1966). Aliquots of the homogenate and mitochondria were stored at −20°C until assay. The experimental procedures and cobalamin extractions were all performed in a dark room using a red safelight and the tissue and plasma samples were kept in foil-wrapped containers.

Operative procedure. Two fasted male guinea-pigs underwent splenectomy and had fine polythene cannulae inserted into their portal veins. They were allowed to recover overnight. Two ml samples of portal venous blood were removed before, and up to 8 hr after, the intragastric administration of 10 ng ^{57}Co-labelled cyanocobalamin. The plasma was separated and stored in foil-wrapped heparinized containers at −20°C until assay and chromatography of the cobalamins. The entire experiment was performed in a dark room using a safelight. Full details of the operative technique are described elsewhere (Peters & MacMahon, 1970).

RESULTS

Forms of Vitamin B_{12} in the Fasting Guinea-Pig

Fig 1 compares chromatograms of the cobalamins extracted from ileal mucosa, ileal mitochondria and portal plasma with those of standard markers. Table I shows the total vitamin B_{12} concentration and the concentration of the different cobalamins in plasma, liver, ileal mucosa and ileal mitochondria. Adenosylcobalamin was the principal form detected in ileum, liver and plasma. Only trace amounts of methylcobalamin were found in liver and ileum but larger amounts of this analogue (approximately 6%) were detected in plasma. In addition considerable amounts of an unidentified cobalamin were detected in all tissues studied.

Forms of Labelled Vitamin B_{12} in Ileum during Absorption of Labelled Cyanocobalamin

Fig 2 shows the specific activity of the different vitamin B_{12} analogues in whole ileal

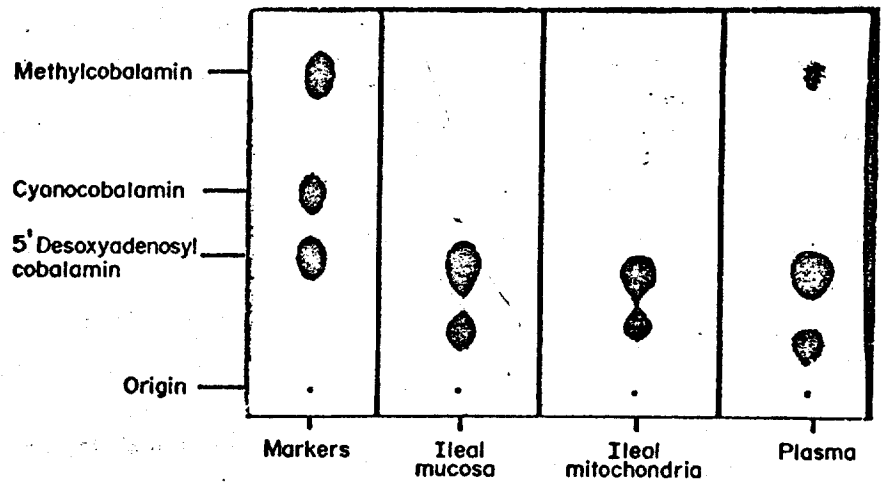


FIG 1. Bioautograms of methylcobalamin, cyanocobalamin, adenosylcobalamin and of the cobalamins extracted from guinea-pig ileum, ileal mitochondria and portal plasma.

TABLE I. The concentrations of the individual cobalamins in guinea-pig liver, ileum, ileal mitochondria and portal plasma

Tissue	*Total vitamin B_{12}** (*pg/mg protein*)	*Methyl-cobalamin*	*Cyano-cobalamin*	*Adenosyl-cobalamin* (*pg/mg protein*)	*Unknown cobalamin*
Ileal mucosa	2080 ± 261 (13)	80	—	1300	700
Ileal mitochondria	5840 ± 180 (4)	—	—	3540	2300
Liver	4910 ± 585 (4)	50	245	2450	2165
Portal plasma	1480 ± 297 (7)	90	—	780	616

* Mean ± SE. Number of observations in parentheses.

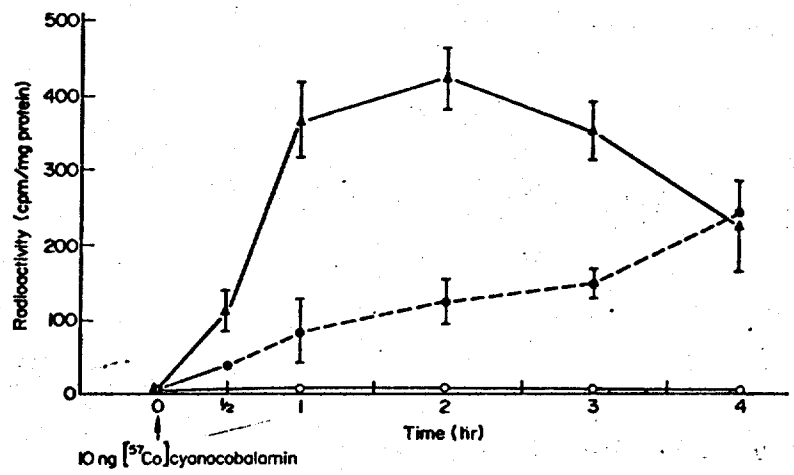


FIG 2. The specific activity of cyanocobalamin (▲), 5'desoxyadenosylcobalamin (●) and methylcobalamin (○) in ileal mucosa at timed intervals after feeding radioactive cyanocobalamin.

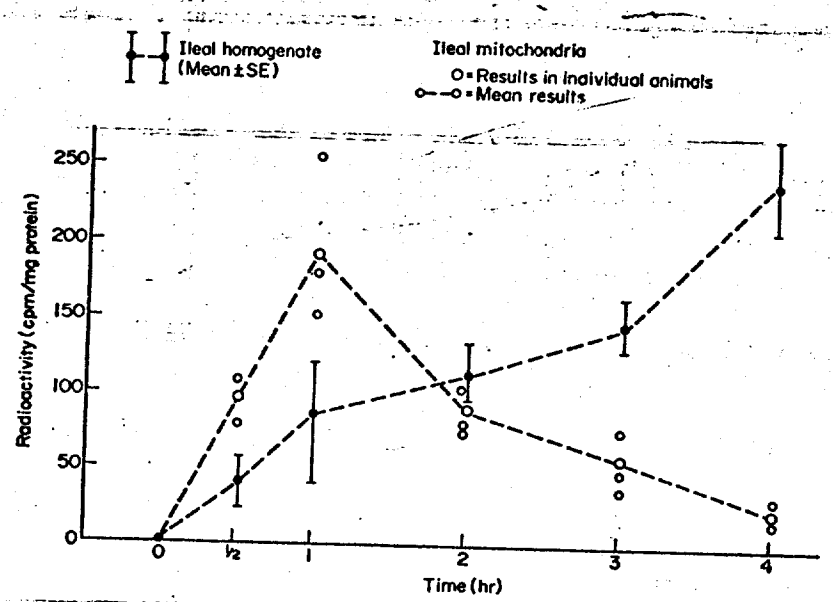


FIG 3. The specific activity of adenosylcobalamin in ileal mucosa and ileal mitochondria at timed intervals after feeding radioactive cyanocobalamin.

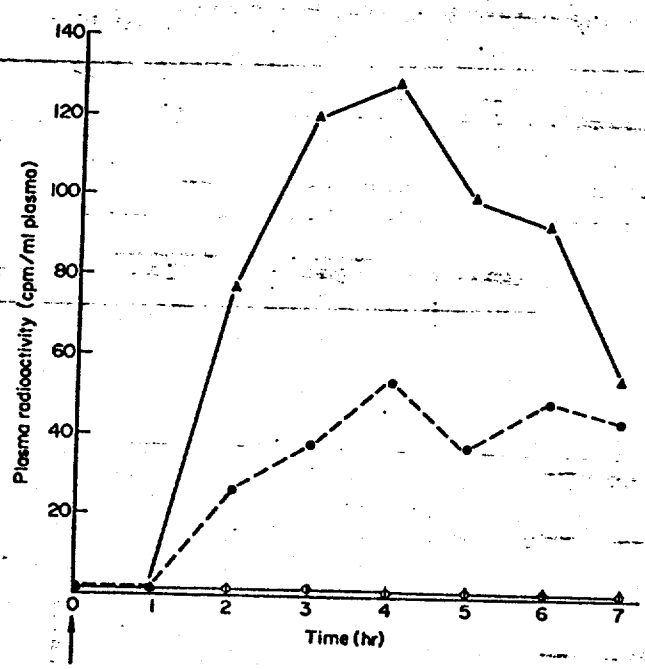


FIG 4. The concentration of labelled cyanocobalamin (▲), 5′ desoxyadenosylcobalamin (●) and methylcobalamin (○) in portal blood at timed intervals after feeding radioactive cyanocobalamin.

mucosa at timed intervals after feeding the labelled cyanocobalamin. Each point is the mean ± SE of duplicate estimations in at least three animals.

No significant radioactivity was detected in either the methylcobalamin or unidentified cobalamin zones at any time during the study. There was, however, a sharp rise in the amount of labelled cyanocobalamin in the mucosa which reached a peak at 1–3 hr and then declined. There was also a progressive rise in radioactive adenosylcobalamin in the mucosa throughout the 4 hr period of study. It is important to note that labelled adenosylcobalamin appeared in the mucosa within 1 hr of feeding the cyanocobalamin, i.e. before any radioactivity had left the mucosa and entered portal blood (Fig 4).

Fig 3 shows the specific activity of labelled adenosylcobalamin in the whole ileal homogenate and in the mitochondrial fraction after feeding labelled cyanocobalamin. Each point is the mean of duplicate estimations in at least two animals. There was a rise in the mitochondrial adenosylcobalamin radioactivity which reached a peak at 2 hr and then declined. In contrast, adenosylcobalamin activity in the whole homogenate increased slowly throughout the experimental period.

Forms of Labelled Vitamin B_{12} in Portal Plasma during the Absorption of Labelled Cyanocobalamin

Fig 4 shows the different radioactive cobalamins in the portal plasma of two guinea-pigs fed 10 ng of ^{57}Co-cyanocobalamin. After the period of mucosal delay, there was a rise in both labelled adenosyl- and labelled cyano-cobalamin in portal plasma. The concentration of labelled adenosylcobalamin increased up to 4 hr and then remained constant throughout the experimental period but the concentration of labelled cyanocobalamin reached a broad plateau between 3 and 5 hr and then declined. No radioactivity was detected in either the methylcobalamin or the unidentified cobalamin zones.

DISCUSSION

These experiments show that the principal form of vitamin B_{12} in guinea-pig ileal mucosa and liver is 5′ deoxyadenosylcobalamin. This is in agreement with observations of other workers on the forms of vitamin B_{12} in mammalian liver (Pawelkiewicz *et al*, 1960; Toohey & Barker, 1961). Significant amounts of methylcobalamin were only detected in plasma and in a much lower proportion than in human plasma (Lindstrand & Stahlberg, 1963; Linnell *et al*, 1969).

The localization of the adenosylcobalamin in the mitochondria is in agreement with the previous report that vitamin B_{12} is found in the highest concentration in the mitochondrial fraction of ileal cells (Peters & Hoffbrand, 1970). It is also consistent with the known mitochondrial localization of methylmalonyl Co-A mutase, the only mammalian enzyme known to require adenosylcobalamin as coenzyme (Gurnani *et al*, 1960; Stadtman *et al*, 1960).

The nature of the unidentified cobalamin is uncertain. The guinea-pig has no foregut and gains its vitamin B_{12} from its food and by coprophagy so the unidentified cobalamin cannot have been made by bacteria in the animal's small intestine. The R_f differs from that of hydroxocobalamin in the chromatography system used here. Several naturally occurring vitamin B_{12} analogues are known but they are generally found only in micro-organisms (Lester Smith, 1965). The low R_f value suggests that the unidentified cobalamin is relatively polar,

JENRYCH, W., PAWELKIEWICZ, J. & MAGAS, S. (1962) Conversion of cyanocobalamin, in vivo, into its coenzyme form in the rabbit. *Bulletin de l'Academie Polonaise des Sciences*, 10, 117.

GORNA, M. (1965) Enzymatzycne przeksztalcenie witaminung B_{12} io jej forme koenzymatyczna in vitro. *Rocziki Naukowe WSWF W Poznaniv*, 10, 9.

GURNANI, S., MISTRY, S.P. & JOHNSON, B.C. (1960) Function of vitamin B_{12} in methylmalonate metabolism. I. Effect of a cofactor form of B_{12} on the activity of methylmalonyl-CoA isomerase. *Biochimica et Biophysica Acta*, 38, 187.

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. (Abstract). *Journal of Physiology*, 208, 66P.

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

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. (Abstract). *Journal of Clinical Pathology*, 22, 742.

LINNELL, J.C., MACKENZIE, H.M. & MATTHEWS, D.M. (1969b) Normal values for individual plasma cobalamins. (Letter). *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.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, 193, 265.

MAHLER, H.R. & CORDES, E.H. (1966) *Biological Chemistry*, p 224. Harper & Row, New York.

PARSONS, D.F., WILLIAMS, G.R. & CHANCE, B. (1966) Characteristics of isolated and purified preparations of the outer and inner membranes of mitochondria. *Annals of the New York Academy of Sciences*, 137, 643.

PAWELKIEWICZ, J., BARTOSINSKI, B. & WALERYCH, W. (1960) A new class of derivatives of vitamin B_{12} group: cobalto-corrin compounds. *Bulletin de l'Academie Polonaise des Sciences*, 8, 123.

PETERKOFSKY, A. & WEISSBACH, H. (1964) Enzymatic synthesis of coenzyme B_{12}. *Annals of the New York Academy of Sciences*, 112, 622.

PETERS, T.J. & HOFFBRAND, A.V. (1970) Absorption of vitamin B_{12} by the guinea-pig. I. Subcellular localization of vitamin B_{12} in the ileal enterocyte during absorption. *British Journal of Haematology*, 19, 369.

PETERS, T.J. & MACMAHON, M.T. (1970) The absorption of glycine and glycine oligopeptides by the rat. *Clinical Science*, 39, 811.

PETERS, T.J., QUINLAN, A. & HOFFBRAND, A.V. (1971) Absorption of vitamin B_{12} by the guinea-pig. II. The subcellular localization of cyano-, methyl- and adenosyl-cobalamins and the effect of fluoroacetate on cyanocobalamin absorption. *British Journal of Haematology*, 20, 123.

SMITH, E. LESTER (1965) *Vitamin B_{12}*, 3rd edn. Methuen, London.

STADTMAN, E.R., OVERATH, P., EGGERER, H. & LYNEN, F. (1960) The role of biotin and vitamin B_{12} coenzyme in proprionate metabolism. *Biochemical and Biophysical Research Communications*, 2, 1.

TAKEYAMA, S. & BUCHANAN, J.M. (1961) Enzymatic synthesis of the methyl group of methionine. III. Spectral and electrophoretic studies of the prosthetic group of the B_{12} enzyme. *Journal of Biochemistry (Tokyo)*, 49, 578.

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

VITOLS, E., WALKER, G.A. & HUENNEKENS, F.M. (1966) Enzymatic conversion of vitamin B_{12s} to a cobamide coenzyme, α-(5,6-dimethylbenzimidazolyl deoxyadenosylcobamide) (adenosyl-B_{12}). *Journal of Biological Chemistry*, 241, 1455.

THE INSTITUTE OF FORENSIC MEDICINE AND INSURANCE OF THE UNIVERSITY OF TURIN

Director: Professor R. Gilli

AGAIN WITH REGARD TO INCIDENTS OF VITAMIN THERAPY. DEATH FOLLOWING PARENTERAL ADMINISTRATION OF VITAMIN B-12

Author: M. Portigliatti Barbos, Ass. and L.D.

In a preceding article (1961) I have already had the occasion to ~~work~~ give attention to the problem of abnormal reactions after administration of Vitamin preparations of group B and to gather and comment upon the cases, indicated in the literature, of death following the parenteral use of aneurin.

A similar occurrence concerning Vitamin B-12 and which therefore fits into the subject matter of the same heading was presented relatively recently ~~reasonably~~ for our consideration and appears worthy of mention because ~~up~~ until now we have not known of such cases with a fatal outcome although some of them with a more or less serious symptomatology have been described, ~~they~~ resulting ~~resulted~~ in a fortunate outcome (i.e., the patients survived).

The patient was a woman about 34 years old, named Amelia P., with a case history that was not noteworthy, the mother of two children and in the eighth month of a pregnancy that had gone on without complications except for an anemic state and a recurring painful symptomatology of neuritic type in the right leg. She was advised to take injections of 100 micrograms of Vitamin B-12 for the treatment of the anemia. After the taking of the third phial, a picture suddenly ~~suddening~~ appeared of immediately serious collapse with cyanosis, dyspnea, hypotension, retrosternal pains and an urticarioid rash. In view of the lack of response to the

analeptic therapy that was at once applied ~~apparent~~ and the obvious aggravation of her general condition, the patient was taken to the hospital where, despite all effort at treatment, she died within a half-hour. A ~~XXX~~ Caesarean section was carried out at once, and a female foetus was removed, weighing about 3 kg., that showed symptoms of asphyxial suffering and that died within about ten minutes.

The autopsy carried out at the order of the judicial authority did not bring to light any noteworthy anatomo-pathological finding except for an acute generalized stasis that was particularly evident in the pulmonary region, with a moderate degree of edema. I omit the description of the autopsy record as well as of the histological findings of all the viscera (heart, lungs, liver, spleen, kidneys, hypophysis [the pituitary glands], the adrenals, thyroid, pancreas, ovary, uterus, brain) of the cadavers both of the mother and of the foetus because this would mean only a repetition of description of a normal morphological picture, there being noted ~~note~~ only the state of pregnancy in the mother, and incomplete foetal maturity in the product of conception and an acute generalized stasis in both of them.

A chemical-toxicological examination was also carried out of the ~~XXXXX~~ phials contained in the same box and not yet used, and it was noted that their ~~the~~ composition corresponded entirely to the requirement of the formula.

The clinical and autopsy findings, the lack of any kind of anatomo-pathological change that could in itself explain ~~justify~~ the

result, the occurrence of death shortly after the administration of the drug, the ability of the drug (already known in the literature) to produce abnormal reactions, the rapid development of the disease picture according to the procedure (collapse, itching, urticarioid rash, etc.) that repeated the characteristics already known of other cases that resulted fortunately less tragically, were all elements that lead us to attributing death to intolerance to the drug.

Now, in order to explain the mechanism of the fatal effect, two hypotheses could be considered, the first being of toxic nature, the second of allergic type.

The toxic mechanism has mostly been excluded by the authors who were concerned with the problem. The only exception is that of Traina who studied it experimentally.

He administered this product in very high doses to mice, noting a toxic effect when it was given intraperitoneally in a dose of 1.5 mg. per kg. of weight, and noting a lethal effect in 100 per cent of the animals treated (either intraperitoneally or sub-cutaneously) when the dose was increased to 3 mg. per kg. He therefore came to the conclusion that the abnormal reactions following the use of this vitamin are to be attributed to a true toxic effect, and he was strengthened in this conviction also by the result of the experiments undertaken to demonstrate a possible sensitivization. Using the technique of Gruhzit, O. M. and of Fisken, R.A. (Journ. Pharmacol. & Exper., Therap., 89, 3, 1947), he administered sub-cutaneously to twelve guinea pigs 5 mg. of Vitamin B-12 for 21 days, then injecting each animal, after an interval of 37 days with 15 mg. intraperitoneally without results. Traina therefore concluded that

Vitamin B-12 did not have sensitivizing power but, according to him it had probably an anti-anaphylactic and anti-histaminic effect.

In the cases observed by us there could be noted against the toxic mechanism both the very small dose used (see for example the statements by Rotting E., Martinelli M., Pugliese F., by Cairella M., by Hadnagy and Norvath regarding the use of shock doses) and the actual allergic response characteristic of the symptomatology under consideration.

In reality the majority of the authors have indicated the anaphylactic nature of the abnormal responses to the administration of this Vitamin.

Joung (sic), Ulrich, Houts in 1950 described the clinical picture of a patient in whom appeared itching and edema at the site of injection after the sixth administration, the symptoms being punctually repeated at each of the five successive administrations.

Arnlers, after four subcutaneous injections noted similarly transitory allergic reactions.

Meyer, Schmid, Metreau, and Lambert (cited by Duchesnay) indicated erythemas on the ninth day and reactions of eczematous type.

Borodulin furthermore completely described an interesting clinical observation. The patient in question was about 29 years old, received, in July, 1960 at the Medical Clinic of Voronezh with a diagnosis of sub-acute reticular endotheliosis and treated with Prednisone, hepatic extracts, Vitamin B-12, massive transfusions, and being discharged April 15 (sic) of that year; on July 11, the

patient had to return because of a high fever (39.4° C), moderate sub-edema, slight cyanosis, tachycardia (102), enlargement of the liver and enlargement of the spleen. He was further treated with the same preparation, cure being effected within one month and a half: his pulse had, in fact, returned to normal, the enlargement of the liver was reduced, and only a slight eosinophilia persisted (6%). On November 9th, two or three minutes after a sub-cutaneous injection of Vitamin B-12, there occurred suddenly a painful retrospernal symptomatology, radiating toward the base of the neck, a dyspnea, vertigo, visual dimness, intense itching; a serious cyanosis and a generalized urticaria occurred, accompanied by tachycardia (120), tachypnea (30), hypotension (P.A.O.S.; 90/40).This group of symptoms began to regress after six-seven minutes and after twenty minutes, the figures for the pulse and pressure had returned to normal, there remaining only an intense itching and a burning in the neck, in the face, and in the limbs with pains in the abdomen (abdominal seat). After 50 minutes, intense shivering occurred, lasting about 30 minutes, with the whole symptomalogical picture returning to normal within two hours. An examination of the blood carried out three days later did not show any eosinophilia. The author considers that any connection between the group of symptoms and the administration of cortisone can be excluded (also because this drug was administered over a period of time without any harmful effect) and he thought of sensitivization by Vitamin B-12. In order to test this assumption,

he made a sub-cutaneous test using a dose 20 times smaller than the one that had in~~sighted~~cited the reaction. This resulted in the appearance of a generalized urticaria with itching in the upper limbs and in the face and also a picture of collapse lasting about 10 minutes that repeated the same characteristics as in the first episode (tachycardia, hypotension, cyanosis, shivering lasting about 10 minutes) with the appearance of hyperemia and edema on a surface of 15 cm by 5 cm, corresponding to the site of the injection.

The case observed by us presents, as is immediately evident, noteworthy similarities with those described above, so that we are led, without substanial reserve to interpret it on the same basis.

It is to be taken into account that, according to some authors, the sensitivizing effect may not be due to the Vitamin B-12 in itself and through itself but rather to impurities that could not be eliminated when this Vitamin was prepared, not from hepatic extracts (according to the most widely used technique) but rather from bacterial cultures.

Bedford, in a ~~xxxxxxxxxxxxxx~~ research series on the secondary effects ~~following~~ of the use of Vitamin B-12 preparations, prepared from cultures of Streptomyces, Griseus, took note of positive results in fourteen out of one hundred cases and sensitivization reactions in six subjects. The same author in 1952 reported a patient in whom the ~~xxxxxxxxx~~ periodic ~~xxxx~~ appearance of an urticaria and of a ~~clearly~~ well defined angioneurotic edema corresponded to a series of injections of Vitamin B-12. In his opinion an allergizing effect with Vitamin B-12 prepared from

hepatic extracts would never have been observed.

Similarly, Joung (sic), Ulrich, and Houts, in the case mentioned, stated that the allergic symptoms appeared only when use was made of Vitamin B-12 prepared from bacterial cultures and disappeared when they returned to using a preparation obtained from hepatic extracts.

It therefore appears advisable, in order to avoid the indicated incidents, however rare they may be, for a lengthy generalized therapy, to use cutaneous tests to determine the possible sensitivization of the subject vis-à-vis the drug, with obvious suspension of this therapy if the result is positive. The cases mentioned above lead us in fact not to underestimate the risks inherent in abnormal responses to this Vitamin treatment since the treatment can cause, as in our case, lawsuits.

BIBLIOGRAPHY

Borodylin Iud., Anaphylactic shock as a complication in a treatment ~~of~~ with Vitamin B-12, "Clin. ~~MEDI.~~ Med.", Moscow, 39, 8, 139, 1961 (in Russian).

Cairella M., Absorption, distribution and elimination of Vitamin B-12. Physio-pathological-therapeutic considerations. "Clin. Ter.", 22, 425, 1962.

Chevallier, P.: Excellence of very large doses of Vitamin B-12~~M~~. "Semaine des Hôpitaux", 39, 1953, 1953. (sic)

Hadnagy, C., Norvath, E.: Our studies on the pharmacodynamics of Vitamin B-12. "Klin. Med.", Moscow, 43, 25, 196~~3~~2 (In Russian).

Lereboullet, J., Plu~~d~~inage, R.: The Use in neurology of massive doses of Vitamin B-12 (100 gamma [sic] per injection). "Sem. Hôp.", 37, 1849, 1953.

Mallarme, J., Schneider J. and Nicolle: The treatment of anemias by Vitamin B-12. "Le Sang", 1, 33, 1951.

Portigliatti, Barbos M.: ~~XXXXX~~ Incidents after administration of Vitamin B-1 "Minerva medico-legale", 81, 239, 1961.

Rostenberg, A., Perkins A.J.: Dermatites from nic~~hol~~kel and cobalt. "Journ~~al~~ of Allergy", ~~25x~~ 22,5, 466, 1951.

Wolff R., Royer P. and Karlin, : Rate (proportion) of Vitamin B-12 in the liver of animals poisoned by carbon tetrachloride. "C.R. ~~SOC.~~ Soc. ~~BIOL.~~ Biol." 145,13-14, 991, 1951.

2187

recei.
received 5/6
1148
MINERVA MEDICO-LEGALE 83: 117-118, (1963)

Istituto di Medicina Legale e delle Assicurazioni
dell'Università di Torino
Direttore Prof. R. GILLI

Ancora in tema di incidenti da terapia vitaminica. Morte consecutiva a somministrazione parenterale di vitamina B_{12}

M. PORTIGLIATTI BARBOS, *Ass. e L. D.*

In una precedente nota (1961) ebbi già occasione di occuparmi del problema delle reazioni abnormi alla somministrazione di preparati vitaminici del gruppo B e di raccogliere e commentare i casi segnalati nella letteratura di morte consecutiva all'uso parenterale dell'aneurina.

Analoga osservazione concernente la vitamina B_{12}, e che pertanto si inquadra nella trattazione del medesimo capitolo, si è presentata in data relativamente recente al nostro esame e par degna di menzione, anche perchè non ci consta che a tutt'oggi siano stati segnalati casi siffatti ad esito letale, pur essendone gli stati descritti alcuni dalla sintomatologia più o meno grave, risoltasi in modo fausto.

Si tratta di una donna trentaquattrenne: tale Amelia P., della mannese indifferente, già madre di due figli ed all'ottavo mese di una gravidanza decorsa senza complicanze fatta eccezione per uno stato anemico di una recente sintomatologia dolorosa di tipo nevritico dell'arto inferiore destro. Per la cura dell'anemia le furono consigliate iniezioni da 100 microgrammi di vitamina B_{12}. Alla terza fiala comparve improvvisamente un quadro di collasso immediatamente grave, con cianosi, dispnea, ipotensione, dolori retrosternali ed un rash urticarioide. Data la mancata risposta alla terapia analettica messa subito in atto e l'aggravarsi manifesto delle condizioni generali, la paziente fu trasportata in ospedale, dove, nonostante ogni cura, morì nel volgere di neppur mezz'ora. Fu eseguito immediatamente un taglio cesareo e venne estratto il feto di sesso femminile, del peso di circa 3 kg, che mostrava segni di sofferenza asfittica e che venne a morte entro una decina di minuti.

L'esame necroscopico eseguito per ordine dell'autorità giudiziaria non mise in luce alcun reperto anatomo-patologico degno di nota, fatta eccezione per una stasi acuta generalizzata e particolarmente accentuata in ambito polmonare, con modico stato di edema. Ometto la descrizione del protocollo di autopsia nonchè dei reperti istologici di tutti i visceri (cuore, polmone, fegato, milza, reni, ipofisi, surreni, tiroide, pancreas, ovaia, utero, encefalo) del cadavere sia della madre che del feto, perchè significherebbe soltanto il ripetere la descrizione di un quadro morfologico normale, essendosi constatato soltanto uno stato gravidico nella madre, una incompleta maturità fetale nel prodotto del concepimento ed una stasi acuta generalizzata in entrambi.

Venne eseguito anche un esame chimico-tossicologico delle fiale contenute nella stessa scatola e non ancora utilizzate, e si constatò che la loro composizione corrispondeva in tutto e per tutto a quanto risultava dalla formula.

I reperti clinici e necroscopici, la mancanza di qualsiasi alterazione anatomo-patologica che potesse di per sè riuscir giustificativa nell'exitus, il sopravvenire della morte in stretto rapporto cronologico con la somministrazione del farmaco, la idoneità di questo (già nota in letteratura) a produrre reazioni abnormi, il divenire rapido del quadro morboso secondo modalità (collasso, prurito, rash urticarioide, ecc.) che ripetevano le caratteristiche già note di altri casi risoltisi fortunatamente in modo meno tragico, furono elementi tutti che indussero ad attribuire la morte ad un fenomeno di intolleranza al farmaco.

Ora, per spiegare il meccanismo di azione letifera potevano essere prospettate due ipotesi, di natura tossica la prima, di tipo allergico la seconda.

Il meccanismo tossico è stato per lo più escluso dagli AA. che si sono occupati del problema. Unica eccezione è quella del Traina che lo studiò specificatamente.

Egli somministrò in dosi molto elevate nei topi tale prodotto, constatando un'azione tossica, quando veniva raggiunta per via intraperitoneale una dose di 1,5 mg per kg di peso, l'effetto letale nel 20% degli animali trattati (sia per via intraperitoneale che sottocutanea) quando si aumentava la dose a 3 mg pro kg. Concluse pertanto affermando che le abituali reazioni secondarie all'uso di tal vitamina sono da addebitare ad una vera azione tossica, in tal convincimento confortato anche dal risultato degli esperimenti intesi a dimostrare una eventuale sensibilizzazione. Ricorrendo alla tecnica di Grubzit O. M. e di Fisken R. A. (« Journ. Pharmacol. & Exper. Therap. », 89, 3, 1947) egli somministrò sotto cute a 12 cavie 5 mg di vitamina B_{12} per 21 giorni, iniettando quindi a ciascun animale, dopo un intervallo di 37 giorni, 15 mg intraperitoneale, senza conseguenze. Il Traina concluse pertanto che la vitamina B_{12} non aveva un potere sensibilizzante ma, a suo dire, sarebbe stata fornita di una azione antianafilattica ed anti-istaminaica.

Nel caso da noi osservato deponevano contro il meccanismo tossico sia la minima dose usata (si veda ad esempio quanto è stato scritto da Rottini E., Martinelli M., Pugliese F., da Cairella M., da Hadnagy e Norvath circa l'uso di dosi urto) sia il carattere di risposta allergica proprio della sintomatologia obbiettivata.

In realtà la maggioranza degli AA. ha segnalato proprio la natura anafilattica delle risposte abnormi alla somministrazione di tal vitamina.

Joung, Ulrich, Houts nel 1950 descrissero il quadro clinico di un paziente in cui comparvero prurito ed edema nel luogo dell'iniezione a partire dalla 6ª somministrazione, ripetendosi puntualmente per ognuna delle cinque successive.

OR PURPOSES OF STUDY OR RESEARCH IN LIEU OF LENDING THE ORIGINAL.

2187

Arnlers, dopo quattro iniezioni sottocutanee, constatò del pari reazioni allergiche transitorie.

Meyer, Schmid, Metreau e Lambert (citati da Duchesnay) hanno segnalato eritemi di 9ª giornata e reazioni di tipo eczematoso.

Borodulin ha inoltre descritto compiutamente una osservazione clinica interessante. Si trattava di una paziente ventinovenne ricoverata nel luglio 1960 nella Clinica Medica di Voronez con la diagnosi di endoteliosi reticolare subacuta e curata con Prednisone, estratti epatici, vitamina B_{12}, trasfusioni massive, venendone dimessa il 15-4 di quell'anno; l'11-7 dovette farvi ritorno per un elevato stato febbrile (39°,4), modico subedema, lieve cianosi, tachicardia (102), epatomegalia e splenomegalia. Fu ulteriormente curata con i medesimi preparati, raggiungendo la guarigione nel volgere di un mese e mezzo: il polso era infatti ritornato alla norma, la epatomegalia si era ridotta e persisteva soltanto una lieve eosinofilia (6 %). Il 9 novembre, due o tre minuti dopo la iniezione sottocutanea di vitamina B_{12}, insorse improvvisamente una sintomatologia dolorosa retrosternale con irradiazione verso la base del collo, una dispnea, oltre a vertigini, annebbiamento visivo, forte prurito; si fecero manifeste una grave cianosi ed un'orticaria generalizzata, accompagnate da tachicardia (120), tachipnea (30), ipotensione (P.A.O.S.: 90/40). Detto quadro morboso prese a regredire dopo 6-7 minuti e dopo 20 minuti i valori del polso e della pressione eran ritornati alla norma residuando soltanto un forte prurito ed un bruciore al collo, al viso ed agli arti con dolori in sede addominale. Dopo 50 minuti comparvero forti brividi della durata di circa 30 minuti con ritorno alla norma di tutto il quadro sintomatologico nel volgere di due ore. Un esame del sangue eseguito dopo tre giorni non dimostrò più alcuna eosinofilia. L'A. ritiene di poter escludere che il quadro morboso potesse essere messo in rapporto causale con la somministrazione di cortisone (anche perchè tale farmaco venne somministrato in prosieguo di tempo senza alcun inconveniente) e pensò ad una sensibilizzazione da vitamina B_{12}. Per provare tale assunto eseguì un test sottocutaneo usando una dose 20 volte minore di quella che aveva scatenata la reazione. Si ebbe la comparsa di un'orticaria generalizzata con prurito agli arti superiore e al volto ed anche un quadro collassiforme della durata di circa 10 minuti che ripetè gli stessi caratteri del primo episodio (tachicardia, ipotensione, cianosi, brividi durati 10 minuti circa) con comparsa di iperemia ed edema su una superficie di 15 cm per 5 in corrispondenza del luogo della iniezione.

Il caso da noi osservato presenta, come è immediatamente evidente, notevoli analogie con quelli dianzi descritti, sì da indurre senza sostanziali riserve ad interpretarlo sulla medesima base.

E' da tener conto che secondo alcuni AA. l'azione sensibilizzante non sarebbe dovuta alla vitamina B_{12} in sè e per sè quanto piuttosto ad impurità che non si riesce ad eliminare allorchè detta vitamina venga preparata non già da estratti epatici (secondo la tecnica più diffusa) bensì da culture batteriche.

Bedford in una serie di lavori sugli effetti secondari dei preparati di vitamina B_{12}, allestiti partendo da culture di Streptomyces Griseus constatò test positivi in 14 su 100 casi e reazioni di sensibilizzazione in 6 soggetti. Lo stesso A. nel 1952 riferì di un paziente in cui la comparsa periodica di una orticaria e di uno spiccato edema angioneurotico corrispondeva a serie di iniezioni di vitamina B_{12}. A suo dire mai sarebbe stata notata efficacia allergizzante con la vitamina B_{12} allestita da estratti epatici.

Analogamente Joung, Ulrich e Houts, nel caso menzionato, constatarono che le manifestazioni allergiche comparvero soltanto allorchè si fece uso di vitamina B_{12} preparata da culture batteriche e scomparvero allorchè si ritornò all'uso di un preparato ottenuto da estratti epatici.

Appare pertanto consigliabile, ad evitare i sia pur rari incidenti segnalati per una terapia largamente generalizzata, di ricorrere all'uso dei tests cutanei per saggiare la eventuale sensibilizzazione del soggetto nei confronti del farmaco, sospendendo ovviamente tale terapia qualora la risposta abbia a risultare positiva. I casi dianzi menzionati inducono infatti a non sottovalutare i rischi insiti nelle reazioni abnormi a tale cura vitaminica, potendo essi dar luogo, come nel nostro caso, a vertenze giudiziarie.

M. Portigliatti Barbos
Istit. Med. Leg. Univ. - Torino

BIBLIOGRAFIA

Bedford P. D.: Side-Effects of a preparation of Vitamin B_{12}. « Brit. Med. Journ. », 1, 690, 1952 (Dello stesso autore vedi anche lavori in argomento in: « Lancet », 1, 1951, e « Brit. Med. Journ. », 1, 1428, 1951).

Bernard J.: La vitamine B_{12} in: « Les Acquisitions Médicales récentes ». Ed. Méd. Flammarion, Paris, 1950.

Boivin P.: Indications and limitations of the use of vit. B_{12} in therapy (with the exception of neurological disorders). « Rev. Prat. », Paris, 11, 3212, 1961.

Borodylin Iud.: Schock anafilattico come complicazione di un trattamento con vit. B_{12}. « Clin. Med. », Mosca 43, 39, 8, 139, 1961 (in russo).

Cairella M.: Assorbimento, distribuzione ed eliminazione della vitamina B_{12}. Considerazioni fisio-patologiche e terapeutiche. « Clin. Ter. », 22, 425, 1962.

Chevallier P.: Excellence de très hautes doses de vitamine B_{12}. « Semaine des Hôpitaux », 39, 1953, 1953.

Duchesnay G.: « Le risque thérapeutique ». Ed. Doin & C., Paris, 1954.

Hadnagy C., Norvath E.: Nostri studi sulla farmacodinamica della vit. B_{12}. « Klin. Med. », Mosca, 43, 25, 1962 (in russo).

Lereboullet J., Pluvinage R.: L'utilisation en neurologie des doses massives de vitamine B_{12} (100 gamma par injection). « Sem. Hôp. », 37, 1849, 1953.

Mallarme J., Schneider J. e Nicolle: Le traitement des anémies par la vitamine B_{12}. « Le Sang », 1, 33, 1951.

Noren B.: Allergic reaction in parenteral liver therapy and vitamina B_{12}. « Acta Medica Scandinavica », CXXXVII, 4, 1950.

Portigliatti Barbos M.: Incidenti da somministrazione di vitamina B_1. « Minerva Medico-legale », 81, 239, 1961.

Rostenberg A., Perkins A. J.: Dermatites au nickel et au cobalt. « Journ. of Allergy », 22, 5, 466, 1951.

Traina V.: Toxycity studies on vit. B_{12} in albino mice. « Arch. of Pathology », 49, 278, 1950.

Wolff R., Royer P. e Karlin: Taux de la vitamine B_{12} dans le foie d'animaux intoxiqué par le tétrachlorure de carbone « C.R. Soc. Biol. », 145, 13-14, 991, 1951.

HIS IS A SINGLE PHOTOCOPY MADE BY THE NATIONAL LIBRARY OF MEDICINE FO

649

Brit. J. Haematol. 18(4): 425-433

2246

British Journal of Haematology, 1970, 18, 425.

The Content of Vitamin B_{12} in Adult and Foetal Tissue: A Comparative Study

MARY E. RAPPAZZO, HEIKKI A. SALMI AND CHARLES A. HALL

Department of Physiology, University of Turku, Turku, Finland, and the Veterans Administration Hospital, Albany, New York

(*Received* 3 *September* 1969; *accepted for publication* 23 *September* 1969)

SUMMARY. The vitamin B_{12} content of 17 tissues in 28 adults and 17 foetuses was measured by a *Euglena gracilis* bioassay. The former were taken after sudden deaths, many accidental, and the latter after therapeutic abortions for mild psychiatric reasons. In the adult, the liver had the highest level of vitamin B_{12} followed by the kidney, adrenal, pancreas and heart. The tissues of greatest vitamin B_{12} concentration in the foetus were the liver, adrenal, kidney and lung. Foetal spleen, lung and colon were significantly higher than their adult counterparts, while foetal liver, kidney, heart and brain were significantly lower in comparison with the adult. Differences in vitamin B_{12} levels may reflect adult and foetal differences in circulation, metabolic maturity or plasma vitamin B_{12} transport.

Vitamin B_{12} is found in almost all tissues and body fluids. The concentration of vitamin B_{12} in different organs varies. The liver is of primary concern in vitamin B_{12} metabolism and various authors give adult mean values ranging from 460 to 1940 pg/mg wet weight of tissue with obvious decreases due to vitamin B_{12} deficiency diseases, cirrhosis, pernicious anaemia, nutritional deficiencies and hepatitis but with little correlation to age (Girdwood, 1952; Swendseid, 1955, 1957; Nelson & Doctor, 1958; Anderson, 1965; Ståhlberg *et al*, 1967). Most studies have involved only one or a few tissues although Hsu *et al* (1959) reported values for eight different tissues from the same person. In the human adult, values are highest in the liver, followed by the kidney and adrenal gland (Ross & Mollin, 1957; Halsted *et al*, 1959). Vitamin B_{12} in the foetus can be distinguished from vitamin B_{12} in the adult by several factors. The serum level of the vitamin B_{12} is higher in the foetus and newborn (Boger *et al*, 1957; Baker *et al*, 1958; Luhby *et al*, 1961; Zachau-Christiansen *et al*, 1962) and an additional binding protein not found in the adult was recently identified in cord serum (Kumento, 1969). In the foetus, the highest tissue level of vitamin B_{12} is represented by the kidney, adrenal gland and liver (Killander, 1958; Salmi, 1963) with no correlation between foetal development and tissue levels (Salmi, 1963).

No investigator studied both adult and foetal tissues but the combined data from several sources suggested different tissue distribution at these two periods of life. The objectives of the present investigation were to obtain first a complete set of adult tissue levels of vitamin B_{12} and subsequently to compare adult and foetal values in an attempt to elucidate further the role of vitamin B_{12} in each. The design of the experiment assured the homogenicity of the samples since all were obtained from a population of similar racial origin, socio-economic

650

status and nutritional conditions. Further, both the foetal and adult material was assayed by the same technique in the same laboratories.

MATERIALS AND METHODS

Tissue selection and preparation. Adult tissue samples were taken from identical sites of the same organ at the time of autopsy from bodies which were freshly received into the morgue at the Department of Forensic Medicine, University of Turku, Turku, Finland. Approximately 200 mg pieces were cut from the samples, weighed on a Sartorius-Semimicro-Selecta balance and immediately frozen at −15°C. No samples were frozen longer than 8 mth (Salmi, 1963). The foetal material collected from therapeutic abortions was obtained in the same manner as that described by Salmi (1963) and frozen as above until needed.

The complete autopsy data from the adult cases was examined as was the maternal case history of the foetuses. There was no indication of abnormalities in vitamin B_{12} metabolism. All cases of chronic alcoholism, liver disease, diabetes or haematological disorders were excluded. The 17 foetal cases came from pregnancy interruptions due to mild reactive depression from otherwise healthy women. All of the adult cases died suddenly; nine of the deaths could be considered purely accidental. The remaining 19 were due to diseases not involved with vitamin B_{12} metabolism, mostly myocardial infarctions and cerebrovascular accidents.

Vitamin B_{12} assay. Euglena gracilis, 'Z' strain, was used for the determination of vitamin B_{12} levels by microbiological assay. The method employed generally followed that of Salmi (1963) with some modifications by Hall & Allen (1964). To prepare the sample, the weighed, frozen piece was homogenized in water as a 1% suspension using an Ultra-Turrax homogenizer. The tissues were digested by a freshly prepared solution of papain (Nutritional Biochemicals Corp.) 1.5 mg and KCN 2 mg in 100 ml of 0.1 N acetate buffer, pH 5.0. The KCN was added to stabilize the vitamin B_{12} during the extraction. This solution was added to the homogenate in a ratio of 1:2, i.e. 5 ml of digestion solution added to 10 ml of homogenate to give a final volume of 15 ml. The mixture was incubated in a 56°C water bath for 3 hr. After cooling, the mixture was brought to pH 3.5–4.0 with 1 N HCl, heated in a boiling water bath for 20 min and allowed to cool. The digestion product from the adult tissues was diluted with distilled water in the ratio of 1:10 and 1:20 except for liver, renal cortex and adrenal which were diluted 1:500, 1:50 and 1:50 respectively. The foetal dilutions were the same except that the liver, kidney and adrenals were diluted 1:100, 1:40 and 1:40. The assay samples were prepared from these dilutions. Three different dilutions were used for each tissue and duplicate determinations were made at each dilution level.

The standard vitamin B_{12} was obtained from Sigma Chemical Co. and a fresh standard series was prepared in triplicate for each complete assay. The concentrations of the standard series ranged from 0.7 to 12.5 pg/ml. One ml of inoculum was added to all tubes. The inoculum was prepared by suspending a 3 times washed 4-day culture of *Euglena gracilis* in approximately 250 ml (No. 0532-15, Bacto Euglena B_{12}) medium (Difco). The assay tubes were then incubated in an environment of uniform and constant light and temperature (29°C) for 4 days. After the 4-day incubation period, the growth was measured, following vigorous shaking to resuspend the growth, by means of a Beckman DU Spectrophotometer

at 450 nm. A standard curve was drawn using the readings of the standard series and from this the vitamin B_{12} values of the test samples were calculated.

Statistical analysis. The statistical analysis applied to the data included the Student's *t*-test and parametric analysis by product moment correlation coefficients. Dr Paul Levy of the Eastern Research Support Center, Veterans Administration Hospital, West Haven, Connecticut, made the analysis and was of great assistance in the statistical aspects of the study. Mrs Sisko Asp, Institute of Occupational Health, Division of Statistics, Helsinki, Finland, made some of the preliminary analysis using the Wilcoxon's S-test and coefficients of rank correlation by the method of Spearman. These results supported the final conclusions of Dr Levy.

RESULTS

The data are expressed in pg/mg of wet weight. Salmi (1963) used both this method of expression and calculation based on tissue nitrogen in his studies of foetal tissues. Nitrogen (N) was also measured in preliminary studies of a small group of adult tissues. Calculations related to tissue N did not contribute to either the comparison of tissue vitamin B_{12} within an age group or between adult and foetal tissues. Therefore, tissue N was not measured in the tissue analysed in the present study.

Adult Values

The results of the adult tissue assays are shown in Table I. Of the 17 tissues analysed, the liver was always highest in vitamin B_{12} content. The kidney, adrenal, pancreas and heart follow although considerably lower in content than the liver. The jejunum, stomach, colon, gonads, thyroid, brain, spleen and muscle have comparable low values with the lowest concentration in the lung and bone marrow. Statistical analysis was applied to the data to determine whether there was a consistency with respect to vitamin B_{12} values for all the tissues analysed from any one individual. Product moment correlation coefficients were computed between pairs of organs. There were 48 correlation coefficients which were statistically significant at the 5% level. This large number gives strong support to the hypothesis that there is an association among organs with respect to vitamin B_{12} levels, i.e. in analysing the 17 different tissues from any one individual, all the values had a tendency to be above, below, or near the median value for that tissue. Fig 1 illustrates the point. Case 94 was a 38-yr-old woman whose direct cause of death was an accidental drowning. She had vitamin B_{12} values which were consistently higher in all tissues. Case 58, a 23-yr-old woman had disseminated lupus erythematosus. Her vitamin B_{12} values were much lower in all tissues. The only organ whose vitamin B_{12} levels showed significant correlation with age was the pancreas which indicated lower levels of the vitamin among the older adults.

The matched *t*-test was applied to different parts from the same organ and to similar tissues from different organs. The mean level of the adult stomach was significantly higher at the 2% level than that of the adult colon and the concentration in the heart muscle was significantly higher at the 1% level than that of the skeletal muscle (Fig 2a). Other differences also found were: adult brain white matter was significantly lower at the 1% level than the grey matter, and adult jejunum was significantly higher at the 2% level than the adult colon. All other comparisons studied were not significant. The adrenal cortex and medulla, the

brain, both white and grey matter, and the stomach, jejunum and colon have values which fall within their respective ranges. Furthermore, an additional analysis was performed to

TABLE I. Adult and foetal levels of vitamin B_{12} recorded as pg/mg wet wt of tissue

Source	Adult		Foetal	
	Median	Range	Median	Range
Liver	698	1137–345	220	369–100
Kidney	161	367– 43	109	141– 65
Adrenal medulla	120	252– 55	128	183– 68
Adrenal cortex	115	195– 43	128	183– 68
Pancreas	98	250– 13	58	67– 42
Heart muscle	92	179– 56	42	55– 27
Jejunum	51	97– 13	58	94– 29
Stomach	47	154– 20	55	67– 27
Colon	39	100– 15	64	101– 37
Gonads	40	134– 20	37	72– 25
Thyroid	39	116– 21	57	101– 20
Brain—grey	39	74– 15	18	36– 11
Brain—white	28	50– 16	18	36– 11
Spleen	38	67– 21	57	88– 34
Skeletal muscle	25	51– 17	33	57– 18
Lung	19	43– 11	80	136– 47
Bone marrow	16	33– 7	20	52– 13

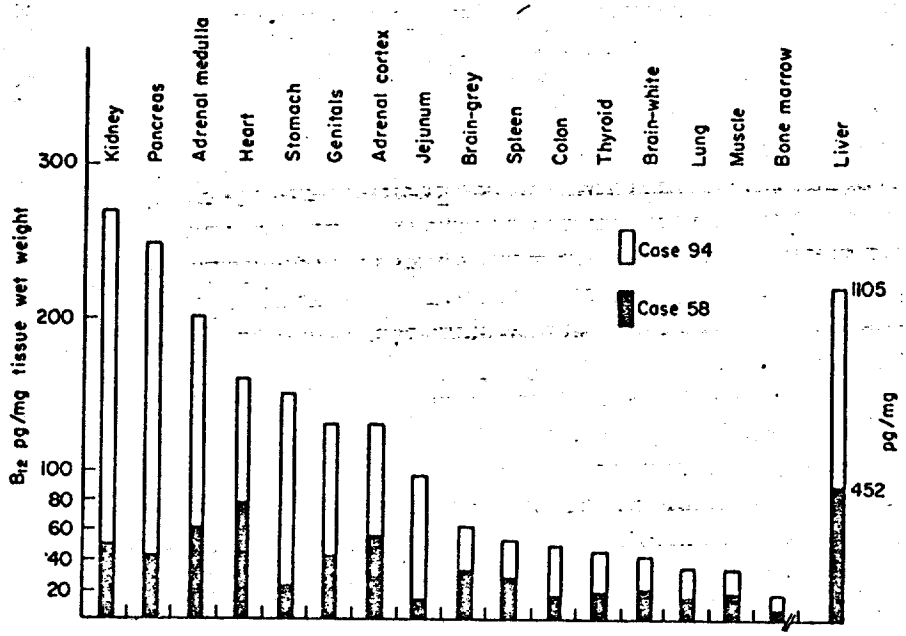


FIG 1. Comparison of median values for all tissues assayed from two individuals.

localize more specifically vitamin B_{12} in the kidney. Seventeen samples of the renal medulla and cortex were taken from a separate group of adults whose deaths were purely accidental.

The *t*-statistic for the comparison between renal medulla and renal cortex in the 17 specimens is highly significant with $t = 8.57$ and $P < 0.01$. The range of values for the cortex was 309–73 pg/mg with a mean value of 203 pg/mg and SD of 71.2. In the medulla the range was 148–8 pg/mg with a mean of 79 pg/mg and SD of 44.5.

Foetal Values

The results of the present foetal study are shown in Table I. In the foetus, vitamin B_{12} values in the liver, adrenal and kidney are highest, followed by the lung. Those next in decreasing order are the colon, jejunum, pancreas, thyroid, spleen and stomach. Still lower

TABLE II. Vitamin B_{12} levels in adult tissues as compared with foetal tissues

Tissue	*Adult*			*Foetal*			*Significance of difference between adult and foetal tissue vitamin B_{12} levels*
	N	*Mean*	*SD*	*N*	*Mean*	*SD*	
Liver	28	746.07	232.74	17	229.94	71.89	$P < 0.01$
Kidney	28	174.54	65.87	17	105.59	24.26	$P < 0.01$
Adrenal*	27	120.56	42.61	15	133.47	30.38	NS
Pancreas	27	103.41	64.21	5	55.00	10.63	NS
Heart muscle	28	99.93	36.17	13	42.08	9.24	$P < 0.01$
Jejunum	28	51.57	21.23	17	60.76	18.50	NS
Stomach	28	58.11	35.23	13	48.46	14.31	NS
Colon	26	38.54	18.79	17	63.82	16.16	$P < 0.01$
Gonads	27	45.81	26.76	3	44.67	24.42	NS
Thyroid	28	40.18	20.27	12	62.08	22.43	NS
Brain—grey	28	44.11	16.36	12	19.25	6.31	$P < 0.001$
Brain—white	27	29.56	9.12	12	19.25	6.31	$P < 0.01$
Spleen	27	40.22	11.44	12	56.00	14.22	$P < 0.01$
Skeletal muscle	28	26.93	7.92	16	31.56	10.49	NS
Lung	28	22.18	9.45	14	84.43	21.40	$P < 0.01$
Bone marrow	28	16.79	6.29	17	21.88	8.94	$P < 0.05$

Mean values = pg/mg tissue; Significance of difference by matched *t*-test.
* Foetal adrenal compared with adult adrenal cortex. Foetal adrenal and brain not separated into its components.

values are found in the heart muscle, gonads, skeletal muscle and bone marrow. The correlation coefficients gave 55 paired foetal tissues which were significant at the 5% level. This indicates that in the prenatal individual there is also a tendency to have either high or low vitamin B_{12} values in all tissues. The matched *t*-test and the illustrations of Fig 2 show levels and differences of paired tissues. The mean B_{12} value in the foetal stomach was significantly lower at the 1% level than the mean values of the colon and jejunum and the mean value for the heart was significantly higher at the 1% level than that of the skeletal muscles.

The foetal work done by Salmi in 1963 under the same conditions but using the 'T' strain of *Euglena gracilis* reports broader ranges and higher mean values than the present study. However, all of the values reported here fall within his range of values. Both studies show that the foetal liver, kidney and adrenal have the greatest concentration of B_{12} per mg of

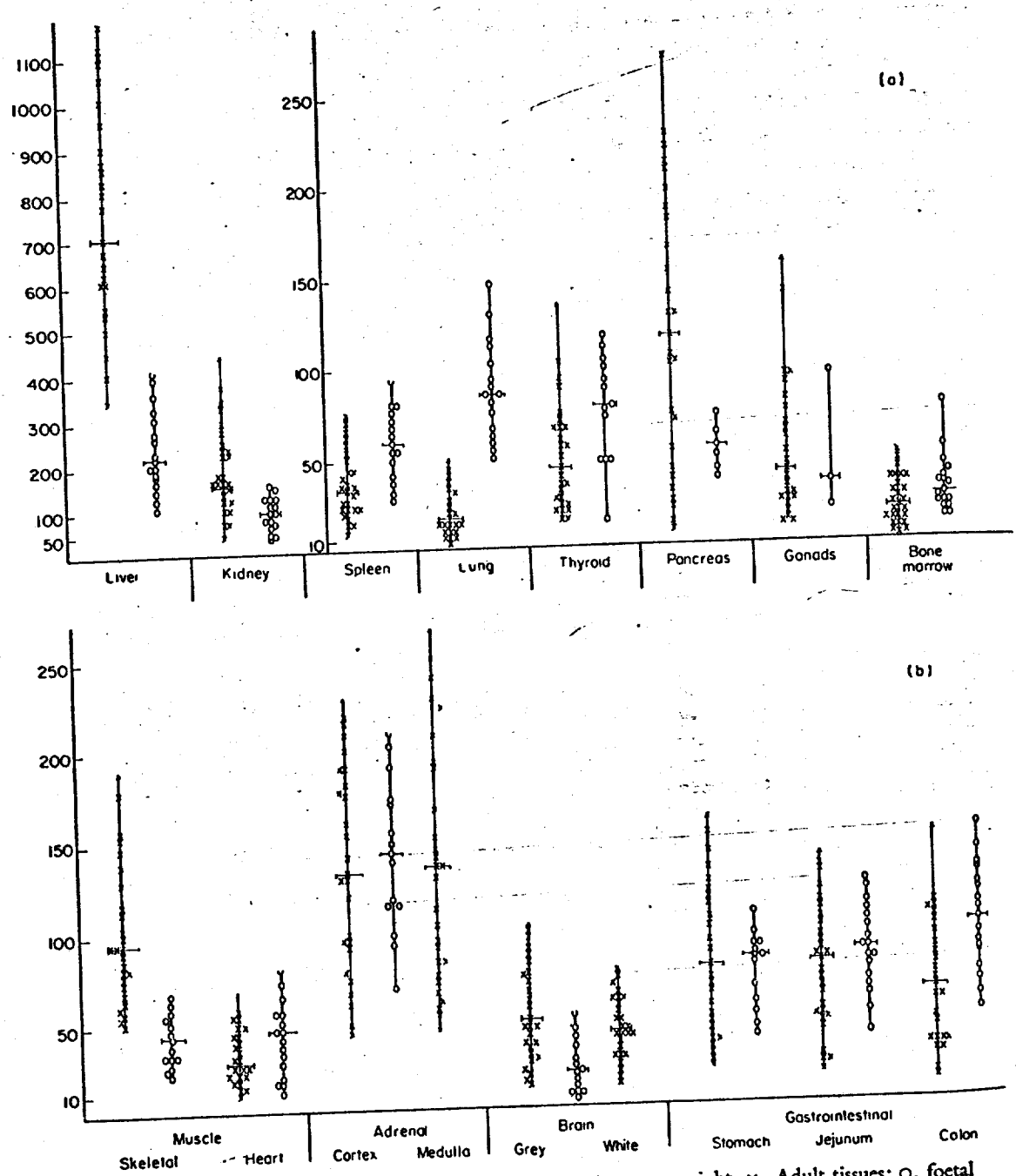


FIG 2. Individual and median vitamin B_{12} levels in pg/mg wet weight. ×, Adult tissues; ○, foetal tissues. (a) Isolated tissues. (b) Parts of some organ or system and closely related tissues.

sue and that an individual is consistent throughout in his B_{12} values; that is, he is either high, low, or near the median value in all his tissues.

Adult Values versus Foetal Values

Adult and foetal values are compared in Table II and Figs 2(a) and 2(b). The mean vitamin B_{12} levels in the adult liver, kidney and heart were significantly higher than the mean vitamin B_{12} levels in the corresponding foetal organs. On the other hand, the mean vitamin B_{12} levels of the foetal spleen, lung, thyroid, bone marrow and colon were higher than the mean vitamin B_{12} levels of the corresponding adult organs. All other differences were not significant. The range of the foetal values was usually smaller than the adult ranges.

DISCUSSION

Vitamin B_{12} levels vary greatly among human tissues. The adult liver is the primary location of the vitamin because of the high concentration in this organ and its large mass. Other tissues have much lower contents. The foetal values show less of a difference in distribution although the liver is still the tissue with the greatest concentration. The smaller variation among the foetal levels might be expected since the foetus has not been exposed to the biological variations which a mature adult would normally encounter. As previously stated, all autopsy data indicated a normal vitamin B_{12} metabolism. In comparing tissue values from those adults whose death was purely accidental (i.e. suicide, etc.), and those which were not (i.e. cerebral hemorrhage, etc.), no significant difference could be demonstrated. Among the adults was one woman who died in childbirth and a man whose death was caused by a crushed chest but who had a partial gastrectomy. The tissues of neither adult showed any great difference from the mean values. In reviewing the autopsy data from individuals who seemed particularly high or low in any or all tissues, nothing was found to explain these differences.

In both the adult and foetus the liver, kidney and adrenal have the greatest vitamin B_{12} content. However, the foetal spleen, lung and colon are significantly higher than their adult counterparts, while the foetal liver, kidney, heart, and brain are significantly lower. Other values are not significantly different. In the normal adult, vitamin B_{12} is transported in the plasma via two binding proteins, transcobalamin I and transcobalamin II (Hall & Finkler, 1963). Foetal and adult plasma vitamin B_{12} transport differ (Kumento, 1969). Foetal plasma contains a third binding protein and there is increased binding of endogenous vitamin B_{12} to transcobalamin II in the foetus. These differences in the foetal transport of vitamin B_{12} could partly explain its different distribution. Before birth only a small part of the blood flows directly through the ductus venosus while a greater portion is directed through the liver sinusoids and hepatic veins. These and other foetal circulatory differences must also be considered in explaining the adult and foetal vitamin B_{12} differences. The foetal liver is the organ that differs most in vitamin B_{12} content from its adult counterpart. The difference in its circulatory system, the immaturity of the foetal liver, or perhaps the absence of decreased levels of enzymes that contain vitamin B_{12} might account for this difference.

Animal studies on the tissue distribution of vitamin B_{12} have also been done. Luhby *et al* (1959) found the proportional distribution of $Co^{60}B_{12}$ in a bitch and her 6-week-old pups to

be similar with the concentration in the newborn pup being one-fifth to one-eighth that of corresponding maternal tissues. This differs from the human adult–foetal comparisons. However, in Luhby's study the measurement is of injected $Co^{60}B_{12}$ distribution and not of endogenous vitamin B_{12}; the pups were 6 weeks old and the vitamin B_{12} content of liver may be naturally lower in the dog at all ages. Cooperman *et al* (1960) report vitamin B_{12} tissue values in two dogs which fall within the ranges of the human adult except for the liver which is lower.

The greatest tissue concentration of vitamin B_{12} in the rat is in the kidney while that of the liver is significantly lower than in man (Salmi, 1963). The difference in vitamin B_{12} concentration between the renal medulla and cortex is supported by microscopic autoradiographic studies done on rats. These studies localized the vitamin B_{12} in the outer parts of the cortex along the surface of the kidney. As in the human, the foetal rat liver, kidney and brains have vitamin B_{12} values significantly lower than their adult counterparts.

ACKNOWLEDGMENTS

This study was carried out in the Department of Physiology, University of Turku, Turku, Finland, and the Haematology Research Laboratory of the Veterans Administration Hospital and the Albany Medical College of Union University, Albany, New York. It was supported in part by NIH Grant AMO2808 of the U.S. Public Health Service and in part by Sigrid Jusélius Foundation and the National Research Council for Medical Sciences, Finland.

REFERENCES

ANDERSON, B.B. (1965) Vitamin B_{12} concentrations in the serum and liver of control subjects and patients with megaloblastic anaemia. (Unpublished personal communication.)

BAKER, H., ZIFFER, H., PASHER, I. & SOBOTKA, H. (1958) A comparison of maternal and foetal folic acid and vitamin B_{12} at parturition. *British Medical Journal*, i, 978.

BLUM, K.U. & HEINRICH, H.C. (1957) Neoplasma and Organ-Vitamin B_{12}-Gehalt des Menschen. *Vitamine und Hormone*, 7, 486.

BOGER, W.P., BAYNE, G.M., WRIGHT, L.D. & BECK, G.D. (1957) Differential serum vitamin B_{12} concentrations in mothers and infants. *New England Journal of Medicine*, 256, 1085.

COOPERMAN, J.M., LUHBY, A., TELLER, D.N. & MARLEY, J.F. (1960) Distribution of radioactive and nonradioactive vitamin B_{12} in the dog. *Journal of Biological Chemistry*, 235, 191.

GIRDWOOD, R.H. (1952) The occurrence of growth factors for *Lactobacillus leichmannii*, *Streptococcus foecalis* and *Leuconostoc citrovorum* in the tissues of pernicious anaemia patients and controls. *Biochemical Journal*, 52, 58.

HALL, C.A. & ALLEN, E.S. (1964) Improved method for the extraction of vitamin B_{12} from serum. *Journal of Laboratory and Clinical Medicine*, 63, 517.

HALL, C.A. & FINKLER, A.E. (1963) A second vitamin B_{12}-binding substance in human plasma. *Biochimica et Biophysica Acta*, 78, 234.

HALSTED, J.A., CARROLL, J. & RUBERT, S. (1959) Serum and tissue concentrations of vitamin B_{12} in certain pathologic states. *New England Journal of Medicine*, 260, 575.

HSU, J.M., MINER, P., MITCHELL, J.A. & CHOW, B.F. (1959) Vitamin B_{12} content in normal and pathological human tissues. American Chemical Society, Atlantic City, N.J., Sept. 13–18, 1959, Abstracts p 75C–76C.

KILLANDER, J. (1958) The vitamin B_{12} content of human fetal tissues. *Acta Chemica Scandinavica*, 12, 1356.

KUMENTO, A. (1969) Studies on the serum binding of vitamin B_{12} in the newborn human infant. *Acta Paediatrica Scandinavica*, Suppl. 194.

LUHBY, A.L., COOPERMAN, J. & DONNENFELD, A.M. (1959) Placental transfer and biological half-life of radioactive vitamin B_{12} in the dog. *Proceedings of the Society for Experimental Biology and Medicine*, 100, 214.

LUHBY, A.L., FELDMAN, R., MARLEY, J., ODANG, O.

& COOPERMAN, J.M. (1961) Serum vitamin B_{12} in infancy. (Abstract). *Federation Proceedings*, **20**, 451.

NELSON, R.S. & DOCTOR, V.M. (1958) The vitamin B_{12} content of human liver as determined by bioassay of needle biopsy material. *Annals of Internal Medicine*, **49**, 1361.

ROSS, G.I.M. & MOLLIN, D.L. (1957) Vitamin B_{12} in tissues in pernicious anaemia and other conditions. *Vitamin B_{12} und Intrinsic Factor*, Symposium, Hamburg, 1956, p 437. Ferdinand Enke, Stuttgart.

SALMI, H.A. (1963) Comparative studies on vitamin B_{12} in developing organism and placenta. *Annales Academiae Scientarum Fennicae (Medica)*, Suppl. 103.

STÅHLBERG, K.G., RADNER, S. & NORDÉN, Å. (1967) Liver B_{12} in subjects with and without vitamin B_{12} deficiency. A quantitative and qualitative study. *Scandinavian Journal of Haematology*, **4**, 312.

SWENDSEID, M., HVOLBOLL, E.E., LEWIS, P.M. & HALSTED, J.A. (1955) Vitamin B_{12} liver content in old age. (Abstract.) *Federation Proceedings*, **14**, 290.

SWENDSEID, M., HVOLBOLL, E., SCHICK, G. & HALSTED, J.A. (1957) The vitamin B_{12} content of human liver tissue and its nutritional significance. *Blood*, **12**, 24.

ZACHAU-CHRISTIANSEN, B., HOFF-JØRGENSEN, E. & ØSTERGÅRD KRISTENSEN, H.P. (1962) The relative haemoglobin, iron, vitamin B_{12} and folic acid values in the blood of mothers and their newborn infants. *Danish Medical Bulletin*, **9**, 157.

#2287

J. Nutrition 58(1): 135-145 1956

STUDIES OF REPRODUCTION IN RATS USING LARGE DOSES OF VITAMIN B12 AND HIGHLY PURIFIED SOYBEAN PROTEINS

LUTHER R. RICHARDSON AND RUTH BROCK
Department of Biochemistry and Nutrition, Texas Agricultural Experiment Station, Texas Agricultural and and Mechanical College System, College Station

(Received for publication August 15, 1955)

The essential nature of dietary vitamin B_{12} for female rats to wean the maximum number of young and for the young to attain normal weaning weight has been demonstrated by several investigators (Schultze, '49; O'Dell, Whiteley and Hogan, '51; Richardson and Russell, '51; Dryden, Hartman and Cary, '51, '52).

Meyer, Thompson and Elvehjem ('51) determined the effect of various supplements to a corn-soybean soil meal diet on lactation and on the vitamin B_{12} content of milk of rats. The B_{12} activity of the milk varied with that of the diet, but lactation performance did not necessarily parallel the vitamin B_{12} content of the diet. Only fish solubles allowed normal reproduction and lactation. Although the fish solubles increased the B_{12} content of the milk above that of the basal group, the milk from the females receiving fish solubles still did not contain more than one-fifth as much B_{12} as that from females receiving 10 μg of crystalline vitamin B_{12} per 100 gm of diet. It was suggested that the difference in response to fish solubles and vitamin B_{12} was due either to an unidentified factor in the fish solubles or to the toxicity of the high level of vitamin B_{12} in milk of the females which received vitamin B_{12}. Since vitamin B_{12} is administered to practically every

659

pregnant women, sometimes in relatively large amounts, it is desirable to know whether the administration of large amounts of the vitamin to the mother has a detrimental effect on the survival and well-being of the offspring.

Schultze ('50, '53) and Schultze, Liener and Glass ('52) reported that female rats receiving a diet containing purified soybean protein supplemented with vitamin B_{12} failed to rear a normal number of young to weaning age. Piccioni, Rabbi and Moruzzi ('51) reported that crude casein contained a factor (or factors) which was indispensable for normal growth and reproduction in rats. When washed casein was substituted for crude casein, the mortality rates in the first and second generation were 70 and 100% respectively. With crude casein the survival usually was close to 100%. The factor was water soluble and heat stable, and was not identified with any of the recognized B vitamins. Sherman, Schilt and Schaefer ('55) reported that the addition of natural ingredients such as distillers solubles, liver, liver meal and fish meal to a vegetable protein diet produced a growth response in the offspring above that obtained with vitamin B_{12}. Recently Gander and Schultze ('55) questioned whether the results of the Italian investigators, as well as some of their own, should be interpreted as conclusive evidence of the existence of an unidentified "animal protein factor" required for the survival of young rats. In another report Schultze ('55) described the results of a study with a diet containing 16 amino acids as the only source of protein nitrogen. Parent females which had received the amino acid diet from weaning produced 11 litters of 76 young. Seventy-three, or 96%, of the young were weaned, but their rate of growth was subnormal. Furthermore, these observations do not support the claim that an unrecognized "animal protein factor" is required for optimum lactation in the rat.

An investigation has been in progress in this laboratory for several years on the nutritive requirement for reproduction and lactation in rats. In experiments conducted at different times with the same diet the numbers of young that

were weaned varied; frequently it was difficult to interpret the data with regard to the requirement of some of the more obscure nutrients for reproduction in the rat. During the past three years, uniform results have been obtained in studies which were conducted on (1) the effect of large doses of vitamin B_{12} in the diet of the mother on the survival and well-being of the offspring, and (2) on the effect of different protein concentrates including highly purified soybean proteins on reproduction and lactation in rats.

EXPERIMENTAL

Female rats which had received the soybean oil meal diet (diet 2) without added vitamin B_{12} from weaning were given the experimental diets when they weighed 150 to 160 gm. Food and water were supplied ad libitum. The composition of the three basal diets used in these studies is given in table 1.

The temperature of the animal room was automatically regulated at about 75° F. during the cool months, and refrigerated air conditioners kept the temperature below 85° F. during the hot periods. Each female was given an opportunity to produce 4 litters and all litters were reduced to 8 young at birth.

After the females in the parent generation had received the experimental diet for 4 weeks, a mature male from the stock colony was placed in a cage with 5 females. The females in succeeding generations received the experimental diet throughout their life span. They were weighed weekly and a pregnant female, as indicated by a large increase in weight, was transferred to an individual maternity cage of the Wahmann type. A false bottom 6 inches wide and turned up three inches at the outer edge was placed in the back of the maternity cage before the litter was born, and remained there until the young were 8 to 10 days old. The false bottom extended the entire length of the cage and was covered with shredded paper in advance of partuition so that the female might make a nest for the young. When this procedure was followed, consistent results were obtained.

TABLE 1

Composition of diets

CONSTITUENT	DIETS		
	1	2	3
	gm	*gm*	*gm*
Protein concentrate	25.0		
Soybean oil meal		70.0	21.4
Dried skim milk			22.0
Dried yeast			8.0
Alfalfa leaf meal			5.0
Cerelose	62.7	23.0	
Ground milo			35.6
Wood pulp	3.0		
Mazola	5.5	3.0	5.5
DL-methionine	0.3		
Mineral mixture [1]	5.0	4.0	
Steamed bone meal			2.0
NaCl			0.5
A.D.K.[2]	+	+	+
Alpha tocopherol, mg	2.5	1.0	
Vitamin mixture [3]	A	B	

[1] Richardson and Hogan ('46).

[2] A.D.K. mixture supplied the following: vitamin A 3000 I.U., vitamin D 400 I.U. and menadione 0.5 gm per 100 gm.

[3] Vitamin mixture A supplied the following: thiamine · HCl, riboflavin and pyridoxine · HCl 1 mg each, calcium pantothenate 4 mg, niacin 5 mg, choline 100 mg, inositol 10 mg, para aminobenzoic acid 50 mg, folic acid 0.1 mg and biotin 0.02 mg per 100 gm of diet. In vitamin mixture B PABA, folic acid and biotin were omitted.

[4] The following salts were added: ferric citrate 40 mg, $CuSO_4 \cdot 5 H_2O$ 20 mg and $MnSO_4 \cdot H_2O$ 100 mg per 100 gm.

Effect of nesting material at birth on survival of young

Since a large percentage of the young survived when nesting material was placed in the maternity cage before partuition, a test was carried out to determine whether the addition of the nesting material before or immediately after partuition had any effect on the survival and weaning weight of the young.

Two groups of 10 females each were given diet 1 supplemented with 25 μg of crystalline vitamin B_{12} per kilogram of diet. Shredded paper was provided for one group of

2287

females before birth of a litter and for the other immediately after birth. These data are summarized in table 2. When the paper was added before birth, 100% of the litters and 95% of the young were weaned. When it was added after birth, only 78% of the litters and 70% of the young were weaned. These data emphasize that it is essential to maintain uniform conditions in management of the colony in investigations on the nutritional requirement for reproduction and lactation.

Vitamin B_{12} and reproduction

Diets containg soybean protein [1], vitamin-free casein and crude soybean oil meal were used in studies with different amounts of crystalline vitamin B_{12} in the diets. These data are summarized in table 3. Two series of experiments were conducted with soybean protein and different amounts of vitamin B_{12}. In one series (A) three groups of females received 0, 10 and 25 μg of crystalline vitamin B_{12} per kilogram of diet respectively. In a 4th group each female received one dose of 100 μg of vitamin B_{12} per week by subcutaneous injection.

In series B, 4 groups of females received 0, 25, 500 and 1000 μg, respectively, of crystalline vitamin B_{12} per kilogram of diet. In series C, three groups of females received the basal diet containing vitamin-free casein and supplemented with 0, 25 and 500 μg of vitamin B_{12}. And in series D, 4 groups of females received the basal diet containing crude soybean oil meal (diet 2) supplemented with 0, 10, 100 and 500 μg of vitamin B_{12} respectively.

Two groups of females (series E) received a diet composed of natural foodstuffs (diet 3) with and without vitamin B_{12}. When vitamin B_{12} was added, the average weaning weight, the average number of young weaned per litter and the percentage of litters and of young weaned were greater (with the exception of diet 3) than without vitamin B_{12}. Regardless

[1] Drackett 220 or C-1, obtained from The Drackett Company, Cincinnati. Ohio.

663

TABLE 2

Influence of nesting material at birth on the survival of the young

SHREDDED PAPER ADDED [1]	YOUNG			LITTERS		AVERAGE NUMBER OF YOUNG PER LITTER		
	Obs.	Weaned	Av. wt.	Obs.	Weaned	Born	Obs.[2]	Weaned
	no.	*%*	*gm*	*no.*	*%*			
Before birth	279	95	34.4	39	100	8.3	7.2	6.8
After birth	254	70	33.1	41	78	7.5	6.4	5.6

[1] Ten females per group.

[2] Average number observed after the litters containing more than 8 young had been reduced to 8.

TABLE 3

Vitamin B_{12} and reproduction in rats

VITAMIN B_{12}	NO. OF FEMALES	YOUNG			LITTERS		AVERAGE NUMBER OF YOUNG PER LITTER		
		Obs.	Weaned	Av. wt.	Born	Weaned	Born	Obs.[2]	Weaned
		no.	*%*	*gm*	*no.*	*%*			
Soybean protein (diet 1)									
Series A									
None	10	258	43	31.7	41	49	8.1	6.9	5.5
10	9	259	95	37.6	37	95	8.2	7.0	7.0
25	10	243	92	39.3	34	94	8.8	7.1	7.0
100 [1]	10	256	92	35.1	39	92	8.0	6.6	6.5
Series B									
None	10	272	69	28.8	51	67	6.9	5.6	5.5
25	10	274	87	35.6	40	92	7.8	6.9	6.5
500	10	230	96	35.5	30	100	9.1	7.6	7.3
1000	8	86	99	35.0	12	100	8.1	7.2	7.1
Series C									
Vitamin-free casein (diet 1)									
None	21	449	61	29.2	71	68	6.9	6.2	5.9
25	10	302	95	39.0	40	100	9.0	7.6	7.2
500	10	291	93	37.9	40	100	8.8	7.3	6.8
Series D									
Soybean oil meal (diet 2)									
None	30	706	38	25.0	114	51	6.9	6.2	4.6
10	38	492	96	36.1	70	96	8.9	7.1	7.0
100	10	164	99	35.9	21	100	10.0	7.8	7.7
500	10	161	93	34.9	21	95	9.5	7.6	7.4
Series E									
Natural foodstuffs (diet 3)									
None	10	285	95	33.2	38	100	8.7	7.5	7.1
25	10	315	91	34.2	40	98	9.9	7.9	7.3

[1] Injected per rat per week.

[2] Average number observed after the litters containing more than 8 young had been reduced to 8.

2287

of whether the amount of vitamin B_{12} supplied was 10 or 1000 μg, a high percentage of the young and litters was weaned. The young at 21 days were slightly heavier than those which received a practical diet.

The number of young weaned without vitamin B_{12} varied in different experiments. The females which received soybean protein weaned only 43% of the young in series A and 69% in series B. Those in series C, which received vitamin-free casein without vitamin B_{12}, weaned 61% of the young, and those which received soybean oil meal (series D) without vitamin B_{12} weaned 38%. It is obvious that females either had various amounts of the vitamin stored in their bodies or their diets contained different amounts of the vitamin. These data show that the mother can receive a dose of vitamin B_{12} at least 100 times that required for satisfactory reproduction without any ill effect upon the offspring. These observations are in agreement with those of Daniel, Gardiner and Ottey ('53) who found no evidence that milk from females receiving 200 μg of vitamin B_{12} per kilogram of diet was toxic to the young.

Unidentified factors for reproduction and lactation

In the study described in the previous section with large doses of vitamin B_{12}, there was no evidence that the diet was deficient in any factor required for reproduction and lactation in rats. However, in view of the reports in the literature that unrecognized vitamins are required for reproduction in rats, attempts were made to remove possible unidentified factors from two commercial soybean proteins. Soybean protein and sodium proteinate [2] were used for this purpose. The extraction procedure for each protein was as follows: approximately 25 pounds of protein were mixed with 18 to 22 gallons of water in a 30 gallon kettle and boiled gently with constant stirring for 30 minutes. The protein

[2] Archer-Daniels Company, Minneapolis, Minnesota.

665

was allowed to settle and the supernatant liquid was syphoned off. Water was added and the mixture was boiled a second time for 30 minutes. After the protein settled, the supernatant liquid was syphoned off and the protein was filtered in a large Buchner funnel. The water-extracted protein was heated in a steam-jacketed kettle with 8 gallons of 95% isopropyl alcohol and stirred constantly for 30 minutes. The hot mixture was filtered through the Buchner funnel and the residue was

TABLE 4

Purified soybean proteins and reproduction in rats

GENERATION NO.	NO. OF FEMALES	YOUNG			LITTERS		AVERAGE NUMBER OF YOUNG PER LITTER		
		Obs.	Weaned	Av. wt.	Born	Weaned	Born	Obs.[1]	Weaned
		no.	*%*	*gm*	*no.*	*%*			
				Purified soybean protein[2]					
P	19	517	94	35.0	74	100	8.3	7.0	6.0
F_1	15	400	90	35.5	58	89	8.6	7.0	6.9
F_2	15	360	96	35.8	54	100	8.4	6.7	6.4
F_3	15	391	98	34.5	60	98	8.3	6.6	6.6
F_4	15	138	100	37.3	21	100	7.0	6.6	6.6
				Purified sodium proteinate[3]					
P	10	217	82	36.3	32	82	8.6	6.8	6.8
F_1	10	268	94	39.3	38	100	8.3	7.1	6.6
F_2	10	277	94	32.1	40	98	7.7	6.9	6.7
F_3	10	174	94	34.2	28	96	6.5	6.2	6.0

[1] Average number observed after the litters containing more than 8 young had been reduced to 8.

[2] Drackett 220, obtained from The Drackett Company, Cincinnati, Ohio.

[3] Archer-Daniels Company, Minneapolis, Minnesota.

re-extracted 5 times by the same procedure with the exception that 70% isopropyl alcohol was used instead of 95%.

The extracted protein was air-dried, ground in a hammer mill and stored at room temperature. The first alcohol extract had a strong yellow color while the last was almost colorless. The results with the two soybean proteins which were purified by this procedure are summarized in table 4. The composition of the diets containing the purified soybean

2287

proteins was the same as diet 1 supplemented with 25 μg of crystalline vitamin B_{12} per kilogram of diet.

The females in the parent, F_1, F_2 and F_3 generations which received the purified soybean protein weaned 90 to 98% of their young. The females in the F_4 generation were allowed to produce only one to two litters each, but all of 138 young were weaned when the observations were discontinued. The parent generation females which received the diet containing purified sodium proteinate weaned 82% of their young, and those in F_1, F_2 and F_3 generations weaned 94%.

It is evident from these data that the diets were adequate for reproduction and lactation. Furthermore there was no evidence that an unidentified factor or factors required for reproduction in rats was removed from the protein by thorough extraction with boiling water and boiling 70% alcohol. Likewise the females were not depleted of any unidentified factors by maintaining them on the same diet through 4 to 5 generations. These conclusions are in agreement with the most recent observations of Schultze ('55) who reported that 96% of 76 young were weaned by mothers which received a diet containing 16 pure amino acids as the only source of protein.

SUMMARY

Two studies are described concerning reproduction in rats with the use of purified diets. One is the effect of large doses of vitamin B_{12} on the survival of young, and the other is the effect of different protein concentrates, including highly purified soybean protein, on reproduction. When vitamin B_{12} was added, the number of young weaned ranged from 90 to 100% regardless of whether the amount added was 10, 25, 500 or 1000 μg per kilo of diet. There was no evidence that large doses of vitamin B_{12} in the diet of the mother were toxic to the offspring. Without vitamin B_{12}, the average weaning weight and the percentage of litters and of young weaned were less than with vitamin B_{12}.

667

Two commercial soybean proteins were exhaustively extracted with boiling water and with boiling 70% isopropyl alcohol. Five generations of females which received a synthetic diet that contained these purified proteins weaned 90% or more of their young.

These data show that the diet was not deficient in an unidentified factor or factors required for reproduction and lactation in rats, and it is concluded that if unidentified factors are required for reproduction in rats, they are held very tenaciously to the protein concentrates.

ACKNOWLEDGMENTS

Acknowledgment is made to Merck and Co., Inc. for many vitamins, to Lederle Laboratories for folic acid and to Dow Chemical Company for the DL-methionine used in these studies.

LITERATURE CITED

DANIEL, L. J., M. GARDINER AND L. J. OTTEY 1953 Effect of vitamin B_{12} in the diet of the rat on the vitamin B_{12} contents of milk and livers of young. J. Nutrition, *50*: 275.

DRYDEN, L. P., A. M. HARTMAN AND C. A. CARY 1951 The relation of vitamin B_{12} deficiency to fertility of the female and birth weight of the young in rats fed purified casein rations. Ibid., *45*: 377.

——— 1952 The effect of vitamin B_{12} deficiency upon the survival of young born to rats fed purified casein rations. Ibid., *46*: 281.

GANDER, J. E., AND M. O. SCHULTZE 1955 Concerning the alleged occurrence of an "animal protein factor" required for the survival of young rats. I. Studies with unpurified rations. Ibid., *55*: 543.

MEYER, M. L., H. T. THOMPSON AND C. A. ELVEHJEM 1951 The effect of vitamin B_{12} on reproduction and lactation in rats receiving pork or beef diets. Ibid., *45*: 551.

O'DELL, B. L., J. R. WHITELEY AND A. G. HOGAN 1951 Vitamin B_{12}, a factor in prevention of hydrocephalus in infant rats. Proc. Soc. Exp. Biol. Med., *76*: 349.

PICCIONI, M., A. RABBI AND G. MORUZZI 1951 Animal protein factor for the rat present in crude casein and its relationship with vitamin B_{12}. Science, *113*: 179.

RICHARDSON, L. R., AND A. G. HOGAN 1946 Diet of mother and hydrocephalus in infant rats. J. Nutrition, *32*: 459.

RICHARDSON, L. R., AND E. L. RUSSELL 1951 Folic acid, crystalline vitamin B_{12} and penicillin in reproduction in rats. Fed. Proc., *10*: 391.

2287

SCHULTZE, M. O. 1949 Nutritional value of plant materials. II. Prevention of acute uremia of the newborn rat by vitamin B_{12}. Proc. Soc. Exp. Biol. Med., *72:* 613.

——— 1950 Nutritional value of plant materials. V. Reproduction of rats fed purified rations containing soybean protein. J. Nutrition, *42:* 587.

——— 1953 Nutritional value of plant materials. IX. The effect of various supplements on lactation failure of rats fed diets containing purified soybean proteins. Ibid., *49:* 245.

——— 1955 Concerning the alleged occurrence of an "animal protein factor" required for the survival of young rats. II. Reproduction of rats fed protein-free amino acid rations. Ibid., *55:* 559.

SCHULTZE, M. O., I. E. LIENER AND R. L. GLASS 1952 Nutritional value of plant materials. VII. Dietary factors affecting pre-weaning and post-weaning mortality of rats. Ibid., *46:* 171.

SHERMAN, W. C., H. L. SCHILT AND H. C. SCHAEFER 1955 The use of depleted rats for investigations of vitamin B_{12} and unidentified factors. Ibid., *55:* 255.

669

Brit. J. Cancer 17: 90-99

2293

EXPERIMENTAL STUDY OF THE RELATIONSHIP BETWEEN VITAMIN B_{12} AND TWO ANIMAL TUMOUR SYSTEMS

CAROLYN C. RIGBY AND MARTIN BODIAN

From The Department of Morbid Anatomy, The Hospital for Sick Children, Great Ormond Street, London

Received for publication January 18, 1963

THE encouraging results of massive vitamin B_{12} dosage in treating neuroblastoma in children (Bodian, 1959) suggested a parallel investigation of the effects of this vitamin on tumours occurring in experimental animals. Initial attempts to implant tissue from human neuroblastomata were unsuccessful, and therefore a spontaneous retroperitoneal tumour of mice was obtained (C1300, Roscoe B. Jackson Memorial Laboratory, Bar Harbour, Maine). This was originally considered to be a neuroblastoma, and although it has been shown histologically to be an undifferentiated sarcoma, it was decided to test the effects of vitamin B_{12} upon it, together with a fibrosarcoma of rats (PWA2) to broaden the scope of the study.

If vitamin B_{12} did affect tumour tissue, it would be reasonable to expect a relatively high uptake of the vitamin by such cells. The distribution of vitamin B_{12} in neoplastic and other tissues has therefore been observed in mice and rats bearing tumour transplants. As far as can be ascertained the only workers who have reported studies of the relationship of vitamin B_{12} to tumour growth are Oleson and Little (1949), Day, Payne and Dinning (1950), Miller *et al.* (1952), Woolley (1955), Bennett, Ramsey and Donnelly (1956), Miller *et al.* (1956), Georgadze (1960), Cooper and Paranchych (1961), and Nelson and Doctor (1962).

MATERIALS AND METHODS

The mice used in this investigation were of CAF1/JAX strain, obtained from the Jackson Memorial Laboratory, which was also the source of the tumour with which they were inoculated. The rats were of the August strain and were supplied by the Chester Beatty Research Institute together with the fibrosarcoma (PWA2) used for transplantation in these animals. Mice were fed on standard cubes (Diet 41) and rats similarly (Diet 86).

The basic method pursued has been to take tissue from a tumour in one animal and to inoculate this into a number of other hosts, allowing these transplants to grow and repeating the transplantation to further hosts at regular intervals. In each successive group of animals, all affected with the tumour, half received vitamin B_{12} and half acted as untreated controls.

In studying the behaviour of the murine tumour under such conditions, mature mice about 20 grammes in weight were inoculated in batches of 20 (all of the same sex—male or female—in each batch) at a single session. The inoculant was prepared by dissecting out a tumour from an affected animal, placing this in an

670

aqueous solution of penicillin and streptomycin, and removing from it 10 approximately equal samples for subcutaneous injection through a 14 S.W.G. Bashford needle into separate mice. It must be emphasised at this point that the tumours transplanted through control and vitamin-treated series of animals were kept strictly separate. Control mice thus always received tumour transplants from a control animal in the previous batch, and similarly in the case of treated animals. This segregation was essential because the tumour used displayed such a rapid rate of growth in mice that it was impracticable to keep the affected individuals alive and under treatment for more than 10 days. Thus the long-term behaviour of treated and untreated tumour tissues could only be compared by carrying them separately through repeated series of control and vitamin-treated hosts.

On the day after transplantation mice in the vitamin-treated series received their first daily dose, 5 μg. of cyanocobalamin, given subcutaneously. Eight such doses were administered, a total of 40 μg. to each animal. On the tenth day the whole batch of 10 treated and 10 untreated controls were killed and dissected. It was almost always comparatively easy to dissect the tumours, and their individual weights were noted. Control and vitamin-treated series have now been carried in parallel through 100 transplant cycles, spread over a period of nearly 3 years.

The rat fibrosarcoma has been studied in a similar manner, in parallel series of equal numbers of control and treated animals in each transplant cycle. Mature rats of about 200 grammes weight and of the same sex in each batch were inoculated with PWA2 fibrosarcoma by the same technique as used in mice, using a 6 S.W.G. needle. Because of the size of the latter, which required a skin incision, and of the greater difficulty of handling the rats, intraperitoneal anaesthesia was adopted. Initially, control and treated batches numbered 7 each in every transplant cycle, but this number has been gradually increased to 10 in succeeding cycles as stocks of rats improved. As in the case of mice the length of the transplant cycle was dictated by the rate of growth of the tumour, which proved much slower in the rat fibrosarcoma. It was found possible to keep rats alive for 5 to 7 weeks, during which period the treated animals received daily individual doses of 30 μg. of cyanocobalamin. At the end of each transplant cycle normal control and treated rats were killed and dissected, like the mice, and the same data recorded. Transplants have now been passed through 15 cycles, during a period of about 2 years.

Radioactive vitamin technique

Radioactive vitamin B_{12} was used as the ^{58}Co isotope for injection in tracer doses into mice and rats to estimate distribution of the vitamin in various tissues, including tumours. It is well to recognise that radioactivity is assessed in such experiments, and that results give a measure of the amounts of cobalt isotope present, not of vitamin B_{12}. If these should be dissociated in a particular tissue, results would be fallacious. Barbee and Johnson (1951) have shown that such dissociation occurs in the gut after oral dosage in rats; but they reported that chromatograms suggested that the ^{60}Co isotope was stored as vitamin B_{12} in the kidney. Rosenblum *et al.* (1952) demonstrated that the radioactivity in urine after parenteral doses of ^{60}Co B_{12} was due to intact radiovitamin, and Adams (1961) has recorded similar findings in man. Gräsbeck *et al.* (1961) concluded

from their experiments in rats that after parenteral doses levels of activity in kidney, liver, spleen, and a number of other tissues could be taken as a measure of the take-up of radiovitamin.

Supplies of ^{58}Co B_{12} were obtained from the Radiochemical Centre, Amersham, having a specific activity of about 1 $\mu c/1$ μg. Since it has been shown that radiovitamin B_{12} is unstable in solution (Smith, 1959), only fresh aqueous solutions were used. Dosage was adjusted between the dictates of counting technique and physiological levels; suitable doses were found to be 0·075 μg. for mice and 0·2 μg. for rats. Animals received a single subcutaneous dose and were killed in batches of 10 at intervals of 4, 24, 48, 72 and 96 hours thereafter. Each batch of 10 consisted of 5 normal and 5 tumour-inoculated animals.

The mouse neuroblastoma is a rapidly growing tumour and tends to ulcerate early. It was therefore necessary to select animals for tracer injections at the right period of tumour growth. This was most conveniently achieved by maintaining an adequate stock of transplanted animals, from which batches of 5 could be taken for injection at the appropriate stage of tumour size. This difficulty did not affect experiments on rats, because of the slower rate of growth of the PWA2 fibrosarcoma.

Batches of 10 animals (5 tumour-transplanted, 5 normal controls) were taken at the intervals noted above, and were anaesthetized, heparinized, and exsanguinated. Individuals in each batch were killed in the same order in which they had been injected to keep survival intervals as uniform as possible. Each mouse or rat was then carefully dissected to remove the tumour (except in the controls), liver, kidneys, spleen, and brain (rats only), and a sample of blood was also collected.

The radioactivity of each specimen from every animal was measured separately in a well-type scintillation counter using a thallium-activated iodide crystal, each organ giving a count of well above 10 times the background activity. Total radioactivity injected into each animal was calculated from a count on a measured specimen of the solution used.

Microbiological assay

For microbiological assay the tumours, livers, kidneys, spleens, and brains were dissected from mice and rats and stored at −10° C., the animals being taken from the main experimental series. From each transplant group assayed the above organs were separately pooled, so that there were 5 pools from the vitamin-treated animals and 5 from the batch controls. Each tissue pool was chopped up and extracted in a buffered (pH 4·8) cyanide-activated papain solution in the ratio of 1 to 20 by volume. The vitamin B_{12} activity of diluted samples of the supernatant extract was assessed by the growth of *Lactobacillus leichmannii* 313 (ATCC7830), using Dano-B_{12} assay medium, after the method described by Spray (1955) and Matthews (1962).

Spray and Taylor (1958) have shown that, using Lactobacillus, 50 per cent of vitamin B_{12} activity is alkali-stable in rat serum, though the fraction in liver tissue was less than 3 per cent. In agreement with this our assays demonstrated a similar low alkali-stable activity in liver and also in the other tissues involved in this study. This fraction was therefore ignored.

Vitamin B_{12} activity of the sample tissue extracts was estimated by photoelectric comparison of the density of bacillus growth in these and in standard

W22293

solutions of the vitamin, after incubation for 20 hours at 37° C. in a uniformly illuminated waterbath. It was found that the stock of test organism could be most conveniently maintained in a gelatin base medium, which produced dispersed cultures easier for sampling.

RESULTS

1. *Effects of Massive Dosage of Vitamin B_{12}*

(a) *C1300 mouse neuroblastoma in CAF1/JAX strain mice*

The effects of prolonged massive dosage of vitamin B_{12} on the murine tumour have been followed continuously by serial transplantation since August, 1959. To date 100 successive batches have been carried through, together with equal numbers of animals which were tumour-inoculated but received no vitamin, thus acting as controls. Initially the mice receiving vitamin B_{12} showed an increase in average tumour weight when compared with the control animals; but an opposite effect soon supervened. This diminution in growth of transplants has persisted in vitamin-treated mice; and while there have been slight vagaries from this trend in individual mice, the overall pattern in the last 88 transplant cycles has shown an average diminution of 23 per cent.

A second and identical tumour transplant series was started later than the above experiment, using a fresh sample of the same tumour. It was carried on in parallel through a further 32 transplantations. At this juncture, about a year after the commencement of this series, the tumour became extinct in the treated mice. At 10 months, shortly before the tumour became extinct, it showed a growth diminution of 60 per cent in the vitamin-treated animals, compared with the controls.

(b) *PWA2 fibrosarcoma in August strain rats*

Long term experiments similar to the above have also been carried out in rats to study the effects of massive vitamin B_{12} dosage on a different tumour, the PWA2 fibrosarcoma. Because of its slower growth rats must be kept alive much longer, and only 15 successive transplants have been carried through in a period of 2 years. Some variation in the behaviour of the tumours in the vitamin-treated rats has occurred, as is also true of the control animals; but in the treated animals, taken as a whole, there has been on average a threefold increase in the weight of tumours, compared with controls.

2. *Relative Uptake of Vitamin B_{12}*

(a) *Distribution of radioactivity after single small injections of cobalt-labelled vitamin B_{12} (^{58}Co B_{12})*

The distribution of radioactivity in certain tissues, including tumours, after tracer doses of vitamin B_{12} is shown in Tables I to IV. Both mice and rats were studied in groups of 5, with an equal number of normal animals. In both series of experiments 5 groups of animals (5 tumour-bearing and 5 normals in each) were killed at intervals of 4 to 96 hours, as the tables show. As will be evident, the distribution of radioactivity in tumours, liver, spleen, brain, and blood has been expressed in both relative and absolute terms. In Tables I and II the results are shown as percentages of the total injected dose, compared on the basis

TABLE I.—*Distribution of Radioactivity after Tracer Doses of* ^{58}Co *Vitamin* B_{12}

Average percentage of injected dose present in 1 gramme of wet tissue

	Time in hours				
	4	24	48	72	96
Tumour-bearing series of mice					
Tumour	3·6	4·4	8·3	2·9	9·0
Range	2·1–5·2	2·5–9·0	4·7–10·1	2·3–3·6	3·4–15·5
Liver	12·2	12·9	14·5	17·6	15·4
Range	9·9–14·3	9·8–15·4	10·9–17·4	14·7–21·6	11·8–17·3
Kidneys	10·1	7·5	4·7	4·8	4·1
Range	7·0–15·3	6·2–10·0	3·9–5·2	4·0–5·5	2·8–5·7
Spleen	0·6	0·7	0·6	0·8	0·7
Range	0·4–0·9	0·6–0·7	0·5–0·8	0·6–1·3	0·5–0·9
Blood (total)	5·6	5·3	3·5	2·6	2·2
Range	3·3–9·4	4·2–6·2	1·6–5·7	2·0–3·6	1·4–3·4
Control series of mice					
Liver	12·9	15·6	15·3	17·0	19·2
Range	12·0–14·1	14·8–16·1	13·2–17·4	15·4–18·8	17·3–21·2
Kidneys	11·0	6·3	6·8	5·5	6·3
Range	8·2–17·2	6·1–6·4	5·3–11·7	4·1–6·7	6·1–6·5
Spleen	0·6	0·8	0·7	0·8	0·6
Range	0·5–0·8	0·6–1·2	0·7–0·8	0·6–1·3	0·1–1·0
Blood (total)	9·2	5·6	2·9	3·1	1·9
Range	7·7–13·0	4·0–8·9	2·4–3·2	1·2–4·4	1·2–2·3

Each mouse received 0·075 μg.

TABLE II.—*Distribution of Radioactivity after Tracer Doses of* ^{58}Co *Vitamin* B_{12}

Average percentage of injected dose present in whole organs

	Time in hours				
	4	24	48	72	96
Tumour-bearing series of rats					
Tumour	1·20	0·26	0·83	0·94	0·60
Range	0·6–1·8	0·1–0·4	0·005–3·1	0·6–1·4	0·3–0·7
Liver	4·18	3·26	4·16	4·16	4·44
Range	4·0–4·3	2·7–3·6	3·6–4·8	3·9–4·8	3·7–4·8
Kidneys	16·88	18·74	17·66	17·88	17·74
Range	16·1–18·4	16·0–20·9	15·6–19·8	15·9–20·0	14·9–20·2
Spleen	1·50	0·76	0·74	0·68	0·80
Range	1·3–1·7	0·7–0·8	0·6–0·8	0·6–0·8	0·6–1·0
Blood (total)	1·56	0·50	0·44	0·32	0·44
Range	1·0–2·0	0·4–0·6	0·3–0·8	0·2–0·5	0·1–0·6
Control series of rats					
Liver	3·92	4·00	4·06	5·10	5·38
Range	3·8–4·3	3·6–4·2	2·6–4·6	4·8–5·6	5·1–5·6
Kidneys	15·24	22·12	18·84	17·86	21·94
Range	14·2–16·6	19·6–25·2	10·8–23·6	14·7–19·3	19·3–25·5
Spleen	1·38	0·84	0·66	0·90	0·54
Range	1·2–1·6	0·6–1·3	0·4–1·0	0·7–1·1	0·4–0·8
Blood (total)	0·96	0·64	0·54	0·60	0·48
Range	0·8–1·1	0·2–0·8	0·3–0·8	0·3–1·0	0·3–0·6

Each rat received 0·2 μg. Batches of five animals killed at the above intervals of time.

2293

TABLE III.—*Distribution of Radioactivity after Tracer Doses of ^{58}Co Vitamin B_{12}*

Average percentage of injected dose present in 1 gramme of wet tissue

	Time in hours				
	4	24	48	72	96
Tumour-bearing series of mice					
Tumour	10·7	17·0	14·6	13·2	12·8
Range	7·4–15·3	14·1–21·4	10·9–18·1	7·3–18·1	10·0–15·9
Liver	12·0	13·0	15·7	15·8	14·8
Range	10·2–14·6	10·6–15·4	11·4–23·2	11·8–19·6	10·8–17·9
Kidneys	39·7	31·7	19·6	18·6	17·0
Range	29·0–58·6	24·2–45·6	17·1–23·5	15·0–23·1	10·4–23·0
Spleen	4·7	5·7	5·4	6·4	5·1
Range	3·3–5·7	5·1–6·3	4·8–6·2	5·2–9·8	4·2–6·5
Blood (total)	5·6	5·3	3·5	2·6	2·2
Range	3·3–9·4	4·2–6·2	1·6–5·7	2·0–3·6	1·4–3·4
Control series of mice					
Liver	13·1	16·0	15·0	18·8	14·9
Range	11·7–14·4	14·2–17·7	12·6–16·8	16·7–20·9	14·5–15·6
Kidneys	53·4	26·6	26·5	24·3	16·0
Range	34·1–101	22·1–33·6	17·6–50·7	20·0–28·1	14·7–18·0
Spleen	4·6	6·1	5·4	5·8	4·1
Range	4·0–5·0	4·9–7·1	4·3–6·6	5·0–7·2	1·0–7·2
Blood (total)					
Range	7·7–13·0	4·0–8·9	2·4–3·2	1·2–4·4	1·2–2·3

Each mouse received 0·075 μg. Batches of five animals killed at varying intervals of time.

TABLE IV.—*Distribution of Radioactivity after Tracer Doses of ^{58}Co Vitamin B_{12}*

Average percentage of injected dose present in 1 gramme of wet tissue

	Time in hours				
	4	24	48	72	96
Tumour-bearing series of rats					
Tumour	0·94	0·76	0·82	0·60	0·48
Range	0·7–1·1	0·6–0·9	0·2–2·3	0·4–1·0	0·3–0·6
Liver	0·34	0·42	0·54	0·48	0·38
Range	0·3–0·4	0·3–0·6	0·4–0·7	0·4–0·5	0·3–0·4
Kidneys	7·9	9·24	8·48	8·42	8·32
Range	6·1–9·3	7·2–10·2	7·1–10·2	7·4–11·7	7·3–9·3
Spleen	1·48	0·96	0·84	0·76	0·66
Range	1·4–1·6	0·9–1·1	0·7–0·9	0·7–0·9	0·6–0·7
Blood (total)	1·56	0·50	0·44	0·32	0·44
Range	1·0–2·0	0·4–0·6	0·3–0·8	0·2–0·5	0·1–0·6
Control series of rats					
Liver	0·4	0·36	0·46	0·52	0·54
Range	..	0·3–0·5	0·4–0·6	0·5–0·6	0·5–0·6
Kidneys	7·82	11·34	8·68	7·9	10·2
Range	7·3–8·5	8·9–14·7	6·1–11·3	6·2–8·8	9·3–11·0
Spleen	1·66	0·98	0·80	0·68	0·76
Range	1·5–1·8	0·6–1·3	0·6–1·1	0·5–0·8	0·6–0·9
Blood (total)	0·96	0·64	0·54	0·60	0·48
Range	0·8–1·1	0·2–0·8	0·3–0·8	0·3–1·0	0·3–0·6

Each rat received 0·2 μg. Batches of five animals killed at above intervals of time.

of counts in unit time; they thus express the amounts found in whole organs. Results set out in this way do not permit direct comparison of the concentrations in individual tissues. This comparison can only be made by calculating the percentages of injected counts taken up by unit mass of the different tissues, as has been done for Tables III and IV.

Regarding the organs selected for examination, the liver is the storage organ in the mouse (Miller *et al.*, 1956) and the kidney in the rat (Okuda, 1962). Since the kidney is an excretory organ with respect to vitamin B_{12} in both species, high concentrations are to be expected in both these organs, in experiments on a short time basis. Originally a considerable variety of other organs was examined, and the spleen was chosen from these as a convenient representative of those showing relatively low radioactivity. Blood concentration was a necessary consideration because it was not practicable to assess the radioactivity of an organ separately from its blood content.

As will be at once apparent from the tables of results the murine tumour showed concentrations much higher than were found in the spleen. Tumour radioactivity reached a maximum in the first 24 hours after injection, showing thereafter a gentle fall. This descent might be due to excretion of the vitamin; but since over the period covered by the experiments the blood concentrations displayed only a slow fall, it is more likely that the diminution in tumour radioactivity reflected its rapid growth—perhaps in conjunction with a falling blood level. Despite this, the tumours showed, at 96 hours after tracer injection, levels of radioactivity not far short of those in the liver.

In contrast to the mouse neoplasm, the fibrosarcoma investigated in rats showed no outstanding concentrations in comparison with other tissues. In comparing the two series of experiments, however, two difficulties must be pointed out. Firstly, the rats received a proportionately smaller dose of radiovitamin (approximately one quarter of the dose given to mice). Secondly, the liver does not act as a storage organ in the rat. For both these resons the levels of radioactivity in rat livers were much lower than in mice. Since the kidney is both a storage and an excretory organ in the rat, it also affords no simple comparison. It will nevertheless be noted that in rats the tumour concentrations of vitamin B_{12} activity were no more than levels found in their spleens.

(*b*) *Distributions of vitamin B_{12} activity after massive and prolonged dosage as estimated by microbiological assay*

In order to examine the distribution of vitamin B_{12} activity following high and prolonged dosage, groups of rats and mice were taken from the main experimental series of the investigation. Five batches of rats and 10 of mice were studied, together with equal numbers of untreated animals as controls. It is to be noted that in these series the control animals had received tumour transplants.

The results of these experiments are shown in Table V. The kidneys, of course, show very large increases in vitamin B_{12} activity in both species, indicative of excretion. Liver levels were much raised in mice but not in rats, corroborating existing views of this organ's different roles in the two animals in relation to vitamin B_{12} storage. This difference is all the more noteworthy in consideration of the fact that the rats lived four to five times as long as the mice. Since daily vitamin dosage was in these experiments approximately proportionate to body weight, the rats received several times more vitamin than the mice, and their

v 2293

TABLE V.—*Distribution of Vitamin B_{12} Activity after Repeated High Doses*

Average vitamin B_{12} activity measured in μmg. per gramme of wet tissue

Mice.—Each mouse in the treated series received 5 μg. cyanocobalamin daily for 8 days; batches (10 mice) were killed on the tenth day.

	Control	Treated	Increase (%)
Tumour	138	262	
Range	95–220	125–562	90
Liver	382	610	
Range	245–500	400–880	60
Kidney	330	7,085	
Range	240–425	4,500–10,000	—
Spleen	323	373	
Range	225–550	275–510	15
Brain	77	91	
Range	65–95	80–110	18

Rats.—Each rat in the treated series received 30 μg. cyanocobalamin daily for 6 to 7 weeks; batches (10 rats) were killed.

	Control	Treated	Increase (%)
Tumour	111	172	
Range	65–170	100–250	55
Liver	192	222	
Range	88–192	125–300	15
Kidney	930	12,750	
Range	326–1,500	7,000–20,000	—
Spleen	248	334	
Range	128–325	160–437	29
Brain	100	138	
Range	65–170	66–250	38

tissues might be expected to show higher levels. In the case of the spleen and the brain this was indeed so, whereas the reverse obtained in the two tumours. It will be seen that the murine tumour showed an average increase of 90 per cent in vitamin B_{12} activity, compared with an increase of 55 per cent in the rat fibrosarcoma, despite the much more prolonged dosage given to rats. However, this difference in dosage should not, perhaps, be unduly stressed, in view of the augmented excretion of vitamin B_{12} which accompanies high dosage. Nevertheless, it is clear from the results of these experiments that while the mouse tumour showed an increase of vitamin B_{12} activity five or six times greater than occurred in the brain or spleen, the increase in the rat fibrosarcoma was no more than doubled in comparison with the same two organs.

DISCUSSION

The original concept which led to clinical trials of vitamin B_{12} in human cases of neuroblastoma, namely that the maturation effect of this vitamin on haemopoietic tissue might also apply to embryonic tumour cells, was only occasionally supported by the results (Bodian, 1959). Yet marked changes did occur. Instead of the expected maturation of neuroblasts into ganglion cells, there appeared to be an actual regression in the size of about 50 per cent of the human neuro-

blastomata treated. In the animal experiments prompted by these clinical results and reported here, the effects have been somewhat different. In the mouse series an inhibition of tumour growth has been observed. Whether regressive effects would have occurred in individual mouse tumours it is impossible to say, their rate of growth being such as to preclude dosage for the periods of time possible in the human neuroblastoma. In the two main series of mice in which the effects of vitamin B_{12} on transmitted C1300 tumours have been studied neoplastic growth has been markedly depressed—to the extent of 23 and 60 per cent in the two series. In the latter one the transplanted tumour eventually succumbed. It is not easy to account for the difference in degree of inhibition in these two series, for the mice in both were derived from the same strain and the experiments were identical in technique, apart from one particular.

The behaviour of the rat fibrosarcoma resembled that of the murine tumour in the latter's initial phase of growth augmentation, but in the rat series this stimulation of growth has persisted through 15 transplant cycles, and has also been more pronounced than was observed in the early stages of the mouse tumour transplants. It is apposite to mention here the early findings of Oleson and Little (1949), who recorded a similar response in Rous sarcoma growth in chicks. Unfortunately they published no metrical data, but merely stated that tumour growth was most noticeably enhanced when vitamin B_{12} dosage was combined with pteroylglutamic acid.

The difference in response of the two tumours so far studied in our experiments presumably indicates a selective action on the part of vitamin B_{12}. It might be objected that the rat fibrosarcoma has been carried through a much smaller number of serial transplantations (15) than the mouse tumour (100). However, the rats lived much longer, so that these numerically very different series in fact occupied comparable lengths of time (2 and 3 years respectively). Such simple comparisons between two different neoplasms in separate series are perhaps unlikely to yield much concrete information. More illuminating are the results of uptake studies.

As has been demonstrated, uptake of vitamin B_{12} was markedly greater in the murine tumour, whether shown by single tracer dose technique or by microbiological assay after massive dosage. With the tracer technique the difference in uptake between unit weights of the mouse and rat tumours was in the ratio of 1 to 17·6, and approximately 1 to 2 after massive dosage. Miller *et al.* (1956) have found uptake of the same order in Sprague-Dawley rats using a Walker carcinosarcoma. They considered that the relative uptake by this neoplasm indicated that vitamin B_{12} was important in the growth of the tumour. Cooper and Paranchych (1961) have observed the specific uptake of vitamin B_{12} *in vitro* by Erlich ascites tumour cells and HeLa cells. Both groups of workers held the view that the vitamin is in some way involved in the growth of some neoplasms, but they gave no indication that it actively augmented the rate of enlargement in their experiments. Day *et al.* (1950), Miller *et al.* (1952), and Georgadze (1960) have all recorded evidence of enhancement of the activity of carcinogens by vitamin B_{12}. On the contrary inhibition of carcinogenetic effect by vitamin B_{12} has been recorded by Bennett *et al.* (1956). So far no other investigators appear to have observed retardation of the growth of transplanted tumours by vitamin B_{12}. It seems likely, therefore, that neoplasms may vary in their type of response to this vitamin, as has been our experience so far. However that

may be, the C1300 tumour studied in this investigation showed not only a clearly marked diminution in growth rate in a prolonged series of transplant experiments under vitamin B_{12} dosage but also a high specific uptake of this agent.

SUMMARY

The effects of massive vitamin B_{12} dosage on serially transplanted tumours were studied in August strain rats (15 series in 2 years, PWA2 fibrosarcoma) and CAF1/JAX mice (100 series in 3 years, 32 series in one year, C1300 neuroblastoma). The vitamin treated rats' tumour growth was increased by 200 per cent, and tumour vitamin content by 55 per cent, whereas in mice corresponding figures were a decrease of 23 per cent in growth (in the shorter series the tumour became extinct after one year) and an increase of 90 per cent in vitamin content.

Estimates of the affinity of the two tumours for vitamin B_{12} using tracer technique showed concentrations in mouse tumours on average 17·6 times that in rat tumours.

These results are interpreted as indicating a selective action of vitamin B_{12} and are discussed in relation to other findings.

We wish to record our indebtedness to Dr. Sephton Smith and Mr. R. Innes for much helpful advice, and to Messrs. Ralph Addison, Michael Bland and Peter Bush. Mrs. King has been responsible for the animals used in this work. It is our pleasure to acknowledge the generous support by the British Empire Cancer Campaign mediated through the Tumour Committee of this hospital.

REFERENCES

ADAMS, J. F.—(1961) *J. clin. Path.*, **14**, 351.

BARBEE, K. W. AND JOHNSON, B. C.—(1951) *Proc. Soc. exp. Biol. N.Y.*, **76**, 720.

BENNETT, M. A., RAMSEY, J. AND DONNELLY, A. J.—(1956) *Int. Z. Vitaminforsch.*, **26**, 417.

BODIAN, M.—(1959) *Pediat. Clin. N. Amer.*, **6**, 2, 449.

COOPER, B. A. AND PARANCHYCH, W.—(1961) *Nature, Lond.*, **191**, 393.

DAY, P. A., PAYNE, L. D. AND DINNING, J. S.—(1950) *Proc. Soc. exp. Biol., N.Y.*, **74**, 854.

GEORGADZE, G. E.—(1960) *Probl. Oncol.*, **6**, 1459.

GRÄSBECK, R., IGNATIUS, R., JÄRNEFELT, J., LINDEN, H., MALI, A. AND NYBERG, W.—(1961) *Clin. Chim. Acta.*, **6**, 56.

MATTHEWS, D. M.—(1962) *Clin. Sci.*, **22**, 101.

MILLER, A., GAULL, G., LEMON, H. M. AND ROSS, J. F.—(1956) *Cancer*, **16**, 842.

Idem, GAULL, G., ROSS, J. F. AND LEMON, H. M.—(1956) *Proc. Soc. exp. Biol. N.Y.*, **93**, 33.

MILLER, E. C., PLESCIA, A. M., MILLER, J. A. AND HEIDELBERGER, C.—(1952) *J. biol. Chem.*, **196**, 863.

NELSON, R. S. AND DOCTOR, V. M.—(1962) *Gastroenterology*, **42**, 414.

OKUDA, K.—(1962) *J. Nutr.*, **77**, 131.

OLESON, J. J. AND LITTLE, P. A.—(1949) *Proc. Soc. exp. Biol. N.Y.*, **71**, 226.

ROSENBLUM, C., CHOW, B. F., CONDON, G. P. AND YAMAMOTO, R. S.—(1952) *J. biol. Chem.*, **198**, 915.

SMITH, E. L.—(1959) *Lancet*, i, 387.

SPRAY, G. H.—(1955) *Clin. Sci.*, **14**, 661.

Idem, AND TAYLOR, K. B.—(1958) *Nature, Lond.*, **182**, 1309.

WOOLLEY, D. W.—(1955) *Proc. nat. Acad. Sci., Wash.*, **41**, 111.

Science (Wash.) 162

Methylmalonic Aciduria: Metabolic Block Localization and Vitamin B_{12} Dependency

Abstract. *Methylmalonic aciduria is an inborn error of metabolism characterized by neonatal or infantile ketoacidosis. Leukocytes isolated from the peripheral blood of a 1-year-old child with this disorder converted negligible quantities of propionate-3-C^{14} to carbon dioxide, but oxidized succinate-1,4-C^{14} normally, an indication of a block in the conversion of propionate to succinate. Parenteral administration of vitamin B_{12} resulted in a reduction in methylmalonic acid excretion and an increase in propionate oxidation by leukocytes in vitro. The results suggest a mutation of methylmalonyl-CoA isomerase, a vitamin B_{12} dependent enzyme which converts methylmalonyl-CoA to succinyl-CoA, and provide the first demonstration of vitamin B_{12} "dependency" in man.*

Methylmalonic acid, barely detectable in the urine of healthy humans (< 2 mg/day), is excreted in large amounts by patients with vitamin B_{12} deficiency (*1*). This reversible biochemical disturbance occurs because methylmalonyl coenzyme A (CoA) isomerase, which catalyzes the conversion of methylmalonyl CoA to succinyl CoA, requires a cobamide coenzyme derived from vitamin B_{12} (*2*). Methylmalonic aciduria has also been observed in newborn infants and young children with severe metabolic acidosis who were not vitamin B_{12} deficient (*3*). The methylmalonic aciduria and acidosis observed in these children has been ascribed to an inborn error in the conversion of methylmalonyl CoA to succinyl CoA due to a mutation of methylmalonyl CoA isomerase.

We have described a 1-year-old boy who excreted 800 to 1000 mg of methylmalonic acid per day and also long-chain ketones during periods of keto-acidosis (*4*). Vitamin B_{12} deficiency was clinically excluded. Ingestion of a high protein diet, valine, or isoleucine increased the excretion of methylmalonic acid and long-chain ketones. Since methylmalonyl CoA is a key intermediate in the catabolism of branched-chain amino acids and fatty acids with an odd number of carbon atoms (Fig. 1), these observations were consistent with the proposed block in the catabolism of methylmalonyl CoA.

We now present evidence in vitro for a defect in propionate catabolism and demonstrate that the administration of vitamin B_{12} modifies significantly the biochemical abnormalities observed in vivo and in vitro.

Leukocytes from fasted patients were isolated from heparinized, venous blood (10 to 30 ml) by differential sedimentation in polyvinyl pyrollidone and subsequent hemolysis of erythrocytes (*5*). The leukocytes were suspended in 1 ml of Krebs-bicarbonate buffer (*p*H 7.4) containing 1.65 μmole of propionic acid and 7.7 μc of

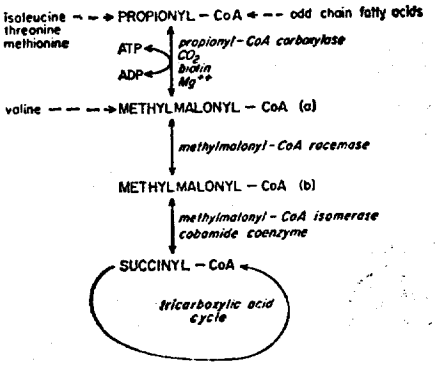


Fig. 1. Pathway of methylmalonic acid formation and catabolism, demonstrating its role as an intermediate in protein and fat metabolism. Broken arrows signify intermediate steps. The cobamide coenzyme active in the isomerization of methylmalonyl CoA to succinyl CoA in mammalian tissue is 5,6-dimethylbenzimidazolyl cobamide 5'-deoxyadenosine (5'-deoxyadenosylcobalamin) (*2*).

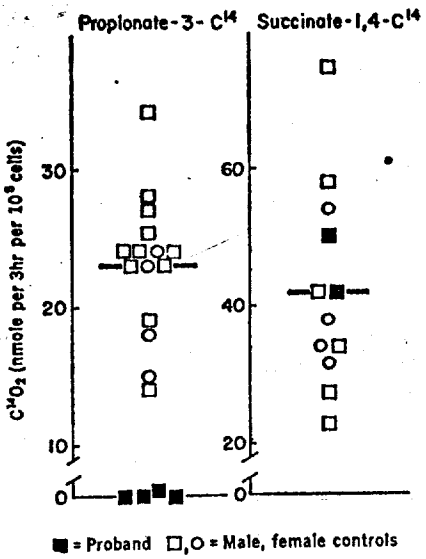


Fig. 2. Formation of $C^{14}O_2$ from propionate-3-C^{14} and succinate-1,4-C^{14} by isolated leukocytes from normal individuals and from a patient with methylmalonic aciduria. The concentration of propionate and succinate in all experiments was 1.65 mmole/liter.

propionate-3-C^{14} or 1.65 μmole of succinic acid and 5.5 μc of succinic acid-1,4-C^{14}. The mixture was gassed with 95 percent oxygen and 5 percent carbon dioxide, sealed, and incubated in modified liquid-scintillation counting vials for 180 minutes at 37°C in a Dubnoff shaker. The reaction was terminated with 1 ml of 6*N* sulfuric acid, and the evolved $C^{14}O_2$ was trapped in hyamine and counted in a liquid-scintillation spectrometer (*6*). Leukocytes from normal subjects oxidized 14 to 30 nmole of labeled propionate to $C^{14}O_2$, but cells from the patient with methylmalonic aciduria produced negligible amounts of $C^{14}O_2$ from propionate in four separate studies over a 3-week interval. The patient's leukocytes oxidized succinate normally, accentuating the significance of the abnormality in propionate oxidation.

These results (Fig. 2) and the observations in vivo (*3, 4*) show that patients with methylmalonic aciduria cannot convert methylmalonyl-CoA to succinyl-CoA, but we are not able to distinguish between an abnormality in the reactions catalyzed by the racemase or the isomerase enzymes (Fig. 1). Additional experiments suggested that methylmalonyl-CoA isomerase is defective in this disease.

On two occasions during a 2-month interval, the patient was hospitalized and placed on a constant diet containing 2 g of protein per kilogram of body weight per day. Urine samples (24-hour collections) were analyzed for methylmalonic acid by a modification of the colorimetric method (*7*) which depends on the diazotization of *p*-nitroaniline yielding a green color in the presence of substituted malonic acids. The patient excreted 800 to 1200 mg of methylmalonic acid per 24 hours (Fig. 3) on this regimen. However, when he was given 1 mg of vitamin B_{12} a day intramuscularly, methylmalonic acid excretion fell to 220 to 280 mg/day, and returned to values before treatment only after the injections were stopped. Coincident with this change in methylmalonic acid excretion was an effect on propionate oxidation by isolated leukocytes (Fig. 3). Whereas $C^{14}O_2$ production from labeled propionate was negligible during control periods, oxidation was observed on four separate occasions while the patient was receiving the vitamin or shortly thereafter.

These effects of vitamin B_{12} are significant for three reasons. First, they provide evidence (albeit indirect) for a defect in methylmalonyl CoA isomerase, because it is the only enzyme in the involved pathway which requires a cobamide coenzyme. Second, they demonstrate a vitamin B_{12} "dependency" state in man, as distinguished from vitamin B_{12} deficiency. There are precedents in microbial and mammalian systems for such coenzyme dependency. Bonner *et al.* (*8*) described tryptophan synthetase mutants in *Neurospora crassa* which could convert indoleglycerolphosphate to indole only in the presence of added pyridoxal phosphate, a cofactor for tryptophan synthetase. They suggested that the mutation affected that portion of the tryptophan synthetase genome concerned with catalytic activities involving pyridoxal phosphate.

Frimpter (see *9*) reported that administration of vitamin B_6 to patients with cystathioninuria, an inborn error due to a defect of a pyridoxal phosphate-dependent enzyme, cystathionase, reduced cystathionine excretion. He also noted that addition of pyridoxal phosphate in vitro enhanced the cystathionase activity of liver homogenates from patients with cystathioninuria. Similar findings in vivo and in vitro have been reported by Tada and associates (*10*) in patients with xanthurenic aciduria, an inborn error due to a mutation of a B_6-dependent enzyme. These and other syndromes which respond to vitamin B_6 administration (*11*) are not due to vitamin B_6 deficiency and may reflect apoenzyme mutations which reduce their affinity for pyridoxal phosphate.

In like manner, the observed vitamin B_{12} dependency in our patient can be explained if the mutant methylmalonyl CoA isomerase apoenzyme has

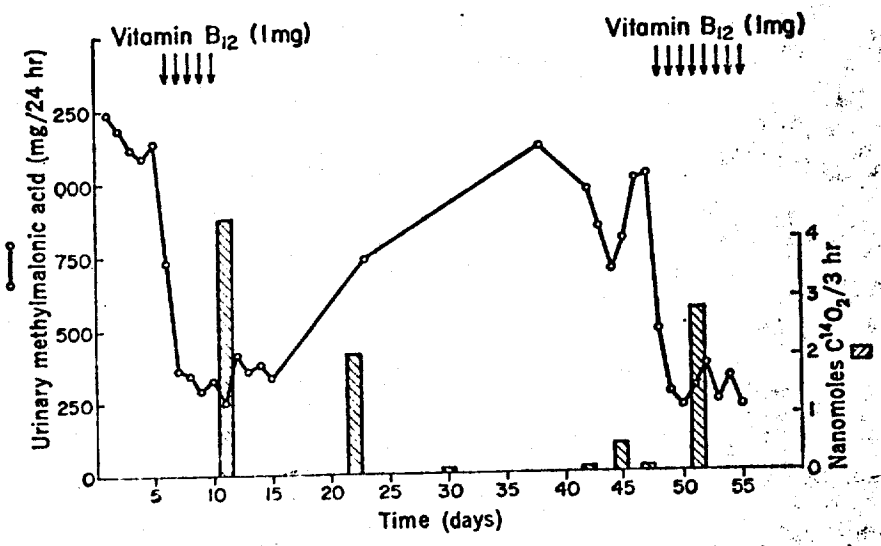


Fig. 3. Effect of parenteral vitamin B_{12} administration on methylmalonic acid excretion (left ordinate) and propionate-3-C^{14} oxidation by isolated leukocytes (right ordinate); nanomoles of $C^{14}O_2$ per 10^6 cells every 3 hours in a patient with methylmalonic aciduria. Each arrow represents single daily injection of vitamin B_{12}. Increasing the amount of vitamin B_{12} administered to 4 mg/day failed to depress urinary methylmalonic acid further.

a very low affinity for its cobamide coenzyme and if, at maximum concentration of coenzyme (as after administration of 1 mg of vitamin B_{12} daily), appreciable binding of coenzyme and of substrate occur with partial restoration of catalytic function. The mechanism of such a proposed apoenzyme-coenzyme interaction is obscure but might involve interaction of enzyme subunits or an allosteric effect on a single polypeptide. The observed vitamin B_{12} dependency could also reflect abnormalities in vitamin B_{12} transport or conversion of the vitamin to its active coenzyme form, but this possibility seems much less likely.

Finally, vitamin B_{12} administration may be useful therapeutically in patients with methylmalonic aciduria, which may not be a rare inborn error, the disorder having been described in three countries in less than 1 year (*3, 4, 12*). This disorder could be detected by urinary screening techniques in newborns, and treatment with large doses of vitamin B_{12} could prevent the characteristic and potentially lethal episodes of keto-acidosis.

LEON E. ROSENBERG
ANNE-CHARLOTTE LILLJEQVIST
YUJEN EDWARD HSIA
Departments of Pediatrics and Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

References and Notes

1. E. V. Cox and A. V. White, *Lancet* **1962-II**, 853 (1962); L. A. Barness, D. G. Young, R. Nocho, *Science* **140**, 76 (1963).
2. E. R. Stadtman, P. Overath, H. Eggerer, F. Lynen, *Biochem. Biophys. Res. Commun.* **2**, 1 (1960); R. M. Smith and K. J. Monty, *Biochem. Biophys. Res. Commun.* **1**, 105 (1959); J. R. Stern and D. L. Friedman, *ibid.* **2**, 82 (1960); P. Lengyel, R. Mazumder, S. Ochoa, *Proc. Nat. Acad. Sci. U.S.* **46**, 1312 (1960).
3. V. G. Oberholzer, B. Levin, E. A. Burgess, W. F. Young, *Arch. Dis. Child.* **42**, 492 (1967); O. Stokke, L. Eldjarn, K. R. Norum, J. Steen-Johnsen, S. Halvorsen, *Scand. J. Clin. Lab. Invest.* **20**, 313 (1967).
4. L. E. Rosenberg, A.-C. Lilljeqvist, Y. E. Hsia, *New Engl. J. Med.* **278**, 1319 (1968).
5. R. Robineaux, J. Lebrun, R. Kourilsky, A. Delauny, *Ann. Inst. Pasteur* **77**, 710 (1949); N. Dioguardi, A. Agostino, B. Lomanto, A. Piccaluga, *Haematol. Lat.* **5**, 99 (1962). Equal volumes of heparinized blood and 1.0 percent polyvinyl pyrollidone (average molecular weight 360,000) in 0.9 percent NaCl were mixed in a syringe and allowed to sediment for 45 minutes. The plasma, containing many leukocytes, was expressed into round-bottom test tubes and centrifuged at 140*g* for 15 minutes. The supernatant was decanted, and the cells were resuspended in 0.8 percent NH_4Cl with a Pasteur pipette. After 5 minutes, the tubes were centrifuged at 100*g* for 15 minutes, and the supernatant was decanted. The pellet was suspended in Krebs-bicarbonate buffer (*p*H 7.4), centrifuged at 100*g* for 15 minutes, and finally resuspended in Krebs-bicarbonate buffer. (All procedures at room temperature in nonwettable glass or plastic; all reagents contained 0.45 mg of *D*-glucose per milliliter.)
6. L. E. Rosenberg, A. N. Weinberg, S. Segal, *Biochim. Biophys. Acta* **48**, 500 (1961); R. L. Stjernholm, *J. Bacteriol.* **93**, 1657 (1967).
7. A. J. Giorgio and G. W. E. Plaut, *J. Lab. Clin. Med.* **66**, 667 (1965). Urine samples were diluted 1:40 to 1:200 with 0.1*N* HCl prior to analysis. After addition of the buffer and diazotized *p*-nitroaniline, the reaction mixtures were heated to 94°C for 3 minutes. The tubes were then stoppered and plunged into an ice bath for 1 minute before the addition of 3*N* NaOH. The tubes were held at room temperature for 10 minutes and optical density was determined. The rapid cooling and 10-minute wait were required for a linear standard curve and proportional color development.
8. D. M. Bonner, Y. Suyama, J. A. DeMoss, *Fed. Proc.* **19**, 926 (1960).
9. G. Frimpter, *Science* **14**, 1095 (1965); ———, in *Amino Acid Metabolism and Genetic Variation*, W. L. Nyhan, Ed. (McGraw-Hill, New York, 1967), p. 315.
10. K. Tada, Y. Yokoyama, H. Nakagawa, T. Yoshida, T. Arakawa, *Tohoku J. Exp. Med.* **93**, 115 (1967).
11. C. R. Scriver, *Amer. J. Dis. Child.* **113**, 109 (1967).
12. G. Morrow, L. A. Barness, V. H. Auerbach, A. M. DiGeorge, *Abstr. Soc. Pediat. Res.* **38**, 20 (1968).
13. Supported by grants from the John A. Hartford Foundation and the USPHS (AM 09527), by a research career development award to L.E.R. (AM 28087), and NIH grant FR 00125. We thank Dr. R. Stjernholm, for suggestions and discussions.

5 August 1968

J. Nat. Cancer Inst.
47(2), 277-287

2350

Some Aspects of the Role of Vitamin B_{12} and Folic Acid in DNA-Thymidine Synthesis in a Neoplastic C3H Mouse Cell Strain [1]

Jean Rotherham, Floyd M. Price,[2] Theodore T. Otani, and Virginia J. Evans,[3] *Laboratory of Biochemistry and Laboratory of Biology, National Cancer Institute,[4] Bethesda, Maryland 20014*

SUMMARY—In chemically defined medium containing adenine, deoxyuridine, and 0.57×10^{-6} M folate, but no vitamin B_{12}, the amount of DNA synthesized by a neoplastic C3H mouse cell strain, NCTC 5905, was comparatively limited. It was increased by substitution of thymidine for deoxyuridine or by addition of B_{12}. When B_{12} was present, but the folate concentration was lowered to 0.57×10^{-7} M and other components were unchanged, less DNA was synthesized; the decrease was partly reversed when deoxyuridine was replaced by thymidine. A further similar limitation was observed when adenine was left out of the B_{12}-containing medium. Incorporation of radioactivity from both ^{14}C-formate and deoxyuridine-2-^{14}C into DNA-thymidine was limited without B_{12}. Under conditions of culture requiring B_{12} for continuous cell proliferation, the requirement was met by B_{12}, methylcobalamin, or hydroxocobalamin at a concentration of 0.7×10^{-6} M. It appeared, however, that methylcobalamin does not provide a pool of methyl groups for DNA-purine or DNA-thymidine synthesis. That B_{12} functions, in part, to increase DNA-thymidine synthesis in 5905 cells in culture by raising limiting levels of folate coenzymes required in the metabolism of "one-carbon fragments" seems the most probable explanation of these findings. Additional data related to our previous studies concerning other aspects of the metabolism of nucleic acid precursors in cells of this strain are included.—J Nat Cancer Inst 47: 277–287, 1971.

IN GROWTH studies of the neoplastic C3H mouse cell strains, NCTC 3681 and 5905, and in precursor incorporation studies reported previously, we found an interrelationship between the folic acid concentration of the medium, the presence of vitamin B_{12} (cyanocobalamin), and the amount of DNA synthesized (*1–5*). Addition of B_{12} to the medium, NCTC 142 (table 1), had no effect on the amount of DNA synthesized when the medium also contained folic acid (0.57×10^{-6} M), thymidine, and either deoxyadenosine or adenine (*3*). However, over the period from 2–4 days after subculture of the cells, if the thymidine

[1] Received October 21, 1970; accepted April 26, 1971.

[2] Laboratory of Biology.

[3] We are grateful for the technical assistance of Mr. Kenneth E. Burdette and the suggestions of Dr. Walter C. Schneider and Dr. Benton B. Westfall.

[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

683

was replaced by cytidine, uridine, or deoxyuridine, the amount of DNA formed was greater when B_{12} was added than in its absence. Although this increase in DNA synthesis was less when deoxyuridine was substituted for thymidine than when cytidine or uridine was substituted, we concluded that the observed increases in DNA synthesis in the presence of B_{12} were partly due to an in-increase in the utilization of deoxyuridine for DNA-thymidine synthesis. Consideration of the additional data we are reporting in the present paper strengthens this conclusion. That this function of B_{12} is mediated indirectly through its effect on the intracellular concentration of folate coenzymes is a tentative explanation in view of the results presented. Methyl-B_{12} (methylcobalamin) and vitamin B_{12} seem to support the growth of 5905 cells equally well at a concentration of 0.7×10^{-6} M, but it appears that methyl-B_{12} does not serve as a substrate pool for methyl groups for the utilization of deoxyuridine in DNA-thymidine synthesis.

MATERIALS AND METHODS

Cell strain NCTC 3681 was derived from minced 12-day C3H/HeN mouse embryos and NCTC 2071 from adult subcutaneous connective tissue of this strain (*4*). These cells have been grown in protein-free medium NCTC 135 (table 1) for about 8–12 years. NCTC 5905 and NCTC 6324 cells were derived from strain 3681 by subculture in medium 142 (table 1) to which folic acid 0.63×10^{-6} M), deoxyadenosine (4.0×10^{-5} M), and thymidine (4.2×10^{-5} M) were added.

To obtain experimental cultures for the growth studies with vitamin B_{12}, methyl-B_{12}, and hydroxocobalamin, stock 5905 cultures were grown for 5 days after the last subculture; then the experimental media were added to these prime, confluent cultures. After 48 hours, and every 7 days thereafter, each culture was subcultured to give two cultures. These were maintained in 3 ml test medium through 10 transplant generations (70 days) with medium renewal on the 2d and 5th day after subculture. A deficient medium was defined as one which, before the tenth generation, gave an insufficient number of healthy cells to serve as an inoculum for the subsequent culture generation. In nondeficient media, cells proliferated to form a continuous sheet comparable in cell number to that of the parent culture. The basal experimental medium was 142 to which 0.57×10^{-6} M folic acid and vitamin B_{12}, hydroxocobalamin, or methyl-B_{12} was added. Medium and experimental cultures containing methyl-B_{12} were not exposed to light except briefly to a diffuse red light.

For the biochemical studies of DNA synthesis, stock cultures were grown in T-60 flasks containing 6 ml of medium: NCTC 135 for 3681 cells and for the other strains, NCTC 142 to which 0.5×10^{-6} M folic acid, 7.4×10^{-5} M adenine, and 4.1×10^{-5} M thymidine were added. They were subcultured every 7 days and the culture medium was renewed on the 2d and 4th days. Experimental cultures were obtained by subculturing in the various media described in the following data tables. Cells were harvested on the 4th and 7th days after subculture.

The methods for the isolation, purification, and determination of the RNA nucleotides of the cells have been described (*3*). However, the purification step was modified by hydrolyzing the LiOH cell extract before paper chromatography. Lithium salts were first extracted (*3*) and a small volume of 1 N HCl was added to the residue which was then heated at 100°C for 1 hour (*5*). The hydrolysis products were separated by chromatography on paper with the solvents: isopropanol-concentrated HCl (65:16.4), water to 100 ml; and isobutyric acid-0.5 N NH_4OH (40:20), *p*H 3.6.

The DNA was isolated, purified, determined, and converted to deoxynucleosides (*3*). These were isolated by paper chromatography by development with the solvents *n*-butanol-H_2O (86:14) and *n*-butanol-concentrated NH_4OH-water (86:4:10), ammonia atmosphere. Deoxynucleosides were determined and radioactivity was measured as described (*3*).

For identification and approximate estimation of degradation products of ^{14}C-labeled components of the culture media, 1.5 ml of medium was applied to an area 6 × 1 cm on a 10-cm wide strip of Whatman 3 MM paper. Various unlabeled compounds applied on top of the medium served as markers. These strips, made in triplicate, were then developed according to the procedure of Reeves *et al.* (*6*). Clear-cut separations of the compounds

TABLE 1.—Composition of NCTC medium 135 *

Component	Molar concentration	Concentration (mg/100 ml)
Amino acids:		
L-Alanine	35×10^{-5}	3.148
L-Amino-*n*-butyric acid	5.3×10^{-5}	0.551
L-Arginine-HCl	15×10^{-5}	3.116
L-Asparagine-H_2O	7.0×10^{-5}	0.919
L-Aspartic acid	7.5×10^{-5}	0.991
L-Cystine	4.4×10^{-5}	1.049
L-Glutamic acid	5.6×10^{-5}	0.826
L-Glutamine	93×10^{-5}	13.573
Glycine	18×10^{-5}	1.351
L-Histidine-HCl-H_2O	13×10^{-5}	2.665
Hydroxy-L-proline	31×10^{-5}	0.409
L-Isoleucine	14×10^{-5}	1.804
L-Leucine	16×10^{-5}	2.044
L-Lysine-HCl	21×10^{-5}	3.843
L-Methionine	3.0×10^{-5}	0.444
L-Ornithine-HCl	7.1×10^{-5}	0.941
L-Phenylalanine	10×10^{-5}	1.653
L-Proline	5.3×10^{-5}	0.613
L-Serine	10×10^{-5}	1.075
L-Taurine	3.3×10^{-5}	0.418
L-Threonine	16×10^{-5}	1.893
L-Tryptophan	8.6×10^{-5}	1.750
L-Tyrosine	9.1×10^{-5}	1.644
L-Valine	21×10^{-5}	2.500
Coenzymes:		
Diphosphopyridine nucleotide (coenzyme I)	$.095 \times 10^{-5}$	0.70
Triphosphopyridine nucleotide sodium salt	$.012 \times 10^{-5}$	0.10
Coenzyme A	0.33×10^{-5}	0.25
Cocarboxylase	0.21×10^{-5}	0.10
Flavin-adenine dinucleotide	0.13×10^{-5}	0.10
Uridine triphosphate sodium salt	0.20×10^{-5}	0.10
Nucleic acid derivatives:		
Deoxyadenosine	4.0×10^{-5}	1.00
Deoxycytidine-HCl	3.8×10^{-5}	1.00
Deoxyguanosine	3.7×10^{-5}	1.00
Thymidine	4.1×10^{-5}	1.00
5-Methylcytosine	$.080 \times 10^{-5}$	0.01
Glucose derivatives and acetate:		
D-Glucuronolactone	1.0×10^{-5}	0.18
Sodium glucuronate-H_2O	1.0×10^{-5}	0.195
D-Glucosamine-HCl	1.8×10^{-5}	0.385
Sodium acetate-$3H_2O$	25×10^{-5}	5.00
Balanced saline:		
NaCl	12×10^{-2}	680.00
KCl	$.54 \times 10^{-2}$	40.00
$CaCl_2$	$.18 \times 10^{-2}$	20.00
$MgSO_4$	$.083 \times 10^{-2}$	10.00
NaH_2PO_4-H_2O	$.10 \times 10^{-2}$	14.00
$NaHCO_3$	2.6×10^{-2}	220.00
Dextrose	$.56 \times 10^{-2}$	100.00
Other compounds:		
Phenol red		2.00
Tween 80		1.25
Ethyl alcohol	$.087 \times 10^{-2}$	4.00
Vitamins:		
Thiamine-HCl	$.0074 \times 10^{-5}$	0.0025
Riboflavin	$.0067 \times 10^{-5}$	0.0025
Pyridoxine-HCl	$.0030 \times 10^{-5}$	0.00625
Pyridoxal-HCl	$.0031 \times 10^{-5}$	0.00625
Niacin	$.0051 \times 10^{-5}$	0.00625
Niacinamide	$.0051 \times 10^{-5}$	0.00625
D-Pantothenate, calcium salt	$.011 \times 10^{-5}$	0.0025
D-Biotin	$.010 \times 10^{-5}$	0.0025
Folic acid	$.0057 \times 10^{-5}$	0.0025
Choline chloride	0.90×10^{-5}	0.125
Vitamin B_{12}	0.73×10^{-5}	1.00
I-Inositol	0.069×10^{-5}	0.0125
p-Aminobenzoic acid	$.091 \times 10^{-5}$	0.0125
Vitamin A (crystalline alcohol)	$.087 \times 10^{-5}$	0.025
Calciferol (vitamin D_2)	$.063 \times 10^{-5}$	0.025
Menadione (vitamin K_3)	$.0055 \times 10^{-5}$	0.0025
α-Tocopherol phosphate disodium salt (vitamin E)	$.0058 \times 10^{-5}$	0.0025
Reducing agents:		
Glutathione, monosodium salt	3.0×10^{-5}	1.00
Ascorbic acid	28×10^{-5}	5.00

* The composition of NCTC medium 142 was the same as NCTC 135 except that the nucleic acid derivatives, folic acid, vitamin B_{12}, the coezymes, sodium acetate, and phenol red were omitted.

2350
685

within each of the 4 groups of degradation products listed in table 4 were obtained (*see* fig. 1). Radioactive compounds were located on the strips with a Baird-Atomic radiochromatogram scanner and identified by comparison with the location of markers seen under ultraviolet light. The compounds were eluted for measurement of radioactivity.

Except for solutions containing ^{14}C-methyl-B_{12}, radioactivity was measured in a windowless gas-flow counter. Self-absorption was corrected from measurements of comparable samples to which known amounts of radioactivity were added. Radioactivity of solutions that contained ^{14}C-methyl-B_{12} was measured by counting samples in 20-ml glass scintillation vials. The samples were incorporated into a mixture of 2 ml of water and 15 ml of toluene scintillation solution containing 333 ml Triton X-100 (Packard Instrument Co., Inc., LaGrange, Ill.) and 42 ml Liquiflor (New England Nuclear Corp., Boston, Mass.) per liter.

Methyl-B_{12} was prepared by methylation of reduced hydroxocobalamin in an inert atmosphere in the dark, as described by Johnson *et al.* (*7*) and Babior (*8*). The method was adapted for the preparation of ^{14}C-methyl-B_{12} as follows. To 20 mg (14.8 μmoles) of vitamin B_{12} in a double-arm Warburg flask, from which the center well had been removed, was added 5 ml of a nitrogen-saturated 1:1 methanol-water solution. One side arm of the vessel contained 20 mg of sodium borohydride and to the other was added 0.2 ml of a cold solution of ^{14}C-methyl iodide (New England Nuclear Corp.) in methanol that contained 8.4 mg (59.1 μmoles) methyl iodide, specific activity 2.0 mc/mmole. The main compartment of the flask was kept immersed in an ice bath and the side arm containing the methyl iodide solution in a "dry-ice"-alcohol bath. After the system was flushed with nitrogen for 20 minutes, the sodium borohydride was washed into the main compartment containing B_{12}. The reduction was carried out for 45 minutes with constant shaking, care being taken to keep both the side arm containing methyl iodide and the main compartment cold. Then, in darkness, except for the occasional use of a diffuse red light about 3 feet away, the methyl iodide solution was tipped into the main flask, and the methylation was continued for 1 hour, with constant shaking and cooling. The solvent was removed by passing nitrogen over the surface while the solution was warmed in a 45°C water bath. The residue was dissolved in 2 ml of water, transferred quantitatively into a glass-stoppered test tube, and extracted with 1 ml portions of a 1:1 phenol-chloroform solution until all the color had been transferred to the phenolic phase (about 3 times). After 2 ml of water was added to the pooled phenolic phase, the mixture was extracted 10–12 times with 1–2 ml portions of a 1:1 chloroform-*n*-butanol system. The aqueous layer, then containing most of the color, was collected, cooled, and washed 2–3 times with ether to remove traces of phenol. Most of the ether in the aqueous phase was removed by passing nitrogen over the surface of the mixture while it was kept in a water bath at 45°C. The bulk of the solution, after addition of 1 drop of *n*-octanol, was concentrated to near dryness under reduced pressure in a flash evaporator at 50°C (bath temperature). The residue was dissolved in 1 ml of water and a large excess of acetone was added; a turbidity developed immediately, and during standing in the dark a crystalline material formed. The product was collected by filtration and kept in the dark. The yield was 12 mg (8.9 μmoles) or 60%. The product gave essentially a single spot after paper chromatography (ascending, 25°C, 18 hr, in the dark) in 2 solvent systems: water-saturated *sec.*-butanol and *n*-butanol:isopropanol:water (10:7:10), with a trace of slower moving materials.

Hydroxocobalamin was prepared by the reduction of vitamin B_{12} with hydrogen in the presence of freshly prepared platinum oxide catalyst (*9*), according to the method of Kaczka *et al.* (*10*). The yield of hydroxocobalamin from 50 mg (36.9 μmoles) of crystalline B_{12}, after two crystallizations, was 22.7 mg (46%). The product yielded a single spot when applied to Baker-flex silica gel 1B-F (J. T. Baker Chemical Co., Phillipsburg, N.J.) and chromatographed in *n*-butanol:isopropanol:-water (10:7:10) (*7*), for 5 hours, ascending at about 25°C. The relative R_f values of the product and vitamin B_{12} in this system were 0.07 and 1.00, respectively.

Methyl-B_{12} solutions were prepared, sterilized by filtration, and stored in a nitrogen atmosphere without exposure to light. Specific activities of

^{14}C-methyl-B_{12} solutions were calculated both on the basis of dry weight of the crystalline material and on spectrophotometric measurement of the concentration of the hydroxocobalamin formed. To study the effect of incubation with the cells on the stability of methyl-B_{12}, samples of media containing ^{14}C-methyl-B_{12} were removed from flasks on the 7th day after subculture and from flasks in which cells had not been planted but that were otherwise similarly handled. These media samples were applied to Whatman 3 MM paper with unlabeled methyl-B_{12} on top as visual markers. The chromatograms were then developed in the dark in *n*-butanol:isopropanol:water (10:7:10). Cell culture and handling of chromatograms were done with minimum exposure to light.[5]

RESULTS

A comparison was made of the amount of DNA synthesized by three long-established neoplastic mouse cell strains, from the 2d–7th day of subculture, when deoxyuridine was substituted for thymidine under the various conditions listed in the first column of table 2. At a folate level of 0.57×10^{-6} M, in the presence of adenine and vitamin B_{12}, the amounts of DNA formed were the same for the 2 deoxynucleosides, both for 5905 and 6658 cells (#4, 7). At the same folate level with adenine present, but no B_{12}, the amount of DNA formed was less when deoxyuridine was substituted for thymidine for these two cell strains (#3, 6). Without B_{12} the percent incorporation of radioactivity from deoxyuridine was also lower than in the presence of B_{12} under conditions #3, 4 and 6, 7. Relatively less DNA was synthesized also when deoxyuridine was substituted for thymidine, if B_{12} was present and the folate level was then decreased from 0.57×10^{-6} M (0.92 μmoles DNA-P for thymidine and 0.96 μmoles DNA-P for deoxyuridine) to 0.57×10^{-7} M (0.47 μmoles DNA-P for thymidine and 0.20 μmoles DNA-P for deoxyuridine) (#4, 2), as well as when adenine was omitted from this medium (#1). On addition of adenine to the low folate medium (#1, 2) containing thymidine, there was an increase in DNA synthesis. For 5905 and, perhaps not significantly, for 6658 cells, there was an increase in DNA formed when B_{12} was added to the medium containing thymidine, adenine, and 0.57×10^{-6} M folate, but the increase was proportionately less than when the thymidine was replaced by deoxyuridine. For 5905 cells a still higher level of folate was tested (#5) with B_{12} absent. The utilization of deoxyuridine was greater than at the folate concentration of one-tenth this value (#3), but under these conditions addition of B_{12} with folate (0.57×10^{-6} M) resulted in comparatively optimal levels of DNA synthesis.

The results obtained when the effect of vitamin B_{12} on DNA synthesis and the incorporation of ^{14}C-formate (sodium ^{14}C-formate, Calbiochem, Los Angeles, Calif.) was studied are shown in table 3. The experimental conditions were the same as in table 2 (#3, 4) except that the labeled precursor was ^{14}C-formate (5.2 mc/mM), and unlabeled deoxycytidine and deoxyuridine were added in place of deoxyuridine alone for comparison with paired separate experiments in which the only deoxynucleoside added was thymidine. Relative differences like those shown in table 2 (#3, 4) were found, both in the amounts of DNA synthesized and in the percentage of radioactivity incorporated into DNA from ^{14}C-formate. After 7 days of subculture, in medium containing deoxyuridine and deoxycytidine but not in that containing thymidine, the amount of DNA synthesized was greater when B_{12} had been added. However, the specific activity of the DNA-thymidine formed from ^{14}C-formate was the same as in the absence of vitamin B_{12}.

In tables 4 and 5 data are listed showing some aspects of the nature of the degradation by the cells of 4 nucleoside components of media and the extent of their utilization, by the cells, for DNA synthesis. The percent of radioactivity distributed among the component degradation products was based on the total recovered, as 100, which actually varied from about 70–100%. When expressed as percent of total cpm in DNA (table 5), the extent of utilization of

[5] Exposure to red light at 3 feet was momentary and limited to the following operations: addition of methyl-B_{12} stock solutions to the medium and of the medium to the cultures, application of medium to paper chromatograms, and removal of areas from chromatograms for transfer to vials containing liquid scintillation counting fluid.

TABLE 2.—Effects of adenine, vitamin B_{12}, and folate concentration on DNA synthesis and on the utilization of deoxyuridine for DNA-thymidine synthesis

Cell strain and medium* for subculture	Days after subculture	Number of experiments	Thymidine added: μmoles DNA-P	Deoxyuridine-2-^{14}C† added: μmoles DNA-P	Total added cpm ‡ × 10^{-5}	Percent of total cpm in DNA
#1. NCTC 3681 cells	2	6	0.59	0.59		
medium 135 (0–2 days)	4		.58	.54	2.3	0.[illegible]
medium 142 (2–7 days)	7		.72	.64	3.4	[illegible]
+ B_{12} + folate (0.57 × 10^{-7} M)	7–2		.13	.05		
#2. NCTC 3681 cells	2	7	.32	.27		
medium 142 (0–7 days)	4		.54	.32	1.9	[illegible]
+ adenine + B_{12} +	7		.79	.47	2.8	[illegible]
folate (0.57 × 10^{-7} M)	7–2		.47	.20		
#3. NCTC 5905 cells	2	8	.48	.43		
medium 142 (0–7 days)	4		.85	.56	3.0	1.[illegible]
+ adenine + folate	7		1.2	.69	4.5	1.[illegible]
(0.57 × 10^{-6} M)	7–2		.72	.26		
#4. NCTC 5905 cells	2	4	.58	.54		
medium 142 (0–7 days)	4		1.1	1.0	1.9	2.5
+ adenine + B_{12}	7		1.5	1.5	2.8	2.2
+ folate (0.57 × 10^{-6} M)	7–2		.92	.96		
#5. NCTC 5905 cells	2	8	.44	.44		
medium 142 (0–7 days)	4		.88	.76	2.4	2.1
+ adenine	7		1.1	.85	3.6	1.6
+ folate (0.57 × 10^{-5} M)	7–2		.66	.41		
#6. NCTC 6658(2071) cells	2	3	.42	.42		
medium 142 (0–7 days)	4		.77	.48	2.1	1.5
+ adenine	7		1.2	.55	2.8	1.2
+ folate (0.57 × 10^{-6} M)	7–2		.78	.13		
#7. NCTC 6658(2071) cells	2	3	.48	.38		
medium 142 (0–7 days)	4		.79	.64	1.6	2.5
+ adenine + B_{12}	7		1.3	1.2	2.7	3.7
+ folate (0.57 × 10^{-6} M)	7–2		.82	.82		

*Concentrations of vitamin B_{12} and adenine were 0.73 × 10^{-8} M and 7.4 × 10^{-5} M, respectively, and of thymidine or deoxyuridine, 4.1–4.4 × 10^{-5} M.
†Purchased from New England Nuclear Corp.
‡Counts per minute.

the 5 nucleosides seemed to be similar in view of the fact that radioactivity from uridine and cytidine appeared in both DNA-thymidine and DNA-deoxycytidine, whereas that for uracil was less. [*See also* (*2*) for growth studies in media containing thymine.] Radioactivity from the pyrimidine base of the nucleosides was incorporated in each case into the DNA-pyrimidines.

In table 6 are shown results obtained from experiments comparing utilization of methyl-B_{12} and deoxyuridine as precursors for DNA-thymidine synthesis. The cells grew well in all experiments. The values given for the amount of deoxyuridine incorporated into DNA were calculated from the specific activities and the total cpm found in the DNA.

Under the conditions of the culture methods, the ^{14}C-methyl-B_{12} appeared to be stable in the presence of growing cells, as judged by retention of radioactivity in the medium and by chromatography of the medium after exposure to the cells. The chromatograms were scanned for radioactivity, and a single sharp peak was observed to be coincident with the visible, added, unlabeled methyl-B_{12} both for medium in which cells had been grown and for medium not exposed to cells.

TABLE 3.—Effect of vitamin B_{12} on the utilization of ^{14}C-formate for DNA-thymidine synthesis by NCTC 5905 cells

	Medium*	Medium* with B_{12} added
Thymidine (4.1×10^{-5} M) added:		
μM DNA-P 2 days after subculture	0.45	0.40
4 days	.86	.88
7 days	1.2	1.2
7–2 days	.75	.80
Deoxycytidine (4.4×10^{-5} M), deoxyuridine (4.4×10^{-5} M) and ^{14}C-formate (1.5×10^{-6} M) added:		
μM DNA-P 2 days	.44	.44
4 days	.67	.86
7 days	.78	1.2
7–2 days	.36	.76
total added cpm $\times 10^{-5}$ 4 days	7.1	7.5
7 days	12.	11.
% of total cpm in DNA 4 days	.75	.96
7 days	.54	1.0
Relative specific activities (7 days)†		
DNA-deoxycytidine	6	6
-thymidine	200	230
-deoxyadenosine	3	5
-deoxyguanosine	12	12
RNA-cytidylic acid	6	5
-uridylic acid	3	4
-adenine	28	41
-guanine	25	33
Number of experiments	5	5

*Medium 142 + adenine + folate (0.57×10^{-6} M).
†Specific activity $\times 10^{6}$/total added cpm = relative specific activity. RNA degradation products include those from acid-soluble nucleotides.

Elution of the portions of the chromatograms containing the radioactive component yielded 80–85% of the radioactivity applied.

We measured the radioactivity in an alkaline solution of washed 5905 cells that had been subcultured for 7 days in medium containing 0.1[illegible] $\times 10^5$ cpm/ml ^{14}C-methyl-B_{12}. Assuming a density of 1 g/ml for the cells, their volume, 0.0[illegible] ml, was calculated from the wet weight of packed cells. The radioactivity found in the solution was 26 cpm, whereas an equal volume of medium would have contained about 130 cpm.

The minimum concentration of vitamin B_{12} required to sustain continuous proliferation of 5905 cells (table 7) was determined by the procedure described (*see* "Materials and Methods"). The purity of both the methyl-B_{12} and hydroxocobalamin was 90% or higher. Thus it was found that catalytic levels of all 3 compounds supported cell proliferation well.

DISCUSSION

We concluded from our earlier work that, unlike some species of bacteria (*11*), 5905 cells in culture utilize cytidine for DNA-thymidine synthesis in the absence of vitamin B_{12} (*2*, *3*). However, under the conditions of culture used, we observed a stimulation of cytidine utilization if B_{12} was present in the medium as well as a stimulation of deoxyuridine utilization when deoxyuridine was substituted for cytidine (*3*). The B_{12} stimulatory effect on deoxyuridine incorporation was small and less than for cytidine. Now that our observations have been extended from a period of 4 to a period of 7 days after subculture, an effect of B_{12} on deoxyuridine incorporation into DNA has become more clearly apparent.

This decrease in DNA synthesis in the absence of vitamin B_{12} might be explained as an effect on the methylation reaction involved, since the

TABLE 4.—Relative approximate extent of degradation of some ^{14}C-labeled pyrimidine nucleosides during growth of certain neoplastic murine cell strains

Labeled nucleoside added and cell strain*	Cell density (μmoles DNA-P/ml medium)	Days after subculture	Days exposure of medium to cells	Distribution of radioactivity among products (%)	
Deoxyuridine-2-^{14}C					
5905	0.05	7	3	Deoxyuridine	54
				Uracil	46
3681	.02	7	3	Deoxyuridine	85
				Uracil	15
6658 (2071)	.02	7	3	Deoxyuridine	58
				Uracil	42
Thymidine-2-^{14}C					
5905	.06	7	3	Thymidine	56
				Thymine	44
3681	.02	7	3	Thymidine	85
				Thymine	15
6658 (2071)	.05	7	3	Thymidine	61
				Thymine	39
Cytidine-^{14}C (U)					
5905	.01	1	1	Cytidine	30
				Uridine	39
				(Unidentified)	16
				Uracil	13
5905 (6324)	.01	1	1	Cytidine	25
				Uridine	43
				(Unidentified)	20
				Uracil	12
5905 (6324)	.02	7	3	Cytidine	17
				Uridine	14
				(Unidentified)	30
				Uracil	38
Deoxycytidine-^{14}C (U)					
5905 (6324)	.03	7	3	Deoxyuridine	71
				Uracil	31

* Medium for subculture of cell strain 3681: 142 + adenine + vitamin B_{12} + folate (0.57×10^{-7} M); cell strains 6658 and 5905: 142 + adenine or deoxyadenosine + vitamin B_{12} + folate (0.57×10^{-6} M).

TABLE 5.—Relative utilization of some ^{14}C-labeled pyrimidine compounds for synthesis of DNA-thymidine by NCTC 5905 cells

Labeled compound	μmoles DNA-P	Days after subculture	Total cpm $\times 10^{-5}$	% Total cpm in DNA	Relative specific activities			
					RNA		DNA	
					Uridine	Cytidine	Thymidine	Deoxycytidine
Uracil-2-^{14}C	1.3	7*	4.6	0.60	140	90	—	—
Uridine-2-^{14}C	1.4	7*	5.0	6.7	840	990	900	1000
Uridine-^{14}C(U)	1.1	4†	3.9	5.8	1200	1300	1000	1100
Cytidine-^{14}C(U)	1.1	4†	7.9	5.2	1000	1300	1000	1400
Deoxycytidine-2-^{14}C	1.4	7*	5.9	4.1	—	—	810	370
Deoxycytidine-^{14}C(U)	0.9	4†	6.0	3.8			1300	380
Thymidine-2-^{14}C	0.91	4†	9.4	3.3			1500	—
Deoxyuridine-2-^{14}C	1.5	7*	2.8	2.2	—	—	—	—

*Medium for subculture: 142 + adenine + vitamin B_{12} + folate (0.57×10^{-6} M) (labeled compounds, 3.3–4.4 $\times 10^{-6}$ M).
†Medium for subculture: 141 + deoxyadenosine + vitamin B_{12} + folate (0.57×10^{-6} M) (labeled compounds, 3.7–4.4 $\times 10^{-6}$ M). *See* (5).

TABLE 6.—Comparison of incorporation by NCTC 5905 cells of radioactivity into DNA from ^{14}C-methyl-B_{12} and deoxyuridine-2-^{14}C

Experiment No.	1*	2*	3†	4‡	5†	6‡	7*	8§
μmoles DNA-P 7 days after subculture	1.4	1.6	.87	1.2	1.1	1.0	1.1	.95
Total added cpm × 10^{-5}								
methyl-B_{12}	3.8	3.8	8.9	8.9	8.9	8.9	12	14
deoxyuridine	15	15	—	—	—	—	—	—
Specific activities, cpm/μmole × 10^{-5}								
methyl-B_{12}	21	21	10	10	10	10	13	13
deoxyuridine	3.2	3.2	—	—	—	—	—	—
Total cpm × 10^{-5} in DNA								
methyl-B_{12}	0	0	0	0	0	0	0	0
deoxyuridine	0.52	0.72	—	—	—	—	—	—
Calculated μmoles deoxyuridine incorporated into DNA	0.16	0.23	—	—	—	—	—	—

*Medium 142 + adenine + folate (0.57×10^{-6} M) + deoxyuridine (4.8×10^{-5} M).
†Medium 142 + folate (0.57×10^{-6} M) + vitamin B_{12} (0.73×10^{-8} M).
‡Medium 142 + folate (0.57×10^{-6} M).
§Medium 142 + adenine + folate (0.57×10^{-6} M) + deoxyuridine (4.8×10^{-5} M) + vitamin B_{12} (0.73×10^{-8} M).

TABLE 7.—Effects of vitamin B_{12}, methyl-B_{12}, and hydroxocobalamin on the proliferation of NCTC 5905 cells*

		Survival of cultures for 10 generations
Vitamin B_{12}	$.73 \times 10^{-6}$M	+
	$.73 \times 10^{-7}$M	−
	$.73 \times 10^{-8}$M	−
Methyl-B_{12}	$.73 \times 10^{-6}$M	+
	$.73 \times 10^{-7}$M	+
	$.73 \times 10^{-8}$M	+
	$.73 \times 10^{-9}$M	−
Hydroxocobalamin	$.73 \times 10^{-6}$M	+
	$.73 \times 10^{-7}$M	+

*Medium 142 + folate (0.57×10^{-6} M).

limitation was overcome by substitution of thymidine for deoxyuridine when other factors remained the same. This suggestion is compatible with the results of *in vitro* studies of chick embryo mitochondria reported by Bolinder and Reichard (*12*). Since a decrease in DNA synthesis was also observed when B_{12} was present, but the folate concentration was lowered tenfold and the decrease was partly reversed by the substitution of thymidine for deoxyuridine, it appears that the observed B_{12} effect on the methylation reaction might be an indirect effect of B_{12} on the synthesis of certain folate coenzymes. If this were due to the function of B_{12} in the formation of a coenzyme for homocysteine-methionine methyl transferase (*13*), or for another methyl transferase system, an increase in critical levels of tetrahydrofolate or other folate coenzymes might be a factor contributing to the observed increase in DNA-thymidine synthesis (*13, 14*).

This concept is supported by the observation that when adenine was omitted from the medium containing B_{12} and a tenfold lower concentration of folate, DNA synthesis was limited still further, a limitation again partly overcome by substitution of thymidine. As shown in (*2*), B_{12} was required for continuous cell proliferation in medium #1 (table 2) but could be replaced by adenine. The specific activity of the DNA-thymidine formed by 5905 cells in the presence of ^{14}C-formate was the same whether or not the medium contained B_{12}, but the amount of DNA synthesized was greater in the B_{12}-containing medium. Probably the observed B_{12} stimulation involves both the utilization of deoxyuridine and formate for DNA-thymidine synthesis.

Stahlberg suggested (*15*) that methyl-B_{12} might act as a carrier or be the form in which the cobalamin is active as a coenzyme for methyl-group transfer in methyl transferase systems. The occurrence of methyl-B_{12} in human plasma has been reported (*15, 16*). It appears from our results that it

2350

does not act as a substrate pool for methyl groups either for DNA-thymidine or DNA-purine synthesis by 5905 cells in culture. However, it may be the prosthetic group of a methyl transferase in these cells, possibly a part of a homocysteine-methionine methyl transferase system. The experiments in table 7 were conducted to determine whether a contamination of the methyl-B_{12} or hydroxocobalamin with 10% vitamin B_{12} might account for the ability of the former 2 compounds to support proliferation of these cells. This possibility appears to be unlikely. The effects noted with vitamin B_{12} and methyl-B_{12} were observed in two different experiments and with hydroxocobalamin in only one. Therefore, we believe the question of significance of the observed 100-fold difference between methyl-B_{12} and B_{12} requires further testing.

There was a more extensive degradation of cytidine than deoxyuridine in the cell strains examined and a relatively lower extent of utilization of uracil for nucleic acid synthesis. Deoxycytidine was readily converted to deoxyuridine, but from a few observations of the ability of these two precursors to support continued growth of NCTC 3681 cells, in what seemed to be a limiting concentration of folate (*2*), the two behaved differently. It appeared that deoxycytidine supported a higher rate of proliferation.

Contrary to a report concerning DNA-synthesis by HeLa cells, in which tritium-labeled deoxycytidine was used in protein-containing medium (*17*), ^{14}C was incorporated from the base of deoxycytidine into the DNA-thymidine of 5905 cells.

REFERENCES

(*1*) Rotherham J, Price FM, Evans VJ: Some aspects of the role of folic acid in nucleic acid synthesis in a neoplastic C3H mouse cell strain. J Nat Cancer Inst 41:781–788, 1968

(*2*) Price FM, Rotherham J, Evans VJ: Some aspects of the role of vitamin B_{12} in nucleic acid synthesis in a neoplastic C3H mouse cell strain. I. Growth studies. J Nat Cancer Inst 44:85–90, 1970

(*3*) Rotherham J, Price FM, Evans VJ: Some aspects of the role of vitamin B_{12} in nucleic acid synthesis in a neoplastic C3H mouse cell strain. II. Incorporation of precursors. J Nat Cancer Inst 44:91–97, 1970

(*4*) Evans VJ, Bryant JC, Kerr HA, et al: Chemically defined media for cultivation of long-term cell strains from four mammalian species. Exp Cell Res 36:4[illegible]–474, 1964

(*5*) Yoshida H, Shibata M: A rapid method for [illegible] determination of the major-base composi[illegible] of ribonucleic acid. J Biochem 66:737–738, 19[illegible]

(*6*) Reeves WJ Jr, Seid AS, Greenberg DM: A new paper chromatography solvent system [illegible] pyrimidine-pyrimidine riboside-pyrimidine [illegible]oxy-riboside mixtures. Anal Biochem 30:474–47[illegible]

(*7*) Johnson AW, Mervyn L, Shaw N, et al: A [illegible]rial synthesis of the vitamin B_{12} coenzyme and [illegible] of its analogues. J Chem Soc 1963:4146–4156, [illegible]

(*8*) Babior BM: The mechanism of action of eth[illegible]ola-mine deaminase. III. Inhibition by coenzy[illegible] B_{12} analogues. J Biol Chem 244:2917–2926, 1969

(*9*) Adams R, Voorhees V, Shriner RL: Platinum catalyst for reductions (platinic oxide). *In* Organic Synthesis, collective vol I, 2d ed. (Gilman H, ed.). New York, John Wiley & Sons, Inc, 1941, pp 463–470

(*10*) Kaczka EA, Denkewalter RG, Holland A, et al: Vitamin B_{12}. XIII. Additional data on vitamin B_{12a}. J Amer Chem Soc 73:335–337, 1951

(*11*) Spell WH Jr, Dinning JS: A requirement for vitamin B_{12} in the conversion of ribose to deoxyribose by *Lactobacillus leichmanii*. J Amer Chem Soc 81: 3804–3805, 1959

(*12*) Bolinder A, Reichard P: The biosynthesis of deoxyribonucleic acid by the chick embryo. III. Influence of vitamin B_{12} deficiency. J Biol Chem 234:2723–2727, 1959

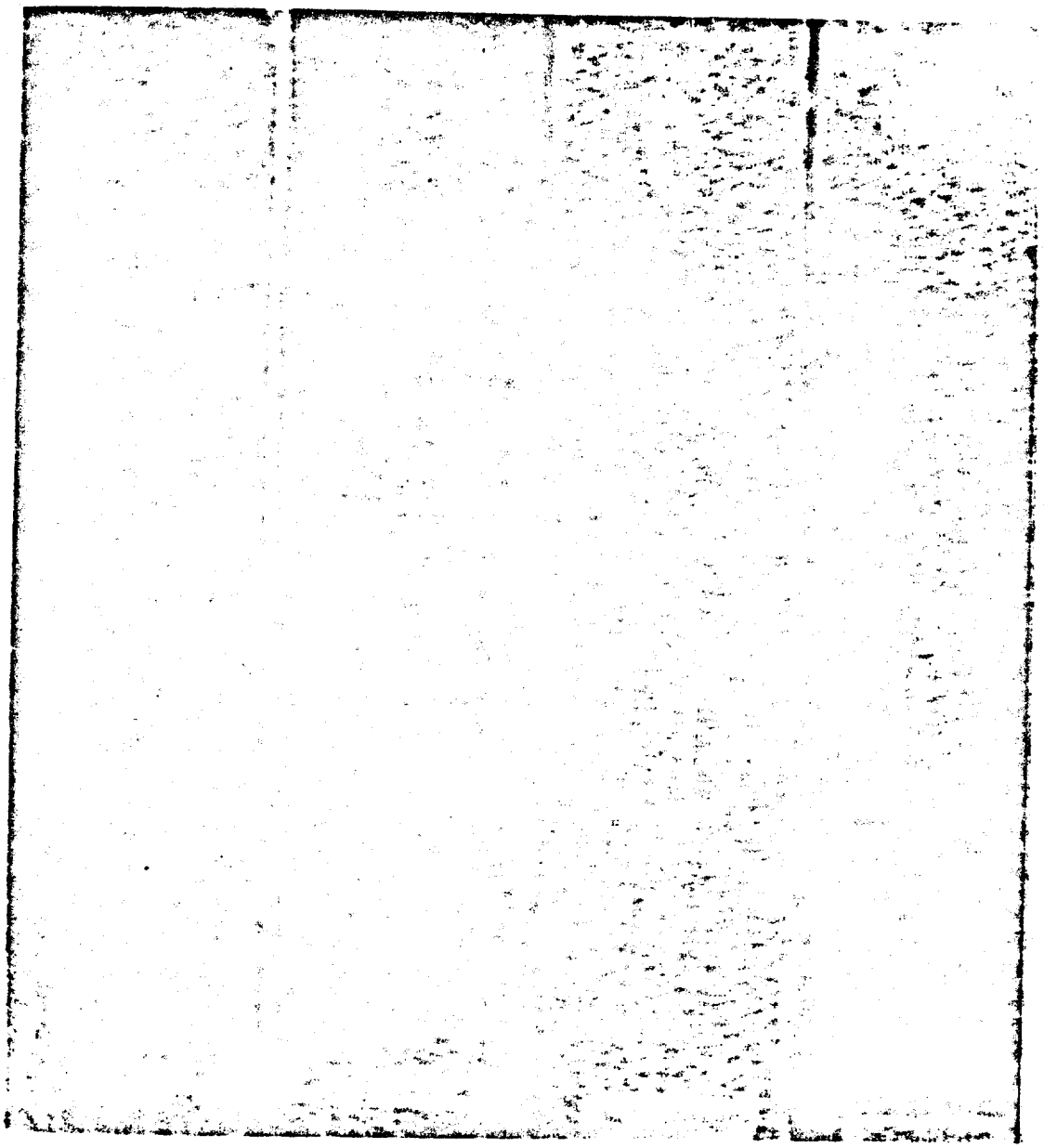
(*13*) Weissbach H, Taylor RT: Metabolic role of vitamin B_{12}. Vitamins & Hormones (NY) 26:395–412, 1968

(*14*) Friedkin M: Enzymatic aspects of folic acid. Ann Rev Biochem 32:185–214, 1963

(*15*) Stahlberg K: Studies on methyl-B_{12} in man. Scand J Haemat Suppl 1:1–99, 1967

(*16*) Linnell JC, Mackenzie HM, Wilson J, et al: Patterns of plasma cobalamins in control subjects and in cases of vitamins B_{12} deficiency. J Clin Path 22:545–550, 1969

(*17*) Mueller D: Percentage participation of exogenous deoxycytidine in the synthesis of DNA cytosine and DNA thymine in HeLa cells. Virchows Arch B 2:62–73, 1969



[illegible]

J. Indian Med. Assoc.
36: 262

V 235-2

CORRESPONDENCE

The Editor is not responsible for the views expressed by correspondents

Death After An Injection of Vitamin B_{12}

SIR,—A Bata employee, Hindu, male, aged [illegible] under insulin therapy for diabetes. Injection of thiamine hydrochloride, 100 mg., was advised for associated peripheral neuritis. He received two injections of the latter drug but each time he had mild reactions in the form of urticaria which was treated with adrenaline by injection and an antihistaminic by mouth. Injection of thiamine hydrochloride was stopped and the patient was advised a course of vitamin B_{12}, 500 mcg. intramuscularly.

On 15-12-60 he worked in the factory from 6 P.M. to 8-30 A.M. and went to the outpatient department (O.P.D.) for the injection. His urine which was examined the same morning contained 3·5 per cent sugar. At about 9-30 A.M. he received his first intramuscular injection of vitamin B_{12} at the O.P.D. Macrabin, 500 mcg. in 0·5 ml. dose (from a freshly purchased vial of 5 ml. containing 1000 mcg. per ml.) was injected into the deltoid muscle.

Immediately after the injection the patient became very restless and collapsed. The pulse became imperceptible, and there was respiratory difficulty. The symptoms resembled those of anaphylactic shock. Adrenaline was injected immediately. Percorten and antihistaminic drugs were also injected. But all attempts at resuscitation failed and the patient expired within 15 minutes.

Anaphylactic shock after injection of vitamin B_{12} resulting in death must be very rare. Comments throwing light on this subject will be highly appreciated.

I am, etc.,

Batanagar, 24 Parganas — A. N. ROY

Medical Facilities in Tea Plantation

SIR,—It is gratifying to note that our National Government is taking active interest as regards the health of the tea garden population, which was utterly neglected during alien rule. Under the purview of the Plantation Act of 1951 many welfare measures are going to be implemented in plantations in the best interest of the workers.

J. Lab. Clin. Med. 42:860

#2425

INTRINSIC FACTOR STUDIES

II. The Effect of Gastric Juice on the Urinary Excretion of Radioactivity After the Oral Administration of Radioactive Vitamin B_{12}

Robert F. Schilling, M.D.
Madison, Wis.

THE simultaneous oral administration of normal human gastric juice and small quantities of vitamin B_{12} results in hematopoiesis in patients with pernicious anemia in relapse, while oral vitamin B_{12} alone in similar amounts is usually ineffective.[1, 2, 3, 4, 8] The hematopoietic activity of intravenously administered vitamin B_{12} was not enhanced by the simultaneous intravenous administration of sterile Berkefeld filtered gastric juice in amounts known to be active as intrinsic factor when given orally.[5] Vitamin B_{12} by parenteral injection regularly corrects the hematologic abnormalities and arrests the neurologic disorder in pernicious anemia. Heinle and associates[6] have reported that the fecal excretion of radioactivity in four pernicious anemia patients receiving 0.5 μg of radioactive vitamin B_{12} ($B_{12}Co^{60}$) by mouth was definitely greater than in normal subjects. They reported that the simultaneous oral administration of an intrinsic factor preparation and $B_{12}Co^{60}$ in three pernicious anemia patients was followed by a definite reduction of fecal radioactivity. The simplest interpretation of the above information is that the function of intrinsic factor is to promote the absorption of vitamin B_{12} from the intestinal tract.

This paper reports observations on the effect of orally administered normal human gastric juice upon the quantity of radioactivity in the urine of pernicious anemia patients who were given a small oral dose of radioactive vitamin B_{12}.[9]

METHODS

A single lot of vitamin B_{12} containing radioactive cobalt (Co^{60}) and having a specific activity of 245 microcuries per milligram (μc/mg.)* was used as the tracer vitamin. In a windowless gas-flow Geiger counter this material contained approximately 280,000 counts per minute per microgram (c/m/μg). Urine samples were collected for twenty-four hours after the oral administration of radioactive vitamin according to the plan detailed below, and the radioactivity in the urine was determined by evaporating 2 ml. to dryness in a cup-type sample pan and counting in a windowless flow counter. All urine samples were counted in duplicate, and the data presented are an average of the two. When agreement between the duplicate counts was not close, two more samples were dried and counted.

After voiding the control specimen of urine, fasting subjects were given 2 μg of $B_{12}Co^{60}$ in fluid by mouth at 8:30 A.M., and one hour later they ate breakfast of coffee, toast, and fruit juice. Two hours after the *oral dose of radioactive* vitamin B_{12} each patient was given a *subcutaneous injection of 1000 μg of nonradioactive* vitamin B_{12}.† Total urine

From the Department of Medicine, University of Wisconsin Medical School.

This investigation was supported by a research grant (A-69) from The National Institute of Arthritis and Metabolic Diseases of The National Institutes of Health, Public Health Service.

Presented before the Twenty-Sixth Annual Meeting of the Central Society for Clinical Research, Chicago, Ill., Oct. 30-31, 1953.

Received for publication July 20, 1953.

*Kindly furnished by Dr. Chas. Rosenblum, Merck and Company, Inc., Rahway, N. J.

†Kindly furnished by Dr. R. A. Peterson, Merck and Company, Inc., Rahway, N. J.

volumes were collected at 10:30 A.M., 12:30 P.M., 2:30 P.M., 4:30 P.M., 8:30 P.M., and 8:30 A.M., so that the radioactivity excreted in twenty-four hours could readily be calculated. Lunch and dinner were allowed as usual. The pernicious anemia patients had all been seen in relapse at one time or another, but when the present observations were made all were in satisfactory hematologic remission because of therapy.

Human gastric juice was collected, neutralized, and filtered as described previously.[10] Only juice secreted in response to histamine was utilized in this study.

When boiled gastric juice was used it was placed in a bath of boiling water for ten minutes and cooled before the $B_{12}Co^{60}$ was added. An exception was the observation of May 13, 1953, in patient A, when a mixture of $B_{12}Co^{60}$ and gastric juice was placed in boiling water.

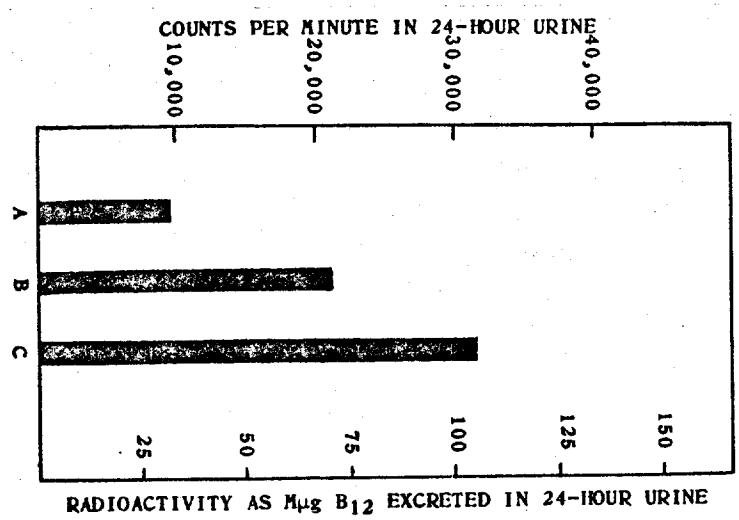


Fig. 1.—Urinary excretion of radioactivity in three normal persons given 2 μg $B_{12}Co^{60}$ orally, and two hours later, 1000 μg nonradioactive B_{12} subcutaneously.

RESULTS

Six normal people all excreted definite amounts of radioactivity in the urine when 2 μg of radioactive vitamin B_{12} were administered orally in 100 ml. of water and 1,000 μg of nonradioactive B_{12} were given subcutaneously under the conditions described above. Fig. 1 shows the urine radioactivity in 3 normal, young, control subjects. Data on 3 other normal persons are not pictured because urine collections were halted at the end of six to eighteen hours, at which time an easily detectable amount of radioactivity was being excreted in the urine. Six patients with pernicious anemia in remission excreted no detectable radioactivity in their urine when the $B_{12}Co^{60}$ was administered by mouth with water or boiled gastric juice. When in a different test period in each patient active gastric juice was administered with the oral $B_{12}Co^{60}$, each excreted easily detectable radioactivity in the urine beginning about four to six hours after the oral dose. The data are shown in Fig. 2 and Table I. Plasma samples showed no detectable radioactivity.

In the observation of April 17, 1953, on patient D, active gastric juice was given twelve hours after the oral $B_{12}Co^{60}$, and ten hours after the injection of nonradioactive vitamin B_{12}. No radioactivity appeared in the urine for thirty-six hours after the oral $B_{12}Co^{60}$.

The lack of effect of oxytetracyclene (Terramycin) upon urine radioactivity is documented in Table I, patient C: observations dated May 1, 1953, and May 5, 1953, were conducted while the patient was receiving 250 mg. of Terramycin orally every six hours; the antibiotic administration was begun on April 27, 1953. It will be noted that no radioactivity appeared in the urine when $B_{12}Co^{60}$ was given in water (May 1, 1953) but when given in active gastric juice (May 5, 1953) definite radioactivity appeared in the urine.

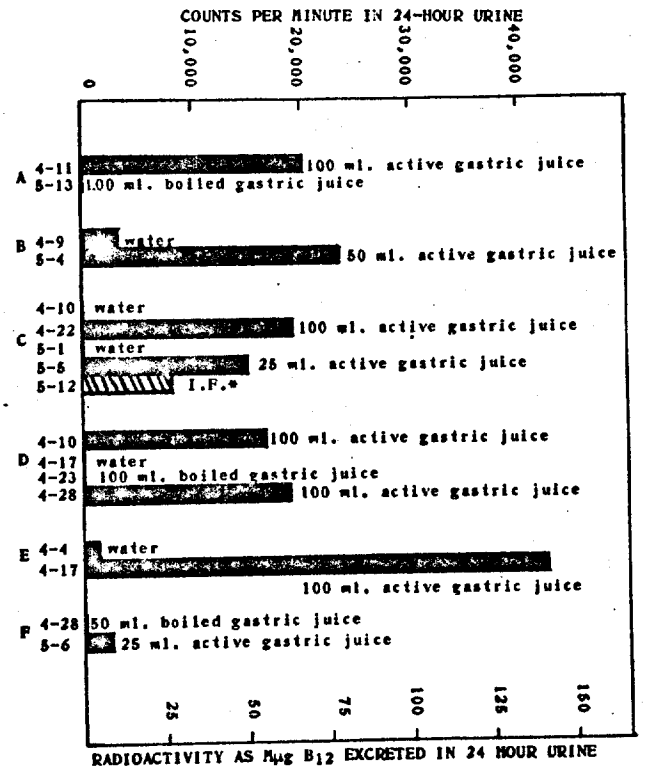


Fig. 2.—Urinary excretion of radioactivity in six pernicious anemia patients given 2 μg $B_{12}Co^{60}$ orally with gastric juice or water. 1000 μg nonradioactive B_{12} was given subcutaneously in each observation two hours after the oral $B_{12}Co^{60}$.
*I.F. = 100 mg. Intrinsic Factor Concentrate prepared from hog duodenum.

Observations on some of these and other subjects showed that when the 1,000 μg injection of nonradioactive vitamin B_{12} was omitted no radioactivity was detected in the urine of normal persons given oral $B_{12}Co^{60}$, or pernicious anemia subjects given normal human gastric juice and $B_{12}Co^{60}$ by mouth.

TABLE I. EFFECT OF GASTRIC JUICE UPON URINE RADIOACTIVITY IN PERNICIOUS ANEMIA PATIENTS GIVEN RADIOACTIVE VITAMIN B_{12} BY MOUTH AND NONRADIOACTIVE B_{12} BY PARENTERAL INJECTION

PATIENT	DATE	ORAL $B_{12}Co^{60}$ MICROGRAMS	NORMAL HUMAN GASTRIC JUICE† (ML.)	RADIOACTIVITY IN URINE COUNTS/MINUTE/ML. HOURS AFTER ORAL $B_{12}Co^{60}$						
				CONTROL	0-2	2-4	4-6	6-8	8-12	12-24
A	4/11/53	2	100	3	1	5	19	22	42	15
	5/13/53	2	100 (Boiled)§	2	4	3	1	2	2	0
B	4/ 9/53	2	0*	2	2	3	2	2	4	1
	5/ 4/53	2	50	1	1	2	26	30	24	30
C	4/10/53	2	0*	2	0	2	1	0	4	1
	4/22/53	2	100	5	4	6	18	46	31	26
	5/ 1/53	2	0*	2	1	1	2	2	2	3
	5/ 5/53	2	25	0	1	3	8	23	36	13
	5/12/53	2	I.F.‖	2	2	1	3	11	10	9
D	4/10/53	2	100	3	1	2	19	35	26	17
	4/17/53	2	100‡	3	1	1	1	2	2	0
	4/23/53	2	100 (Boiled)	3	2	3	2	2	1	1
	4/28/53	2	100	3	3	2	13	20	20	15
E	4/ 4/53	2	0*	1	0	0	2	3	3	1
	4/17/53	2	100	1	5	4	29	27	36	13
F	4/29/53	2	50 (Boiled)	1	2	0	2	5	2	2
	5/ 6/53	2	25	1	2	5	11	1	5	5

*Water used to dilute $B_{12}Co^{60}$.
†Given with oral $B_{12}Co^{60}$ except ‡ which was given twelve hours later.
§$B_{12}Co^{60}$ and gastric juice mixed and placed in boiling water for ten minutes.
‖100 mg. Intrinsic Factor concentrate from hog duodenum.

DISCUSSION

Progress toward the isolation of Castle's intrinsic factor has been hampered by the necessity of a tedious bioassay in patients with pernicious anemia in relapse. Heinle and co-workers[6] observed that pernicious anemia patients in remission who were given $B_{12}Co^{60}$ by mouth simultaneously with intrinsic factor excreted less radioactivity in the stool than when the radioactive vitamin was given without intrinsic factor. The observation was regarded as a new tool for the study of intrinsic factor activity. Meyer[7] has reported data somewhat at variance with those of Heinle and co-workers, but the oral dose of $B_{12}Co^{60}$ administered by Meyer was 1.0 μg, while Heinle and collaborators gave only 0.5 μg. The larger quantity given by Meyer may have obscured the effect of intrinsic factor. Preliminary data collected by Ley[11] show that the simultaneous oral administration of intrinsic factor does cause a decrease in fecal radioactivity in totally gastrectomized patients given radioactive B_{12} by mouth.

Using microbiological methods several observers[12, 13] have failed to demonstrate increased plasma or urine levels of vitamin B_{12} after oral administration of the vitamin to normal subjects or patients with pernicious anemia.

Conley and associates[12] and Mollin and Ross[14] have reported that as the parenteral dose of vitamin B_{12} is increased the percentage of that dose appearing in the urine in the following eight to twelve hours is increased. At the level of 1,000 μg of vitamin B_{12} subcutaneously approximately 80 to 100 per cent of the dose appeared in the urine in twenty-four hours. Because of these findings it occurred to me that a large parenteral injection of nonradioactive vitamin B_{12} might enhance the per cent excretion of radioactive B_{12}. This indeed

appears to be the case, for urine radioactivity was easily detected in normal subjects who received *oral* $B_{12}Co^{60}$ and a saturating parenteral injection of *nonradioactive* B_{12}. Normal subjects who were not given the parenteral vitamin excreted no detectable radioactivity in the urine after an oral dose of 2 μg of $B_{12}Co^{60}$.

The data offer no direct evidence as to the form in which the Co^{60} is excreted by the kidney. A sample of the radioactive urine from one of the control subjects was extracted with N-butanol and the radioactivity in the butanol extract was approximately 60 per cent of that present in the original sample. When $B_{12}Co^{60}$ was added to normal urine and the mixture extracted with N-butanol, a similar percentage of the radioactivity appeared in the extract. The fact that radioactivity is excreted in the urine only when the kidneys are excreting large quantities of nonradioactive vitamin B_{12} suggests that the radioactivity is in a molecule which is in competition with the nonradioactive B_{12} for the renal reabsorptve mechanisms. An attractive alternative explanation is that the nonradioactive B_{12} given by parenteral injection saturates the plasma binding capacity for B_{12} and, therefore, the $B_{12}Co^{60}$ absorbed from the gut is not bound to the plasma protein and hence appears in the glomerular filtrate. Mollin and Ross[14] have reported some observations on the ability of serum to combine with vitamin B_{12} to render the vitamin unavailable to certain microbes. In another study[10] paper electrophoresis of a mixture of serum and $B_{12}Co^{60}$ demonstrated that some of the vitamin moved with albumin fraction while the other part of the vitamin moved as it did when subjected to paper electrophoresis in saline solution.

The only known variable in the observations reported in this paper was the presence or absence of active gastric juice in the upper gastrointestinal tract with the $B_{12}Co^{60}$. Castle and Ham[15] have reported that when a twelve-hour lapse occurred between the administration of the extrinsic and the intrinsic factors no hemopoietic effect was observed in patients with pernicious anemia in relapse. In the present study, when active gastric juice was given 12 hours after the oral dose of $B_{12}Co^{60}$ and ten hours after the parenteral nonradioactive B_{12}, no radioactivity appeared in the urine.

In the observation dated May 12, 1953, in patient C, 100 mg. of intrinsic factor concentrate* prepared from hog duodenum was given with the $B_{12}Co^{60}$, and definite radioactivity appeared in the urine.

The observation of May 13, 1953, on patient A is of interest relative to the recent report of Spies and co-workers[16] that a boiled mixture of intrinsic factor and vitamin B_{12} was hemopoietic in two patients with "typical pernicious anemia." Unfortunately they did not publish data showing whether or not these patients had failed to respond to a similar oral dose of vitamin B_{12} without intrinsic factor. In a single observation in the present study a mixture of 2 μg of $B_{12}Co^{60}$ and 100 ml. of gastric juice was placed in boiling water for ten minutes. When the boiled mixture was administered to patient A on May 13, 1953, no

*Kindly supplied by Dr. K. C. Robbins, Armour Laboratories, Chicago, Ill.

2425

detectable radioactivity appeared in the urine. However, when this patient ingested 2 μg $B_{12}Co^{60}$ with 100 ml. of unboiled gastric juice, the urine contained significant amounts of radioactivity.

Patients with pernicious anemia are reported to have a large number of bacteria in the gastro-duodenal contents,[17] and one theory of the mechanism of the vitamin B_{12} deficiency is that these organisms are effectively competing with the host for dietary vitamin B_{12}. Lichtman and associates[18] have reported data from four patients showing hemopoietic responses to oral Aureomycin. In one patient Ungley[3] found that a single 80 μg oral dose of vitamin B_{12}, after partial sterilization of the intestinal contents with antibiotics, was not followed by a hemopoietic response. However, in a subsequent observation in this patient, definite erythropoietic response ensued when 80 μg B_{12} and 500 ml. gastric juice were given by mouth. In the present study observations made on May 1, 1953, and May 5, 1953, on patient C (Table I and Fig. 1) demonstrate that radioactivity does not appear in the urine of a Terramycin treated pernicious anemia patient given oral $B_{12}Co^{60}$ unless normal human gastric juice is also administered. This observation, like that of Ungley, provides no support for the theory that intestinal microorganisms are depriving the host of dietary vitamin B_{12}.

SUMMARY AND CONCLUSIONS

Normal human gastric juice given simultaneously with an oral dose of radioactive vitamin B_{12} enhances the urinary excretion of radioactivity in pernicious anemia patients. This radioactivity did not appear in the urine unless the patient was given a subcutaneous "flushing" dose of 1,000 μg of nonradioactive vitamin B_{12}. Because the nonradioactive vitamin B_{12} made radioactivity appear in the urine, it is presumed that the radioactivity represents vitamin B_{12} or a closely related metabolite. The data support the theory that Castle's intrinsic factor enhances the intestinal absorption of vitamin B_{12} when given to patients with pernicious anemia.

This observation provides a new method for the study of intrinsic factor activity because observations can be made on pernicious anemia patients in hematologic remission.

It was the author's privilege to have the advice of Dr. Frank C. Larson on the technical aspects of quantitating the radioactivity in the specimens. Mrs. Ethel Slotnik and Mrs. Edith Eisenhauer provided valuable technical assistance.

REFERENCES

1. Berk, L., Castle, W. B., Welch, A. D., Heinle, R. W., Anker, R., and Epstein, M.: Observations on 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, 1948.
2. Schilling, R. F., Harris, J. W., and Castle, W. B.: Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia. XIII. Hematopoietic Activity of (Vitamin B_{12b}), Blood **6:** 228, 1951.
3. Ungley, C. C.: Absorption of Vitamin B_{12} in Pernicious Anemia, Brit. M. J. **2:** 905, 1950.

700

2425

4. 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 Juice: Preliminary Report, Proc. Staff Meet., Mayo Clin. 24: 99, 1949.
5. Wallerstein, R. O., Harris, J. W., Schilling, R. F., and Castle, W. B.: Observations on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia. XV. Hematopoietic Effects of Simultaneous Intravenous and of Simultaneous or Serial Oral Administration of Intrinsic Factor in Vitamin B_{12}, J. LAB. & CLIN. MED. 41: 363, 1953.
6. Heinle, R. W., Welch, A. D., Scharf, V., Meacham, G. C., and Prusoff, W. H.: Studies of Excretion (and absorption) of Co^{60}-Labeled Vitamin B_{12} in Pernicious Anemia, Trans. Assoc. Am. Phys. 65: 214, 1952.
7. Meyer, Leo M.: Oral Administration of Co^{60} Vitamin B_{12} in Pernicious Anemia, Proc. Soc. Exper. Biol. & Med. 82: 490, 1953.
8. Hall, Byron E.: Studies on the Nature of the Intrinsic Factor of Castle, Brit. M. J. 2: 585, 1950.
9. Chaiet, L., Rosenblum, C., and Woodbury, D. T.: Biosynthesis of Radioactive Vitamin B_{12} Containing $Cobalt^{60}$, Science 111: 601, 1950.
10. Schilling, R. F., and Deiss, W. P.: Intrinsic Factor Studies. I. Paper Electrophoresis of a Mixture of Gastric Juice and Radioactive Vitamin B_{12}, Proc. Soc. Exper. Biol. & Med. 83: 506, 1953.
11. Ley, A. B.: Personal communication, 1953.
12. Conley, C. L., Krevans, J. R., Chow, B. F., Barrows, C., and Lang, C. A.: Observations on the Absorption, Utilization, and Excretion of Vitamin B_{12}, J. LAB. & CLIN. MED. 38: 84, 1951.
13. Rosenthal, J. L., and Sarett, H. P.: The Determination of Vitamin B_{12} Activity in Human Serum, J. Biol. Chem. 199: 433, 1952.
14. Mollin, D. L., and Ross, G. I. M.: Vitamin B_{12} Concentrations of Serum and Urine in the First 72 Hours After Intramuscular Injections of the Vitamin, J. Clin. Path. 6: 54, 1953.
15. Castle, W. B., and Ham, T. H.: Observatians on the Etiologic Relationship of Achylia Gastrica to Pernicious Anemia, J. A. M. A. 107: 1456, 1936.
16. Spies, Tom D., Stone, R. E., Suarez, R. M., Garcia-Lopez, Guillermo, Lopez-Toca, Ruben, and Reboredo, Alfredo: Antianemic Properties of Reaction Products of Vitamin B_{12} and the Intrinsic Factor, J. A. M. A. 151: 1264, 1953.
17. Davidson, L. S. P.: The Gastro-intestinal Flora in Pernicious Anemia, J. Path. & Bact. 31: 557, 1928.
18. Lichtman, H., Ginsberg, V., and Watson, J.: Therapeutic Effect of Aureomycin in Pernicious Anemia, Proc. Soc. Exper. Biol. & Med. 74: 884, 1950.

Scand. J. Gastroenterol. 7(5): 455-461, 1972

The In Vivo Uptake of Vitamin B_{12} by Intestinal Bacteria in Rats with Jejunal Blind Loops

H. SCHJÖNSBY
Chr. Michelsens Institute, Medical Dept. A and
Laboratory of Oral Microbiology, Dept. of Microbiology,
the Gade Institute, University of Bergen, Bergen, Norway

Schjönsby, H. 1972. The In Vivo Uptake of Vitamin B_{12} by Intestinal Bacteria in Rats with Jejunal Blind Loops. *Scand. J. Gastroent.* 7, 455–461

$^{57}CoB_{12}$ was given orally to control rats and to rats with surgically created jejunal blind loops; and the radioactivity in washed small intestinal sediments was determined at timed intervals after the dose. The radioactivity in the sediments as a percentage of recovered radioactivity from small intestinal contents, and the amount of $^{57}CoB_{12}$ per mg dried sediment were markedly increased in untreated blind-loop rats compared with control rats and with blind-loop rats treated orally with tetracyclin and lincomycin. In blind-loop rats there was a delay in gastric emptying and also a stagnation of radioactivity in the blind loop.

Key-words: Intestinal microorganisms; intrinsic factor; vitamin B_{12}

H. Schjönsby, M. D., Medical Dept. A, Haukeland Hospital, Bergen, Norway.

It has been proposed that malabsorption of vitamin B_{12} in rats with experimental intestinal blind loops is due to bacterial uptake of the vitamin (5). Although certain microorganisms are capable of utilizing vitamin B_{12} in vitro, even when the vitamin is bound to intrinsic factor (4, 6, 12, 13), it is nevertheless uncertain whether bacteria are able to utilize vitamin B_{12} in vivo in the lumen of the small intestine.

In the present study, ^{57}Co-labelled vitamin B_{12} was therefore fed to rats with surgically created jejunal blind loops and to unoperated control rats, the amount of radioactivity recovered from washed small intestinal sediments (presumably bound to bacteria) being measured at timed intervals following the dose. The results indicate that bacteria in the small intestinal contents of blind-loop rats were able to bind or take up high quantities of the radioactive vitamin when compared to control rats and to blind-loop rats given tetracyclin or lincomycin orally.

MATERIALS AND METHODS

Experimental animals. Male Wistar rats weighing 150 to 200 g were fed commercial pellets. Self-filling blind jejunal segments 9–10 cm long were created surgically by the method of Cameron, Watson & Witts (3). No experiments were performed until at least 4 weeks after the operation.

For 48 hours some blind-loop rats were given ground pellets containing 1 per cent (w/w) tetracyclin chloride or 1 per cent (w/w) lincomycin hydrochloride. The intake of antibiotics was roughly estimated by weighing the food cups daily. The rats consumed 120 to 160 mg tetracyclin and 55 to 175 mg lincomycin daily.

Reagents. ^{57}Co-labelled cyanocobalamin (specific activity 15 μc per μg) was obtained from the Radiochemical Centre, Amersham, England.

Rat-gastric-juice-(RGJ-)bound $^{57}CoB_{12}$ was prepared with a concentration of 10 ng $^{57}CoB_{12}$ per ml, as described elsewhere (11).

Distribution and concentration of ^{57}Co-vitamin B_{12} in contents of gastrointestinal tract. Rats were lightly anaesthetized with ether, and given 10 ng $^{57}CoB_{12}$ through a fine polyethylene tube into the stomach. The animals were sacrificed 1, 2, 4, and 8 hours after feeding. Animals with signs of intestinal obstruction were excluded. The stomach and the colon, and the proximal, middle, and distal thirds of the small intestine were isolated between ligatures. The proximal small intestinal segment was ligated at the pylorus and 1 cm distal to the anastomosis of the blind loop. The contents of each segment were rinsed out with 15 ml cold buffered saline. Any faeces passed after the administration of the radioactive dose were combined with the colonic contents.

To determine the amount of $^{57}CoB_{12}$ bound to intestinal sediments, the contents of the small intestinal segments were centrifuged at 12000 g × 30 min at 4 °C in an MSE High Speed 18 centrifuge. The sediments were washed twice in saline and recentrifuged, and then homogenized for 1 minute in an MSE top-drive macerator. The saline washings were combined with the supernatants, and the radioactivity in the combined supernatants and in the sediments was counted for 600 sec in a Nuclear Enterprise 8312 Automatic Spectrometer.

The distribution of $^{57}CoB_{12}$ in the gastrointestinal contents was assessed by calculating the amount of $^{57}CoB_{12}$ recovered from each part of the gastrointestinal tract. The percentage radioactivity in the washed small intestinal sediments was determined by dividing the radioactivity in the sediment by the total radioactivity recovered from the contents of the segment. The concentration of $^{57}CoB_{12}$ in the sediments was found by dividing the amount of $^{57}CoB_{12}$ in the sediments by the dry weight. (Dry weight was determined by heating the sediments in an oven at 95 °C for 24 hours).

Vitamin B_{12} absorption. The percentage of $^{57}CoB_{12}$ absorbed from test doses of $^{57}CoB_{12}$ by the rat was estimated by means of the faecal excretion test (9). Under light ether anaesthesia the doses were inserted into the stomach through a fine polyethylene tube. The animals were placed in separate metabolic cages, and faeces were collected for 5 consecutive days. The faeces were then diluted with water, and 1 ml Triton x-100 was added. The samples were homogenized for one minute, and the radioactivity was counted in identical containers and identical volumes in a scintillation counter with a cylindrical Na I crystal 3 × 2 inches (Frieseke & Hoepfner, Germany). The counting error was less than 1 per cent. The radioactive counts were the same when standard doses of $^{57}CoB_{12}$ were homogenized in water or in rat feces. The absorption was calculated by assuming that radioactivity not recovered from faeces had been absorbed.

To determine the reproducibility and the accuracy of the absorption method, eight rats were given two doses of 10 ng $^{57}CoB_{12}$ with a 14-day interval. Mean absorption after the first dose was 55.7 per cent, and after the second dose 53.9 per cent. The difference between the two means was not significant ($p > 0.5$). Standard deviation of the mean difference was 15.2.

Vitamin B_{12} measurements. Serum vitamin B_{12} was estimated by microbiological assay, using *Lactobacillus leichmannii* 313 (ATCC 7830) as test organism (10). Eight rats were divided into two groups of four animals using a table of random sampling numbers (2). A sham operation was performed in one group and a blind-loop operation in the other. Twenty-eight to 33 days after the operation the abdominal wall was opened, and blood was collected with a syringe from the abdominal aorta. The sera were stored at − 20 °C and analysed 9 days later.

To prepare extracts of the pellet diet for vitamin B_{12} assay, 10 g of pellets were homogenized for 2 min in 60 ml 0.1 M phosphate buffer (pH 5.6) containing 0.4 mg per cent KCN. The homogenate was boiled at 100 °C for 30 min, cooled and centrifuged, and the clear supernatant was poured off into sterile tubes. The vitamin B_{12} content recovered from the pellets was measured by microbiological assay, and was 0.3 ng vitamin B_{12}/gram (Dr. E. Magnus, personal communication). The intake of vitamin B_{12} was then estimated by weighing the food cups daily. The group of sham-operated rats consumed 6.7 ng vitamin B_{12} daily (range 6.2–6.8), and the group of blind-loop rats 6.4 ng vitamin B_{12} daily (range 6.3–6.6).

Table I. Distribution of $^{57}CoB_{12}$ in contents of the gastrointenstinal tract at timed intervals after feeding 10 ng $^{57}CoB_{12}$ to control rats and blind-loop rats (B.L.).

Anatomical segment	Ng $^{57}CoB_{12}$ in gastrointestinal contents							
	1 hour		2 hours		4 hours		8 hours	
	Control	B.L.	Control	B.L.	Control	B.L.	Control	B.L.
Stomach	1.97	2.92	0.54	1.85	0.05	2.11	0.01	0.03
Proximal small int.	0.61	1.95	0.04	1.99	0.03	0.76	0.01	1.44
Middle small int.	0.95	1.11	0.30	0.53	0.16	0.23	0.04	0.10
Distal small int.	0.18	0.17	1.71	0.60	0.30	0.34	0.14	0.10
Colon + faeces	0.00	0.01	0.85	0.40	1.19	2.14	1.91	3.32
Ng $^{57}CoB_{12}$ recovered	3.71	6.16	3.44	5.37	1.73	5.58	2.11	4.99
No. of animals	2	3	4	4	2	2	2	2

Binding of $^{57}CoB_{12}$ by rat gastric juice. The binding of $^{57}CoB_{12}$ by rat gastric juice in vivo was determined by dialysis. The pylorus was ligated in control rats and in blind-loop rats, and 10 ng $^{57}CoB_{12}$ was fed into the stomach through a polyethylene tube. Thirty minutes later the gastric contents were washed out with buffered saline and dialysed in cellophane bags for 42 hours against running tap water. The percentage of the dose bound to vitamin B_{12}-binding substances was assessed by dividing the final radioactivity retained in the bags by the radioactivity of the undialysed solutions.

Control experiments showed that 0.5 per cent of free $^{57}CoB_{12}$ and 87.9 per cent of RGJ-bound $^{57}CoB_{12}$ were non-dialysable.

Statistical methods. Student's test (2) was used for comparison of experimental groups with control groups. The difference between two means was considered significant at p-values below 0.05.

RESULTS

Distribution and concentration of $^{57}CoB_{12}$ in contents of gastrointestinal tract.

Distribution. Table I shows the distribution of ^{57}Co-labelled vitamin B_{12} in the different parts of the gastrointestinal tract at timed intervals after oral feeding. In blind-loop rats there was an appreciable delay in gastric emptying of the vitamin one, two, and four hours after the dose. Nevertheless, after eight hours nearly all the radioactivity had passed through the stomach. There was stagnation of radioactivity in the proximal blind-loop, and considerable amounts of $^{57}CoB_{12}$ were still being recovered eight hours after the dose. After 2 hours a small part of the dose had entered the colon, and at 4 and 8 hours more radioactivity was found in the colon of the blind-loop rats than in the controls.

It was not possible to calculate true recoveries due to absorption of radioactivity.

Percentage radioactivity in small intestinal sediments. The percentage of recovered radioactivity in sediments of small intestinal contents at timed intervals after feeding $^{57}CoB_{12}$ is shown in Table II. At each interval after the dose the percentage radioactivity was much higher in sediments from blind-loop rats than from control rats.

Table III shows the percentage radioactivity in the small intestinal sediments two hours after the dose. Following oral administration of free $^{57}CoB_{12}$, there was a marked increase in the radioactivity in the sediments from untreated blind-loop rats, compared with control rats and with blind-loop rats treated with tetracyclin or lincomycin. After feeding $^{57}CoB_{12}$ bound to rat gastric juice to untreated blind-loop rats, the mean percentage radioactivity in the sediments was less than the mean percentage after administration of free $^{57}CoB_{12}$; but the difference between these means was not significant.

703

Table II. Percentage radioactivity recovered from sediments of intestinal contents from different segments of the small intestine at timed intervals after feeding 10 ng $^{57}CoB_{12}$ to control rats and to untreated blind-loop rats (B.L.)

Small intestinal segment	1 hour		2 hours		4 hours		8 hours	
	Control	B.L.	Control	B.L.	Control	B.L.	Control	B.L.
Proximal	7.4	45.4	10.0	87.3	15.6	88.6	8.3	71.9
Middle	11.1	45.6	6.9	72.8	11.5	45.0	16.5	50.3
Distal	9.4	54.8	8.1	70.7	11.1	49.0	4.6	50.1
No. of rats	2	3	4	4	2	2	2	2

Table III. Percentage radioactivity in sediments of small intestinal contents two hours after feeding 10 ng $^{57}CoB_{12}$ or 10 ng $^{57}CoB_{12}$ bound to rat gastric juice. The results are given as mean percentage radioactivity in the sediments ± S.E.

Experimental group	Dose	No. of animals	% $^{57}CoB_{12}$ in intestinal sediment		
			Proximal intestine	Middle intestine	Distal intestine
Control rats	$^{57}CoB_{12}$	4	10.0 ± 0.9	6.9 ± 2.5	8.1 ± 2.9
B.L. rats, untreated	$^{57}CoB_{12}$	4	87.3 ± 3.3	72.8 ± 1.1	70.7 ± 1.6
B.L. rats, untreated	$^{57}CoB_{12}$ + RGJ	6	58.8 ± 12.4	47.8 ± 14.9	55.7 ± 11.7
B.L. rats, tetracyclin-treated	$^{57}CoB_{12}$	4	28.1 ± 8.6	17.6 ± 4.5	30.9 ± 11.8
B.L. rats, lincomycin-treated	$^{57}CoB_{12}$	4	8.8 ± 2.2	18.4 ± 10.1	7.5 ± 2.9

Table IV. Concentration of radioactivity in intestinal sediment at 1, 2, 4, and 8 hours after feeding 10 ng $^{57}CoB_{12}$ to control rats and to blind-loop rats (B.L.). The results are expressed as pg $^{57}CoB_{12}$ per mg sediment

Small intestinal segment	1 hour		2 hours		4 hours		8 hours	
	Control	B.L.	Control	B.L.	Control	B.L.	Control	B.L.
Proximal	0.43	1.90	0.05	4.13	0.06	0.79	0.01	3.04
Middle	0.68	4.72	0.14	17.75	0.10	0.77	0.05	0.72
Distal	0.04	0.43	1.01	24.25	0.18	0.97	0.08	0.74
No. of rats	2	3	4	4	2	2	2	2

Table V. Concentration of radioactivity in small intestinal sediments two hours after feeding 10 ng $^{57}CoB_{12}$ to control rats and to blind-loop (B.L.) rats. All data are expressed as mean pg $^{57}CoB_{12}$ per mg sediment ± S.E

Experimental group	Dose	No. of animals	pg $^{57}CoB_{12}$ per mg sediment		
			Proximal intestine	Middle intestine	Distal intestine
Control rats	$^{57}CoB_{12}$	4	0.05 ± 0.03	0.14 ± 0.03	1.01 ± 0.37
B.L. rats, untreated	$^{57}CoB_{12}$	4	4.13 ± 1.25	17.75 ± 6.90	24.25 ± 12.50
B.L. rats, untreated	$^{57}CoB_{12}$ + RGJ	6	3.21 ± 0.82	1.45 ± 0.37	2.59 ± 0.90
B.L. rats, tetracyclin-treated	$^{57}CoB_{12}$	4	0.87 ± 0.52	1.13 ± 0.34	2.08 ± 1.80
B.L. rats, lincomycin-treated	$^{57}CoB_{12}$	4	0.63 ± 0.53	0.83 ± 0.52	0.27 ± 1.12

Table VI. Absorption of $^{57}CoB_{12}$ in unoperated rats and in blind-loop (B.L.) rats, and the effect of rat gastric juice (RGJ) and of oral antibiotic therapy on the absorption

Experimental group	Dose	No. of rats	Percentage of dose absorbed
			% ± S.E.
Unoperated rats	$^{57}CoB_{12}$	15	56.6 ± 2.9
B.L. rats, untreated	$^{57}CoB_{12}$	15	22.3 ± 5.1
B.L. rats, untreated	$^{57}CoB_{12}$ + RGJ	6	18.6 ± 8.3
B.L. rats, tetracyclin-treated	$^{57}CoB_{12}$	6	49.0 ± 4.4
B.L. rats, lincomycin-treated	$^{57}CoB_{12}$	6	68.4 ± 2.9

Concentration of $^{57}CoB_{12}$ in small intestinal sediments. The concentration of $^{57}CoB_{12}$ in small intestinal sediments at 1, 2, 4, and 8 hours after feeding $^{57}CoB_{12}$ is shown in Table IV. The blind-loop rats showed a marked increase in the concentration of $^{57}CoB_{12}$ compared with control rats. The concentration increased from 1 to 2 hours, but was much less 4 and 8 hours after the dose.

Table V shows the concentration of $^{57}CoB_{12}$ two hours after the radioactive dose. After oral administration of free $^{57}CoB_{12}$, the concentration in the sediments was much higher in untreated blind-loop rats than in blind-loop rats treated orally with tetracyclin or lincomycin, and in control rats.

The mean concentration was also higher after feeding free $^{57}CoB_{12}$ to untreated blind-loop rats than after feeding RGJ-bound $^{57}CoB_{12}$; the difference in mean concentration was not significant when the sediments were obtained from the proximal and distal part of the small intestine, but significant when obtained from the middle part ($p < 0.05$).

Absorption of $^{57}CoB_{12}$.

The absorption of $^{57}CoB_{12}$ is shown in Table VI. Mean absorption of $^{57}CoB_{12}$ in blind-loop rats (22.3 per cent) was significantly lower than mean absorption in unoperated rats (56.6 per cent); $p < 0.01$. Mean absorption in untreated blind-loop rats was not corrected when the dose of 10 ng $^{57}CoB_{12}$ was given together with 0.7 ml rat gastric juice. In contrast, the absorption increased significantly in blind-loop rats treated orally with tetracyclin ($p < 0.01$) and lincomycin ($p < 0.01$).

Serum vitamin B_{12}

Table VII shows vitamin B_{12} concentrations in sera from control rats and blind-loop rats. Serum vitamin was significantly reduced in the blind loop rats. ($p < 0.01$).

Binding of $^{57}CoB_{12}$ by rat gastric juice

The percentage of $^{57}CoB_{12}$ bound to rat gastric juice is shown in Table VIII. Rat gastric juice

Table VII. Serum vitamin B_{12} levels in control rats (sham operated) and in blind-loop rats.

Status	No. of rats	Days after operation (range)	Vitamin B_{12} activity
			pg B_{12}/ml serum ± S.E.
Control rats	4	28–34	595 ± 32
Blind-loop rats	4	28–33	269 ± 46

704

1 2429

Table VIII. Binding of $^{57}CoB_{12}$ by rat gastric juice collected 30 minutes after feeding 10 ng $^{57}CoB_{12}$

Dialysed sample	No. of rats	Percentage binding of $^{57}CoB_{12}$
		% ± S.E.
Gastric juice of control rats	4	87.4 ± 1.8
Gastric juice of blind-loop rats	4	88.1 ± 2.0

from control rats bound 87.4 per cent of $^{57}CoB_{12}$, and a similar amount was bound by rat gastric juice from blind-loop rats.

DISCUSSION

The results show that following an oral dose of $^{57}CoB_{12}$ a markedly higher percentage of radioactivity was recovered from small intestinal sediments from untreated blind-loop rats than from sediments from blind-loop rats treated orally with tetracyclin or lincomycin, and from control rats (Tables II and III). This suggests that particles in intestinal fluid from untreated blind-loop rats were able to bind or take up considerable amounts of radioactive vitamin B_{12}. Since malabsorption of $^{57}CoB_{12}$ (Table VI) and also reduced serum-vitamin B_{12}-like activity (Table VII) was found in these rats, it is possible that $^{57}CoB_{12}$ was unavailable for intestinal absorption when bound to particles in the small intestinal fluid. Furthermore, Donaldson (5) showed that when $Co^{60}B_{12}$ bound to rat gastric juice was injected into the blind-loop, most of the radioactivity was recovered from the washed sediment of the loop contents. After feeding $^{57}CoB_{12}$ bound to human gastric juice, much higher amounts of radioactivity were recovered from sediments of ileal aspirates from patients with the stagnant-loop syndrome than from sediments from control subjects (13).

High concentrations of anaerobic and aerobic microorganisms have been cultured from the small intestinal fluid of blind-loop rats (7, 8, 11), and bacteria seem to be the major constituent of the blind-loop contents (5). Yet the small intestinal fluid also contains other particulate material which is more difficult to identify, but presumably consists of food residue, desquamated intestinal epithelial cells, and dead bacteria. It has not been directly shown whether the high uptake by the small intestinal sediment of $^{57}CoB_{12}$ is due to bacteria or other particles. Nevertheless, it is probable that the high percentage of radioactivity in the intestinal sediments was due to bacterial uptake of $^{57}CoB_{12}$. The percentage and concentration of $^{57}CoB_{12}$ in the small intestinal sediments decreased when blind-loop rats were treated orally with antibiotics (Table III, Table V). Furthermore, the results in this paper corroborate those of previous in vitro studies which have shown that the in vitro uptake of intrinsic-factor-bound $^{57}CoB_{12}$ is much higher in blind-loop sediment obtained from untreated blind-loop rats than in blind-loop sediment from antibiotic-treated blind-loop rats (11), and that certain bacterial strains are able to take up significant amounts of intrinsic-factor-bound vitamin B_{12} in vitro (4, 6, 12, 13).

The results also indicate that malabsorption of $^{57}CoB_{12}$ was not due to reduced gastric secretion of intrinsic factor; malabsorption of $^{57}CoB_{12}$ was not corrected when the oral dose of $^{57}CoB_{12}$ was given together with rat gastric juice (Table VI); following an oral dose of free $^{57}CoB_{12}$, the major part of the radioactivity in gastric contents was non-dialysable (Table VIII). Furthermore, gel-filtration studies suggest that the radioactivity in centrifuged small intestinal fluid from blind-loop rats is bound to protein (Schjönsby, unpublished). Donaldson (5) showed that the concentration of vitamin B_{12}-binding substances, and the intrinsic factor activity were normal in gastric juice from blind-loop rats.

These results suggest that uptake of $^{57}CoB_{12}$ by intestinal bacteria took place in the lumen of the small intestine, not only in the blind intestinal segment, but also in the middle part of the small intestine, which is the site of vitamin B_{12} absorption in the rat (1). The data support the hypothesis that bacteria inhibit the absorption of vitamin B_{12} by their uptake of the vitamin.

ACKNOWLEDGEMENT

I wish to thank Dr. S. Fröland for counting the radioactive samples.

REFERENCES

1. Booth, C. C., Chanarin, I., Anderson, B. B. & Mollin, D. C. 1957. The site of absorption and tissue distribution of orally administrated ^{56}Co-labelled vitamin B_{12} in the rat. *Brit. J. Haemat. 3*, 253–261.
2. Bradford Hill, A. 1961. *Principles of Medical Statistics* (7th ed.) The Lancet Ltd., London.
3. Cameron, D. G., Watson, G. M. & Witts, L. J. 1949. The experimental production of macrocytic anemia by operations on the intestinal tract. *Blood 4*, 803–815.
4. Dellipiani, A. W., Samson, R. R. & Girdwood, R. H. 1968. The uptake of vitamin B_{12} by *E. coli:* Possible significance in relation to the blind loop syndrome. *Amer. J. dig. Dis. 13*, 718–726.
5. Donaldson, R. M., Jr. 1962. Malabsorption of Co^{60}-labelled cyanocobalamin in rats with intestinal diverticula. I. Evaluation of possible mechanisms. *Gastroenterology 43*, 271–281.
6. Donaldson, R. M., Jr., Corrigan, S. & Natsios, G. 1962. Malabsorption of Co^{60}-labelled cyanocobalamin in rats with intestinal diverticula. *Gastroenterology 43*, 282–290.
7. Kent, T. H., Summers, R. W., Den Besten, L., Swaner, J. C. & Hrouda, M. 1969. Effect of antibiotics on bacterial flora of intestinal blind loops. *Proc. Soc. exp. Biol. (N.Y.) 132*, 63–67.
8. Midtvedt, T., Norman, A. & Nygaard, K. 1969. Bile acid transforming microorganisms in rats with an intestinal blind segment. *Acta path. microbiol. scand. 77*, 162–166.
9. Nieweg, H. O., Arends, A., Mandema, E. & Castle, W. B. 1956. Enhanced absorption of vitamin B_{12} in gastrectomized rat by rat intrinsic factor. *Proc. Soc. exp. Biol. (N.Y.) 91*, 328–332.
10. *Pharmacopoea Nordica 4* ed. dan. 1960, 105–109.
11. Schjönsby, H. 1971. The effect of bacteria on intestinal uptake of vitamin B_{12}. I. Effect of cultures of blind-loop contents. In preparation.
12. Schjönsby, H. 1971. Effect of bacteria on intestinal uptake of vitamin B_{12}. II. Effect of pure bacterial cultures. In preparation.
13. Schjönsby, H., Peters, T. J., Hoffbrand, A. V. & Tabaqchali, S. 1970. The mechanism of vitamin B_{12} malabsorption in the blind loop syndrome, (abstract). *Gut 11*, 371.

Received 27 December 1971
Accepted 2 February 1972

705

2429

Scand. J. Haemat. (1968) 5, 35–40

A New Vitamin B_{12} Binding Protein in Serum Causing Excessively High Serum Vitamin B_{12} Values

MICHAEL SCHWARTZ, M.D. & P. BASTRUP-MADSEN, M.D.

Medical Department F (Chief, M. Schwartz), Glostrup Hospital, Copenhagen
and
Medical Department (Chief, P. Bastrup-Madsen), Aarhus Amtssygehus, Aarhus C, Denmark

Following treatment with a vitamin B_{12} preparation with retarded effect, extremely high serum vitamin B_{12} values were observed in several cases. The explanation for this phenomenon is a very high serum vitamin B_{12}-binding capacity, caused by a vitamin B_{12} binding protein located in the γ_G area – presumably an antibody.

In 1961 a new vitamin B_{12} preparation, Betolvex® (B_{12}-TAM), was made in Denmark. The preparation contains 1 mg cyanocobalamin-tannate suspended in an aluminium-monostearate-sesame oil gel. Betolvex proved to be well suited to the treatment of pernicious anaemia (P.A.) both initially and in the long-term treatment.

The serum vitamin B_{12} values in Betolvex-treated patients are usually high, and the mean values higher than those seen following treatment with other preparations for injection. Clinical experience with the preparation has been uniformly good with as few as 4 or 6 injections intramuscularly a year (Schwartz et al. 1962, Gough et al. 1964, Bastrup-Madsen et al. 1966).

In several patients treated with Betolvex very high values of serum vitamin B_{12} have been observed following 1–2 years of treatment. Table I shows serum vitamin B_{12} values in one such patient. This patient was treated with an oral preparation for several years, before put on Betolvex treatment in 1962.

Serum vitamin B_{12} levels as high as those shown in Table I have not been reported previously in man – except during a few hours following injection of conventional B_{12} preparations. Out of a total of 200 P.A. patients treated with Betolvex for more than two years serum vitamin values higher

This work was supported by grants from King Christian X's Fund.

TABLE I

Serum vitamin B_{12} values in a P.A. patient during oral treatment and following treatment with Betolvex (1 mg cyanocobalamin in aluminium-monosterate sesame oil gel every second month)

Two values are given for each year

Year	Serum B_{12} pg/ml	Treatment
1959	246 298	
1960	141 427	Oral (IF + B_{12} preparation)
1961	160 314	
1962	450 4,450	
1963	8,500 85,000	
1964	92,000 114,000	Betolvex®
1965	34,000 100,000	
1966	24,800 30,000	
1967	23,600	

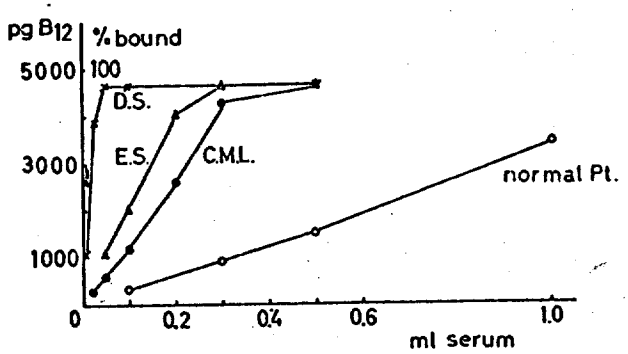


Figure 1. Vitamin B_{12}-binding capacity in two patients with P.A. with very high serum B_{12} values (D.S. and E.S.), one patient with CML and a normal person. The figure shows the B_{12}-binding capacity of increasing amounts of serum following exposure to 5000 pg B_{12}.

TABLE II

Serum vitamin B_{12} values and vitamin B_{12}-binding capacity ('unbound binding capacity') in 41 normal persons, 4 patients with chronic myeloid leukaemia and 4 P.A. patients with high B_{12} values following treatment with Betolvex®

	Serum vitamin B_{12} values (pg B_{12}/ml serum)	Vitamin B_{12} binding capacity (pg B_{12}/ml serum)
41 normal persons (donors)	200–800	900–2,400
Chronic myeloid leukaemia	6,960	4,300
	5,460	3,620
	6,800	6,550
	6,100	7,650
P.A. patients treated with Betolvex	90,000	105,000
	38,000	21,200
	11,100	5,900
	64,000	33,600

than 2,000 pg/ml serum have been observed in 35 patients and seven of these patients had B_{12} values exceeding 20,000 pg/ml.

As vitamin B_{12} in the unbound form has a clearance identical with that of inulin, it was evident that the vitamin B_{12} in our patients must be protein-bound. Investigations of the vitamin B_{12}-binding capacity (the so-called 'unbound binding capacity') proved that this was very high. Figure 1 shows the vitamin B_{12}-binding capacity of the serum from two of our patients with very high serum vitamin B_{12} values, one patient with chronic myeloid leukaemia (CML) and a normal person. The B_{12} binding capacity was measured by a modification of the method of Gottlieb et al. (1965).

It is evident from Figure 1 that the explanation of the extremely high serum vitamin B_{12} values is an excessively high vitamin B_{12}-binding capacity in the serum – only a part of which is used, as the method measures the 'unbound' B_{12} binding capacity.

Table II shows the serum vitamin B_{12} values and the serum vitamin B_{12}-binding capacity in 41 normal persons, 4 patients with CML and 4 patients with P.A. and very high B_{12} values.

The high serum vitamin B_{12} values in CML are well known. Table II shows that also the B_{12} binding capacity in CML is higher than normal, however, the values for the Betolvex-treated patients are still considerably higher.

This abnormally high vitamin B_{12}-binding capacity in serum from P.A. patients treated with Betolvex seems to be due to a γ_G-protein not previously described. Figure 2 shows autoradiographs of paper electrophoresis from a

itself non-antigenic, and a pure B_{12}-antibody is thus hardly not reasonable. The relation between the new B_{12}-binding γ-globulin and the transcobalamins is now being investigated.

Nor is it possible to say whether the presence of the very large amounts of vitamin B_{12} bound to a γ-globulin should be considered an advantage to

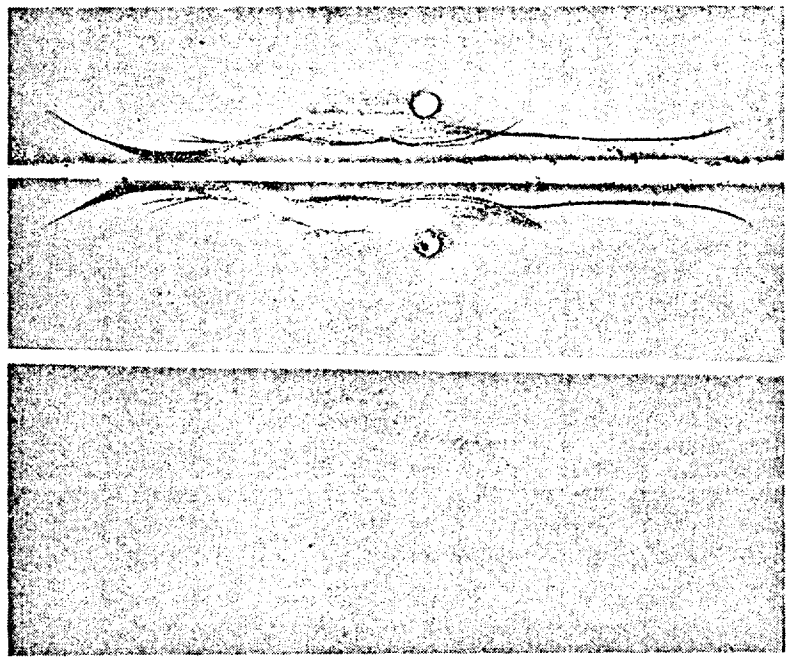


Figure 3. Immunoelectrophoresis (A) and autoradiography of the immunoelectrophoresis (B) of normal serum (lower well) and serum with very high B_{12} value (upper well).

the patient – or the opposite. Cases of concomitant P.A. and CML have been described with the characteristic haematological picture of P.A., in spite of a (low) normal serum vitamin B_{12} value (Britt & Rose 1966). All our P.A. patients treated with Betolvex are in full haematological remission and well – regardles of their having a normal or a high serum vitamin B_{12}.

ACKNOWLEDGEMENT

We wish to thank S. Bryde Andersen, M.D., and Miss Lisbeth Madsen, Department of Clinical Physiology, Glostrup Hospital, for valuable help with the paper electrophoresis and Jørgen Clausen, M.D., Neurochemical Laboratory, Copenhagen, for performance of the immunoelectrophoresis.

REFERENCES

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.

Britt, R. P. & Rose, D. P. (1966) 'Pernicious anemia with a normal serum vitamin B_{12} level in a case of chronic granulocytic leukemia.' *Arch. intern. Med.* **117**, 32–33.

Gough, J., Israëls, M. C. G. & Bottomley, A. C. (1964) 'Treatment of vitamin-B_{12} deficiency with a vitamin-B_{12}-tannin complex.' *Lancet* II, 1311–13.

Gottlieb, C., Kau, K. S., Wasserman, L. R. & Herbert, V. (1965) 'Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated B_{12} binding capacity, antibody to IF, and serum unsaturated B_{12} binding capacity.' *Blood* **25**, 875–84.

Schwartz, M., Bastrup-Madsen, P., Nørregaard, S. & Kristensen, K. (1962) 'A vitamin-B_{12} preparation with retarded absorption.' *Lancet* II, 1181–83.

Accepted for publication August 10, 1967.

Correspondence should be addressed to Michael Schwartz, M.D.
Medical Department F
Glostrup Hospital
Copenhagen, Denmark

J. Bact. 88:581-585 2458

JOURNAL OF BACTERIOLOGY
Vol. 88, No. 3, p. 581-585 September, 1964
Copyright © 1964 American Society for Microbiology
Printed in U.S.A.

MICROBIAL DEGRADATION OF CORRINOIDS

I. VITAMIN B_{12}

W. M. SCOTT, R. C. BURGUS, J. B. HUFHAM, AND J. J. PFIFFNER

Department of Physiology and Pharmacology, Wayne State University College of Medicine, Detroit, Michigan

Received for publication 21 March 1964

ABSTRACT

SCOTT, W. M. (Wayne State University, Detroit, Mich.), R. C. BURGUS, J. B. HUFHAM, AND J. J. PFIFFNER. Microbial degradation of corrinoids. I. Vitamin B_{12}. J. Bacteriol. 88:581–585. 1964.—Microorganisms isolated from a variety of soil, sewage, and mud samples, and stock cultures, were examined for the ability to degrade vitamin B_{12}. More than 200 isolates which attack the vitamin were examined, and they all demonstrated reversible fading of the red vitamin. The color was restored by aeration. Very few microorganisms were able to degrade the vitamin to permanently colorless products, although many were able to degrade it partially, to produce new pigments. Some of these pigments appeared similar, if not identical, although they were produced by different bacteria. Radiotracer and electrophoretic mobility data are presented to show that the transformation products are derived from the vitamin. All the degradative microorganisms isolated were bacteria, and the most active was *Pseudomonas rubescens*.

Numerous studies have been carried out on transformations of vitamin B_{12} (Brown and Reynolds, 1963; Stokstad, 1962), but no information is available on the metabolic degradation of the corrinoid nucleus. Corrinoids occur in the animal body only in trace amounts (Wokes and Smith, 1962); therefore, studies were undertaken on microbial degradation in the hope of obtaining degradation products in sufficient quantities for characterization and structural studies, as well as for use as reference compounds in metabolic studies in animals and man.

Sewage, faunal excreta, and the bodies of dead creatures should enrich the soil and waters with the vitamin, and it seemed likely that organisms capable of degrading the vitamin could be isolated from such sources. While our work was in progress, Helgeland, Jonsen, and Leland (1961) reported the production of two yellow pigments from vitamin B_{12} by a strain of *Aerobacter aerogenes* isolated from river mud. The present report is concerned with our search for microorganisms which have the ability to degrade vitamin B_{12}.

MATERIALS AND METHODS

Culture isolation. Nutrient broth (Difco) containing 1.0% glucose, and minimal salts medium (Davis and Mingioli, 1950), were employed. Vitamin B_{12} (Merck, Sharp and Dohme & Co., Inc., Rahway, N.J., or Squibb Institute for Medical Research, New Brunswick, N.J.) was either autoclaved separately or filter-sterilized. The vitamin was added to 5 to 10 ml of medium in test tubes (16 by 150 mm) to a final concentration of 5 to 10 mg per 100 ml of medium. This concentration was sufficient to give an observable red color.

The media were inoculated with soil, water, or sewage samples and were incubated at 30 C. The disappearance of the red color was used as presumptive evidence that the vitamin had been attacked. After several serial transfers, the cultures were streaked on agar plates and isolated colonies were picked.

In addition, a chemostat was constructed and was employed as a vitamin B_{12}-enrichment chamber. The growth chamber was inoculated with a soil or sewage sample and was continuously flushed with minimal salts medium containing vitamin B_{12} and a limiting concentration of glucose as the carbon sources. The effluent was sampled intermittently and new cultures were isolated and tested.

Cultures from the American Type Culture Collection and many natural isolates were tested for the ability to degrade vitamin B_{12}. The most active cultures were examined for degradation products.

Radiotracers. Co^{57}-labeled vitamin B_{12} was obtained from Abbott Laboratories, North Chicago,

712

III. C^{14}-labeled vitamin B_{12} was prepared by biosynthesis from δ-aminolevulinic acid-4-C^{14} (Miller and Rosenblum, 1960). *Propionibacterium freudenreichii* (ATCC 6207) was employed, and the synthesis was carried out by the method of Speedie and Hull (1960).

Extraction procedure. The test organism was allowed to grow in the presence of Co^{57}- or C^{14}-labeled vitamin until no observable red color remained. The cells were removed by centrifugation, the corrinoids were extracted from ammonium sulfate-saturated broth with two volumes of benzyl alcohol, and the emulsion was separated by centrifugation. Three volumes of diethyl ether and less than one-tenth volume of 0.001 M potassium cyanide were shaken with the alcoholic phase. The corrinoids passed into the aqueous phase. The water was evaporated under reduced pressure, and the residue was extracted with anhydrous methanol to separate the corrinoids from salt.

Paper electrophoresis. Electrophoresis of corrinoids was carried out on strips (1 by 35 cm) of Whatman no. 3 MM paper with free horizontal suspension of the paper. The best separations were obtained with the use of 1 N acetic acid containing 0.001 M potassium cyanide to prevent the formation of hydroxocorrinoids. Most separations took 3 hr at 500 V.

Assay of radioactivity. The electrophoresis strips were dried and taped to strips (2 by 25 cm) of Kodak No-Screen X-ray film, and the film was developed after 5 to 10 days of exposure. As little as 50 counts per min of Co^{57} or 15 counts per min of C^{14} activity could be detected as a spot on the film after 5 days of exposure. For semiquantitative measurements of the relative activities of spots on electrophoresis strips, the strips were cut into 1-cm^2 pieces, and the pieces were placed in planchets for counting. Radioactivity was determined by use of a thin-window, gas-flow, proportional counter (750 PF, Baird-Atomic, Cambridge, Mass.).

RESULTS

Reversible fading. Reversible fading of the red color of vitamin B_{12} occurred in over 200 cultures which attacked the vitamin. If the tube was closed tightly after growth occurred, the red color faded, leaving a medium that varied from brown to pale yellow. When air was present to allow growth, the red color often faded in the butt of the tube, but remained near the surface, where more oxygen was dissolved in the medium. Cultures which grew in a pellicle often faded the color near the surface. The time required for visual observation of the fading depended on the organism and the cultural conditions, but usually the fading occurred after 1 or 2 days.

If the tube was aerated soon after the red color had disappeared, the color was partially, if not completely, restored. The major recolorized product was red and behaved electrophoretically like vitamin B_{12}. A crystalline red product isolated from one recolorized culture was identical to vitamin B_{12} in activity towards *Lactobacillus leichmannii*, ultraviolet and visible spectrum, and electrophoretic mobility in 1 N acetic acid buffer.

Effect of light. The effect of light on recolorization was investigated. Three tubes containing 5 ml of nutrient broth plus 1.0% glucose and 0.25 mg of vitamin B_{12} per tube were inoculated with *Pseudomonas rubescens* (Pivnick, 1955; ATCC no. 12099). Two tubes were incubated in the dark at 30 C. When the control tube had faded, examination of one of the tubes incubated in the dark showed that it, too, had faded. Hence, light is not essential to the fading reaction. The second tube incubated in the dark was aerated in the dark, and on examination the red color had returned. Thus, the oxidation of the reduced product did not require light. The results were the same when the experiment was repeated with the use of culture RS2A1 in minimal salts medium.

Permanent fading. After prolonged incubation (5 days to several weeks), some of the cultures which initially exhibited reversible fading failed to recolorize to a comparable extent upon aeration. Many crude, mixed cultures from lake-bottom mud, sewage effluent or sludge, and various soils enriched with animal feces faded the red color irreversibly in 1 day. Isolates from these sources and stock cultures were much slower in their action than were the crude cultures. No organism able to use vitamin B_{12} as a sole carbon source was isolated from cultures enriched with the vitamin. All the isolates that attacked vitamin B_{12} required an energy source such as glucose. None of the isolates was able to fade the color of the vitamin under conditions of vigorous aeration.

Cultures that did not exhibit degradative activity were *Bacillus stearothermophilus*, *Clostridium haemolyticium*, *C. thermosaccharolyticum*,

Propionibacterium freudenreichii, *Pseudomonas fluorescens*, and *P. riboflavina*. None of the isolated molds was active, and several streptomycetes showed either very little activity or no activity. The cultures which showed the most activity were *P. rubescens* (ATCC 12099); Chemo-11 (soil isolate), D-37, and J-30 (air contaminants); and RS2A1 (sewage isolate).

Radioactive degradation products. Figure 1 shows the location of radioactive spots on electrophoresis strips spotted with extracts from several cultures. Table 1 shows the cultural conditions and percentage of recovered radioactivity found in the electrophoresis fractions. The results with either C^{14} or Co^{57} label demonstrate that the pigment fractions were derived from added vitamin B_{12} and were not synthesized de novo. With *P. rubescens*, comparable results were obtained when either label was used.

In our searches for degradation products, the extracted corrinoids were the most extensively studied, but other fractions of the fermentation broth were checked for radioactivity in a few cases. Among the cultures listed in Table 1, none of the whole-cell fractions or diethyl ether extracts of broth supernatant contained appreciable Co^{57} activity. After saturation of the supernatant fluid with ammonium sulfate and extraction with benzyl alcohol, the aqueous phases usually showed little radioactivity

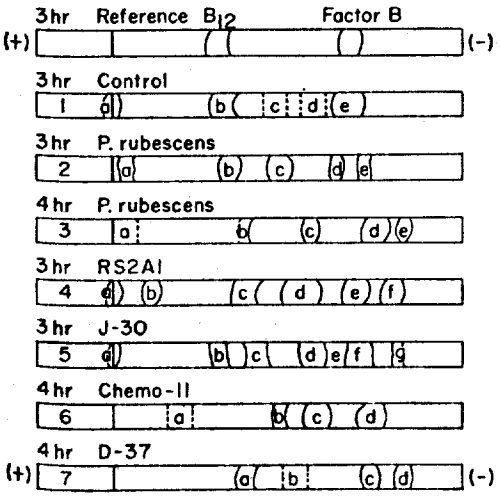


FIG. 1. *Electrophoresis of corrinoids in acetic acid-KCN buffer at 500 v. Time of each run is shown above strip. See Table 1.*

TABLE 1. *Distribution of radioactivity in corrinoid extracts*

Strip[a]	Organism and conditions[b]	Spot[a]	Color	Per cent recovered activity[c]
1	Control; C^{14}-vitamin B_{12}; 2,600 counts per min; nutrient broth plus glucose; 13 days	a	Colorless	1
		b	Red[d]	90
		c	Colorless	3
		d	Yellow?	3
		e	Pink[d]	3
2	*Pseudomonas rubescens;* C^{14}-vitamin B_{12}; 2,600 counts per min; nutrient broth plus glucose; 4 days	a	Colorless	12
		b	Red-orange	36
		c	Light yellow	13
		d	Orange	27
		e	Pink	12
3	*P. rubescens;* Co^{57}-vitamin B_{12}; ca. 200 counts per min; nutrient broth plus glucose; 13 days	a	Colorless	4
		b	Pink	28
		c	Light yellow	25
		d	Orange	34
		e	Pink	7
4	RS2A1; Co^{57}-vitamin B_{12}; 4,000 counts per min; minimal salts medium; 25 days	a	Light yellow	1
		b	Light pink	1
		c	Red	65
		d	Yellow	8
		e	Orange	16
		f	Pink	9
5	J-30; Co^{57}-vitamin B_{12}; 4,000 counts per min; minimal salts medium; 25 days	a	Yellow-brown	2
		b	Yellow	3
		c	Red	65
		d	Yellow	10
		e	Orange	10
		f	Orange	8
		g	Pink	1
6	Chemo-11; Co^{57}-vitamin B_{12}; 4,000 counts per min; minimal salts medium; 55 days	a	Colorless	12
		b	Red-orange	62
		c	Light yellow	20
		d	Light yellow	6
7	D-37; Co^{57}-vitamin B_{12}; 4,000 counts per min; nutrient broth plus glucose; 55 days	a	Red	71
		b	Light yellow	8
		c	Light yellow	11
		d	Pink	9

[a] See Fig. 1.
[b] All cultures were incubated at 30 C.
[c] Per cent total radioactivity counted on each strip. In control experiments, there was a large loss of radioactivity due to absorption by the paper. With C^{14} label, about 20% of the added radioactivity was detected on the strip; with Co^{57} label, about 50%.
[d] Electrophoresis was carried out in the presence of unlabeled vitamin B_{12} and factor B as internal reference compounds.

(RS2A1 was not tested). Levels varied from 1 to 4% of the original Co^{57} activity introduced as vitamin B_{12}. The one exception was one sample of Chemo 11, where the aqueous phase contained 36% of the original activity after 55 days of incubation. In agreement with the results with Co^{57} label, *P. rubescens* acting on C^{14}-vitamin B_{12} also showed little radioactivity in noncorrinoid fractions.

Discussion

Although soil and water enriched with vitamin B_{12} yielded microorganisms capable of degrading the vitamin, some of the best degraders found were *P. rubescens*, which was isolated from cutting oil by Pivnick (1955), and some air contaminants encountered in the laboratory. Some of the degraders thus came from rather unlikely sources.

A common feature of more than 200 active cultures studied was their production of a reduced product(s), derived from vitamin B_{12}, which appears yellow and which turns red with aeration. In all cases, the red product seems to be identical to vitamin B_{12}, as judged by color and electrophoretic mobility. In one instance, the isolated crystalline product was proven to be identical with vitamin B_{12}.

Reports of the widespread occurrence of the coenzyme, and the suggestion of Helgeland et al. (1961) that one of their products might be a coenzyme analogue with increased stability to light, forced us to consider the involvement of vitamin B_{12} coenzyme in the reversible fading. The coenzyme is sensitive to light and remains brown after anaerobic exposure to light; the brown product recolorizes to red when air is admitted (Brady and Barker, 1961). Our cultures decolorized vitamin B_{12} anaerobically without exposure to light. Neither of the yellow-brown pigments that were observed was vitamin B_{12} coenzyme because they both showed slower electrophoretic mobility; furthermore, the faded product could be recolorized by air without exposure to light. Therefore, we concluded that we were not studying the coenzyme in our work.

The reversible fading of color should be distinguished from the permanent fading that occurs after more prolonged incubation. The complete degradation of the vitamin to colorless products was not observed in any of the studies in this report. On the other hand, several cultures that can produce yellow, orange, or red pigments from vitamin B_{12} were isolated. Their vitamin B_{12} origin was confirmed by the use of Co^{57}- or C^{14}-labeled vitamin.

P. rubescens, RS2A1, and J-30, yield two radioactive spots of yellow and orange color between vitamin B_{12} and a pink spot with mobility similar to factor B (Fig. 1). It seems likely, though not certain, that these two pigments, as well as factor B, are common degradation products that result from the action of many different microorganisms. Helgeland et al. (1961) previously reported two yellow-brown pigments, referred to as I and II, derived from vitamin B_{12} by *A. aerogenes*. These pigments have properties similar to the pigment fractions formed by our cultures of *P. rubescens*, RS2A1, and J-30. On the other hand, cultures of Chemo-11 and D-37 form pigment fractions, some of which differ from the above, judging by color and electrophoretic mobility. Of the cultures studied, *P. rubescens* attacked vitamin B_{12} most rapidly and gave the largest yields of degradation products.

Microbiological activities and chemical characterization of degradation products will be reported later.

Acknowledgments

This investigation was supported by Public Health Service grant AM 04302 from the National Institutes of Health. The authors are indebted to Merck & Co. and E. R. Squibb & Sons for supplies of vitamin B_{12}. We would also like to thank W. C. Alegnani, Parke Davis & Co., for microbiological assays.

Literature Cited

Brady, R. O., and H. A. Barker. 1961. Influence of oxygen on the photolysis of coenzyme B_{12}. Biochem. Biophys. Res. Commun. 4:373–378.

Brown, G. M., and J. J. Reynolds. 1963. Biogenesis of water-soluble vitamins. Ann. Rev. Biochem. 32:419–462.

Davis, B. D., and E. S. Mingioli. 1950. Mutants of Escherichia coli requiring methionine or vitamin B_{12}. J. Bacteriol. 60:17–28.

Helgeland, K., J. Jonsen, and S. Leland. 1961. New pigments derived from vitamin B_{12} by a strain of *Aerobacter aerogenes* present in river mud. Biochem. J. 81:260–265.

Miller, I. M., and C. Rosenblum. 1960. The production of vitamin B_{12} using delta-aminolevulinic acid. U.S. Patent 2,939,822.

Rivnick, H. 1955. Pseudomonas rubescens, a new

2458

species from soluble oil emulsions. J. Bacteriol. 70:1–6.

SPEEDIE, J. D., AND G. W. HULL. 1960. Cobalamin producing fermentation process. English Patent 829,232.

STOKSTAD, C. L. R. 1962. Biochemistry of the water-soluble vitamins. Ann. Rev. Biochem. 31:451–490.

WOKES, F., AND A. D. M. SMITH. 1962. Vitamin B_{12} and vegetarians, p. 602–606. *In* H. G. Heinrich [ed.], Vitamin B_{12} and intrinsic factor. Enke Verlag, Stuttgart.

716

Cancer Res. 18(7):849-852

Isotopic and Microbiological Studies of Vitamin B_{12} Distribution in Normal and Hepatoma-bearing Rats*

OLGA STEIN, Y. STEIN, J. ARONOVITCH, N. GROSSOWICZ, AND M. RACHMILEWITZ

(Departments of Pathology, Internal Medicine "B," Bacteriology and Clinical Research, Hebrew University–Hadassah Medical School, Jerusalem, Israel)

Studies on the distribution of labeled vitamin B_{12} in man (3) and in experimental animals (1, 6) have shown the liver to be one of the principal organs of vitamin B_{12} retention. On the other hand, tumor tissues such as methylcholanthrene-induced sarcomas in hamsters and Walker carcinosarcoma in rats have a lower B_{12}-Co^{60} uptake than that of the liver (5). It was of interest to compare the uptake of B_{12}-Co^{60} by neoplastic tissue of the liver with that of normal liver tissue. Concurrently, the content of nonradioactive vitamin B_{12} of normal and neoplastic liver tissues was also studied.

MATERIAL AND METHODS

Eighty-five white, male rats of a local random-bred strain (Hebrew University strain), weighing 110 ± 10 gm., were fed a synthetic diet containing 0.06 per cent of *p*-dimethylaminoazobenzene (DAB) for 6 months (8); twenty control animals were fed the same diet except for the DAB. After that period the DAB was omitted from the diet of all the animals. Another control group was made up of eighteen animals fed the regular laboratory stock diet. In the "Results," the respective three groups will be designated: DAB-fed, diet control, and normal rats.

Six to 8 weeks following the withdrawal of DAB, the animals of the three groups were given injections intravenously or intraperitoneally of 0.1 μc. of B_{12}-Co^{60} in a total volume of 0.4 ml.[1] The animals were sacrificed 1, 2, 7, and 12 days following the injection of the radiovitamin.

The gross appearance of the tumors and the liver was noted, and wherever possible the tumors were dissected free from liver tissue and processed separately. Sections were removed for histological examination, fixed in Zenker acetic acid and neutral formaldehyde, and cut at 6 μ.

Preparation of tissue homogenates.—The determination of B_{12} in organs was made on tissue homogenates prepared in water, either in a Waring Blendor or the Potter-Elvehjem homogenizer. Unless examined immediately, the homogenates were kept at −20° C.

Microbiological assay.—Vitamin B_{12} was determined microbiologically by the *Escherichia coli* assay (4). Total serum B_{12} was estimated after liberation of the vitamin from the serum protein after acid precipitation (10). Tissue B_{12} content was determined in samples of tissue homogenates; 1 gm. of tissue homogenate was added to 10 ml. of 0.002 N acetate buffer, pH 4.5, and autoclaved at 110° C. for 30 min. in the presence of 10 mg. of KCN. After centrifugation the supernatant was neutralized and assayed. The final value is the mean of three determinations of each sample.

Vitamin B_{12}-Co^{60} determination.—The vitamin B_{12}-Co^{60} (specific activity, 0.83 μc/μg B_{12}) was diluted with water, 1:50. Aliquots of this solution were used as standards for radioactivity measurements of the organ homogenates and for microbiological assays. The determinations were made on 10-ml. samples in a liquid scintillation counter with an annular geometry (E. R. D. Engineering Co., Ltd.). B_{12}-Co^{60} in serum was measured in 3–5-ml samples. The radioactivity measurement was continued to give a counting error less than 3 per cent. The B_{12}-Co^{60} uptake by the tissues was expressed in counts/min/gm of wet tissue. The specific activity was expressed as counts/min/mμg B_{12}.

RESULTS

Morphological findings.—Liver tumors developed in 56 per cent of the 85 animals fed DAB during 6 months. The tumors varied in weight from 1 to 35 gm. There were usually multiple tumor

* This investigation was supported by Leukemia Research Foundation, Chicago, Ill.

[1] The B_{12}-Co^{60} was supplied by Merck, Sharp & Dohme, International, through the courtesy of Dr. E. L. Nowicki.

Received for publication February 26, 1958.

717

nodules in the same animal, and occasionally the entire liver was infiltrated by the neoplastic tissue, leaving only a few uninvolved areas. The tumors were mostly solid, and in the largest tumors there were areas of necrosis. Histologically, both hepatocellular carcinoma and cholangio- or adenocarcinoma were found, with frequent transitions from one pattern to the other. In the non-neoplastic part of the liver there were areas where the lobular architecture was normal and areas where pseudo-lobules were formed by thin septa, containing proliferating bile ductules.

The livers of the two control groups showed no gross and histological changes.

the liver and kidney of the three groups of rats, sacrificed 1–12 days following injection.

In the animals of Group III, the B_{12}-Co^{60} uptake was determined in the non-neoplastic part of the liver. This group also included animals which failed to develop tumors during the DAB feeding.

The B_{12}-Co^{60} concentration in the liver was uniformly higher on the 7th and 12th days than 24 hours after injection, in all three groups of animals. Contrariwise, the B_{12}-Co^{60} concentration in the kidney was highest on the 2d day and decreased during the next 12 days.

Vitamin B_{12} content of liver, tumor, and kidney of DAB-fed, diet control, and normal animals.—

TABLE 1

UPTAKE OF $B_{12}Co^{60}$ BY LIVER AND TUMOR TISSUE OF RATS 1–12 DAYS FOLLOWING INTRAVENOUS INJECTION

Rat no.	Day after injection	Weight (gm.) Liver	Weight (gm.) Tumor	Counts/min/gm Liver	Counts/min/gm Tumor	Per cent of injected dose Liver	Per cent of injected dose Tumor
11	1	9.5	6.0	120	76	11.5	4.5
12	1	12.0	0.65	78	88	9.3	0.5
13	1	8.5	5.2	124	117	9.9	6.0
14	1	9.2	8.6	146	87	11.5	6.0
15	2	13.4	1.0	113	87	16.0	8.0
16	2	8.2	0.75	121	132	9.2	0.9
17	7	13.0	16.0	117	94	15.0	13.0
18	7	9.5	10.8	155	70	14.8	7.1
19	7	9.4	22.8	224	45	21.0	10.2
20	7	7.2	23.9	177	40	12.7	9.5
21	7	8.0	31.0	260	30	20.8	9.3
22	12	11.5	13.5	221	82	24.0	10.3
23	12	10.0	14.5	217	63	20.1	8.8
24	12	9.5	7.5	310	78	27.5	5.5
25	12	8.6	7.9	283	151	22.6	11.5
26	12	9.4	1.0	200	61	17.6	0.6
27	12	12.8	8.3	177	118	21.0	9.1
28*	12	9.6		278		24.9	
29*	12	10.9		220		22.3	
30*	12	10.8		217		22.0	

* Animals which failed to develop tumors.

B_{12}-Co^{60} uptake by the liver and tumor tissue of rats, 1–12 days following intravenous injection.—The animals were divided into four groups, sacrificed 1, 2, 7, and 12 days following injection. It can be noted (Table 1) that in animals examined 1 and 2 days after injection the B_{12}-Co^{60} uptake of both tumor and liver tissue did not differ significantly. In animals sacrificed 7 and 12 days after injection the concentration, i.e., counts/min/gm of the radiovitamin by the liver, was about 2–7 times higher than that of the tumor tissue of the same animal except in rat 17.

The same behavior was observed in ten tumor-bearing rats that had been given B_{12}-Co^{60} intraperitoneally and sacrificed 1 and 7 days after injection.

B_{12}-Co^{60} uptake by liver and kidney of DAB-fed, diet control, and normal rats.—In Table 2 a comparison is drawn between the uptake of B_{12}-Co^{60} by

Total vitamin B_{12} content of liver, tumor, and kidney was determined microbiologically, and the results are summarized in Table 3. The vitamin B_{12} content of the tumor tissue was one-fifth that of the liver, while in the kidney the B_{12} concentration was highest.

Specific activity of B_{12}-Co^{60} in liver, tumor, kidney, and serum of DAB-fed rats.—The results are summarized in Table 4.

The specific activity of vitamin B_{12} of the liver rose during the 12 days after injection, that of the tumor did not change appreciably, while that of serum and kidney fell throughout the experimental period.

DISCUSSION

Hepatic tumors produced by prolonged feeding of DAB behave differently toward injected radioactive B_{12} than their parent liver tissue; 24–48

hours after injection, both normal and tumor tissue show a similar B_{12}-Co^{60} uptake, but 7–12 days following injection higher uptake values are found in the non-neoplastic liver tissue than in the tumor.

The low B_{12}-Co^{60} uptake by the tumor cell could be misinterpreted as a high B_{12} turnover. However, the relatively constant B_{12}-Co^{60} concentration in the tumor tissue throughout the experimental period indicates a rather slow B_{12} turnover. Similarly, a slow B_{12}-Co^{60} turnover was found in methylcholanthrene-induced sarcoma in the hamster by Miller *et al.*, during a 45-day period (5).

TABLE 2

UPTAKE OF B_{12}-Co^{60} BY LIVER AND KIDNEY OF NORMAL, DIET CONTROL, AND DAB-FED RATS, 1–12 DAYS FOLLOWING INJECTION

Day	Normal Group I	Diet control Group II	DAB Group III
		Liver (Counts/min/gm)	
1	78±14.8* (8)†	55±11.2 (7)	105±25.4 (8)
2	102±13.8 (7)	120 (1)	129±18.7 (3)
7	124±19.8 (11)	146± 9.6 (4)	170±54.7 (7)
12	124±17.5 (7)		235±47.0 (9)
		Kidney (Counts/min/gm)	
1	777±164 (8)	921±207 (7)	1075±204 (7)
2	693±158 (7)		1114±288 (5)
7	708±126 (11)	750±238 (8)	776±286 (10)
12	543±112 (8)		756±205 (9)

* Mean and S.D.

† Numbers in parentheses represent number of rats.

The lack of fall in the specific activity of B_{12}-Co^{60} in the tumor seems to rule out the possibility that the low B_{12}-Co^{60} uptake could be due to a substantial increase of the tumor mass during the post-injection period.

Necrotic areas of tumors could affect the B_{12}-Co^{60} concentration by either taking up less B_{12}-Co^{60} or, if necrosis developed in the post-injection period, by a release of B_{12}-Co^{60} from the disintegrating areas. Both release of B_{12} from necrotic liver tissue (11) and a low B_{12} uptake by larger tumors, showing areas of necrosis, had been reported (5). However, in the present experiments the low B_{12}-Co^{60} uptake was also observed in the smaller tumors, showing no necrosis.

It seems likely that the high serum/tissues B_{12} gradient occurring during the first 48 hours causes the uptake of considerable amounts of the vitamin into both tumor and liver. This, together with a markedly lower B_{12} content of the tumor tissue, could explain the relatively higher specific activity of B_{12}-Co^{60} in the tumors (Table 4). The subsequent increase of radioactivity, confined to the non-neoplastic liver tissue only, may be due to a continuous exchange between B_{12} of the serum and that of the liver, since the serum B_{12} specific activity continues to be much higher than in normal liver tissue even at 7 days (Table 4). Such an exchange between serum B_{12}-Co^{60} and that of the tumor is evidently lacking, as shown by the constant specific activity of B_{12}-Co^{60} in the tumors throughout the experimental period.

TABLE 3

VITAMIN B_{12} CONTENT OF LIVER, TUMOR, AND KIDNEY OF DAB-FED, DIET CONTROL, AND NORMAL RATS

Group	Vitamin B_{12} content (mμg/gm wet tissue) Liver	Tumor	Kidney
DAB	132±36* (23)†	28±13 (16)	440± 98 (22)
Diet control	113±29 (9)		420±120 (9)
Normal	103±25 (8)		390± 70 (8)

* Mean and S.D.

† Figures in parentheses represent number of rats.

A derangement of the mechanism of B_{12} uptake at normal B_{12} serum levels by the tumor cell is reflected in the low B_{12} concentration of that tissue in comparison with normal liver. The normal daily uptake of B_{12} content may be a function of a specific protein, and a decrease in the synthesis of such a protein in the tumor cell could explain its low B_{12} content. An analogy could be seen in the 50 per cent fall of serum albumin synthesis in hepatoma cells when compared with parent liver tissue as reported by Campbell *et al.* (2). It is of

TABLE 4

SPECIFIC ACTIVITY OF B_{12}-Co^{60} IN LIVER, TUMOR, KIDNEY, AND SERUM OF DAB-FED RATS, 1–12 DAYS AFTER INJECTION

Day	Liver	Tumor	Kidney	Serum
		(Counts/min/mμg B_{12})		
1	0.96±0.104* (10)†	3.8±1.7 (4)	2.81±0.73 (7)	49±16.7 (6)
2	0.98±0.02 (5)	3.2±0.33 (4)	2.90±0.33 (5)	30 (1)
7	1.45+0.07 (5)	2.8±1.05 (5)	2.20±0.91 (5)	14± 6 (5)
12	1.66±0.6 (7)	3.4±1.05 (5)	2.0 ±0.47 (9)	0 (5)

* Mean and S.D.

† Figures in parentheses represent number of rats.

interest that DAB-induced liver tumors contain only very small amounts of the particular protein which binds DAB and which is found in large quantities in normal rat liver tissue (9). Similar to the low B_{12} content of the tumor tissue, a low riboflavin, B_6, and biotin content was reported (7).

A difference in behavior toward injected B_{12}-Co^{60} was noted between the liver and the kidneys. Our results confirm the data of others (6) that in the rat the kidney is capable of a much higher B_{12} uptake per gram of tissue than the liver.

The data presented here indicate that studies with labeled B_{12}, at pharmacologically high levels, do not reflect the normal behavior of tissues toward serum B_{12}. The results of a combined determination of the total B_{12} content by microbiological assay and of the turnover of labeled B_{12} suggest that at normal serum B_{12} levels the tumors would show minimal B_{12} uptake.

SUMMARY

The uptake of parenterally administered B_{12}-Co^{60} and the vitamin B_{12} content were studied in rats bearing liver tumors induced by DAB.

The specific activity of B_{12}-Co^{60} of the serum showed a decline during the 1st week, but was still much higher than that of either liver or tumor tissue 7 days following injection.

The B_{12}-Co^{60} uptakes of the liver and tumor tissue were similar during the first 48 hours; at 7 and 12 days an increase of B_{12}-Co^{60} concentration was found in the liver, but not in the tumor tissue. In contrast to the liver the B_{12}-Co^{60} concentration in the kidney showed a decline with increasing time intervals following injection.

The total B_{12} content of the tumors, as determined by microbiological assay, was about one-fifth of that of the non-neoplastic part of the liver.

ACKNOWLEDGMENTS

We wish to thank Prof. J. Gross for his kind help in the preparation of this manuscript and Prof. H. Ungar for his helpful suggestions. We also acknowledge the skilled assistance of Mrs. Mala Yablonska and Mr. Yehuda Werechson.

REFERENCES

1. Booth, C. C.; Chanarin, J.; Anderson, B. B.; and Mollin, D. L. The Site of Absorption and Tissue Distribution of Orally Administered ^{56}Co-labelled Vitamin B_{12} in the Rat. Brit. J. Haemat., 3:253–61, 1957.
2. Campbell, P. N., and Stone, N. E. The Synthesis of Serum Albumin and Tissue Proteins in Slices of Rat Liver and Liver Tumor. Biochem. J., 66:19–31, 1957.
3. Glass, G. B. J.; Boyd, L. J.; Gellin, G. A.; and Stephenson, L. 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. Arch. Biochem., 51:251–57, 1954.
4. Grossowicz, N.; Aronovitch, J.; and Rachmilewitz, M. Determination of Vitamin B_{12} in Human Serum by Mutant of *Escherichia coli*. Proc. Soc. Exper. Biol. & Med., 87: 513–14, 1954.
5. Miller, A.; Gaull, G.; Lemon, H. M.; and Ross, J. F. Studies on the Uptake of Vitamin B_{12}-CO^{60} by the Hamster Methylcholanthrene-induced Sarcoma and the Rat Walker Carcinosarcoma. Cancer Research, 16:842–47, 1956.
6. Miller, A.; Gaull, G.; Ross, J. F.; and Lemon, H. M. The Distribution of Parenteral Co^{60} Vitamin B_{12} in Mouse, Hamster, Rat and Guinea Pig. Proc. Soc. Exper. Biol. & Med., 93:33–35, 1956.
7. Miller, E. C.; Miller, J. A.; Kline, B. E.; and Rusch, H. P. Correlation of the Level of Hepatic Riboflavin with the Appearance of Liver Tumors in Rats Fed Aminoazo dyes. J. Exper. Med., 89:88–98, 1948.
8. Miller, J. A., and Miller, E. C. The Carcinogenic Aminoazo Dyes. Adv. Cancer Research, 1:339–90, 1953.
9. Sorof, S., and Cohen, P. P. Electrophoretic and Ultracentrifugal Studies on the Soluble Proteins of Various Tumors and Livers of Rats fed 4-Dimethylaminoazobenzene. Cancer Research, 11:376–82, 1951.
10. Spray, G. H. An Improved Method for the Rapid Estimation of Vitamin B_{12} in Serum. Clin. Sc., 14:661–67, 1955.
11. Stein, Y.; Stein, O.; Aronovitch, J.; Grossowicz, N.; and Rachmilewitz, M. Serum Vitamin B_{12} in Experimental Liver Injury. Bull. Res. Counc. Israel, 6E:5–10, 1956.