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LEUKOCYTE PROPIONATE OXIDATION AND SERINE FORMATION IN HUMAN VITAMIN B_{12} DEFICIENCY

James Stephen Robertson

1973

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LEUKOCYTE PROPIONATE OXIDATION AND SERINE FORMATION IN HUMAN VITAMIN B_{12} DEFICIENCY

by

James Stephen Robertson

B.S. Trinity College, 1969

PRESENTED TO THE DEPARTMENT OF HUMAN GENETICS

YALE UNIVERSITY SCHOOL OF MEDICINE IN

PARTIAL FULFILLMENT OF REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF MEDICINE

NEW HAVEN, CONNECTICUT

April, 1973

Abstract

To study the metabolic consequences of cobalamin-folate interactions in vitamin B_{12} deficiency, a simple _in vitro_ assay of folate-dependent ^{14}C-formate incorporation into ^{14}C-L-serine by peripheral leukocytes has been developed and applied to B_{12} deficient and folate deficient patients. In five vitamin B_{12} deficient patients, incorporation was significantly reduced, suggesting that cobalamin deficiency depletes the intracellular pool of free tetrahydrofolate, required in this reaction as carrier of the ^{14}C-formate. The possible mechanisms by which this might occur are discussed. No defect in ^{14}C-formate incorporation into ^{14}C-L-serine was observed in two folate deficient patients, but the significance of this finding is speculative because of the small number of patients studied. Leukocytes from vitamin B_{12} deficient patients also demonstrated a reduced capacity to oxidize ^{14}C-3-propionate to $^{14}CO_2$ but normal $^{14}CO_2$ production from ^{14}C-1,4-succinic acid. These observations suggest a block in the oxidative catabolism of propionate at the B_{12}-dependent methylmalonyl CoA mutase step. No defects in propionate metabolism were observed in the folate deficient patients.

Acknowledgments

My preceptor, Dr. Y. Edward Hsia, has been overly generous of his time and sensitive to my needs for frequent direction. More importantly, he has been an outstanding teacher, not only as a tutor but also as an exemplary model of the effective combination of critical scientist and compassionate physician. Dr. Peter McPhedran has made the study possible by finding most of the patients for me. Miss Kathy Scully has given valued technical suggestions and assistance, and demonstrated remarkable patience with my occasional displays of ineptitude in the laboratory. Finally, Dr. Richard B. Crawford, of the Department of Biology of Trinity College, Hartford, deserves an expression of gratitude for first engendering in me the interest in intermediary metabolism which has led to these efforts.

LEUKOCYTE PROPIONATE OXIDATION AND SERINE
FORMATION IN HUMAN VITAMIN B_{12} DEFICIENCY

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List of Abbreviations

Cobalamins

B_{12}:	Vitamin B_{12} (any form)
$CN-B_{12}$:	Cyanocobalamin
$dA-B_{12}$:	5'-deoxyadenosylcobalamin
CH_3-B_{12}:	Methylcobalamin

Folates

PGA:	folic acid, pteroylglutamic acid
FH_4:	tetrahydrofolic acid
$N^5-CH_3-FH_4$:	N^5-methyltetrahydrofolic acid
$N^5N^{10}-CH_2-FH_4$:	N^5N^{10}-methylenetetrahydrofolate
$N^5N^{10}-CH=FH_4$:	N^5N^{10}-methenyltetrahydrofolate
$N^5-CHO-FH_4$:	N^5-formyltetrahydrofolate, folinic acid
$N^{10}-CHO-FH_4$:	N^{10}-formyltetrahydrofolate

AICAR:	5-aminoimidazolecarboxamide
DNA:	desoxyribosenucleic acid
dTR:	thymidine
dU:	deoxyuridine
FAD, $FADH_2$:	oxidized and reduced flavin adenine dinucleotide
FIGlu:	formiminoglutamic acid
FMN, $FMNH_2$:	oxidized and reduced flavin mononucleotide
HCys:	Homocysteine
HCys-S-Ad:	S-adenosylhomocysteine
IMP:	inosine monophosphate

List of Abbreviations

MCV:	erythrocyte mean cell volume
Met:	methionine
Met-S-Ad:	S-adenosylmethionine
NAD, NADH:	oxidized and reduced nicotinamide adenine dinucleotide
RNA:	ribosenucleic acid
tRNA:	transfer ribosenucleic acid
TCA:	trichloroacetic acid

INTRODUCTION

From little-understood disorders of insidious onset, unknown etiology, and usually fatal outcome, the megaloblastic anemias have evolved over the past fifty years into a well-defined group of eminently treatable diseases due to deficiency of vitamin B_{12} or folates. They have been the subject of increasingly sophisticated investigations, the results of which have had broad implications for the understanding of regulatory mechanisms in intermediary metabolism both at the cellular and whole-organism level. In spite of this probing, however, fundamental questions remain about the mechanisms by which deficiencies of vitamin B_{12} and/or folic acid produce anemia. The objectives of this paper will be twofold: first, to explore the hypotheses which have been advanced to account for the clinical similarities - and differences - between the syndromes produced by lack of these essential nutrients; and second, to review the still-unsettled question of what in fact constitutes folate "deficiency", and how it can best be assessed in the clinical setting. In terms of this second objective, the results of a study by the author of a recently developed *in vitro* assay for detecting folate deficiency[1] will be presented. The data derived from the application of this same assay to the B_{12} deficient patient, and of an indirect assay of vitamin B_{12}-dependent methylmalonyl CoA mutase activity[2] to both the folate and B_{12} deficient patient, will also be presented and discussed in the context of present understandings of cobalamin - folate interdependence.

A comprehensive review of the fascinating but voluminous literature concerning the historical, clinical, morphologic, genetic, and immunologic aspects of the megaloblastic anemias is beyond the scope of the present effort, but the field has recently been surveyed admirably by Chanarin[3] and by Harris and Kellermeyer.[4]

VITAMIN B_{12}

Only five years after Addison's description in 1855 of what may have been what we now recognize as pernicous anemia, Austin Flint offered the following hypothesis concerning its pathogenesis:

> I suspect that in these cases there exists degenerative disease of the glandular tubuli of the stomach. . . . Fatal anemia must follow an amount of degenerative disease reducing the amount of gastric juice so far that assimilation of food is rendered wholly inadequate to the wants of the body. . . . I shall be ready to claim the merit of this idea when the difficult and laborious researches of someone have shown it to be correct.[5]

As predicted, however, little progress was made in either the understanding or therapy of the disease until 1926, with Minot and Murphy's[6] introduction of an effective dietary treatment. Castle's studies,[7] resulting eventually in the isolation of intrinsic factor from gastric secretions, the crystallization of cyanocobalamin in 1948, and the elucidation of its structure in 1955, have led to our present understanding of the nature of the missing vitamin, and the means by which its deficiency can arise in individuals with apparently adequate nutrition.

Structure[8]

Crystalline vitamin B_{12} consists of a planar tetrapyrrole nucleus, with three of the rings joined by α-methene bridges, as in the porphyrins. In contradistinction to the porphyrins, the ring is completed by a direct carbon-to-carbon bond between α-positions of

the pyrroles designated A and D, and the four bases are partially reduced. A trivalent cobalt atom lies at the center of the ring structure; four of its six coordinate binding sites are occupied in one plane by the pyrrole nitrogens. The 5th binds to base nitrogen of a nucleotide lying "below" the planar ring. In addition to the Co-N coordinate bond, the nucleotide, which is a 5,6,-dimethyl-benzimidazole base linked by an α-glycoside bridge to ribose-3-phosphate, is also bound to the tetrapyrrole nucleus through its phosphate group. This ties it, through an aminoisopropanol linkage, to a propionic acid side chain of the D ring. "Above" the nucleus lies a cyano group, which by an ionic bond is tied to the sixth coordinate site of the central Co atom. Cyanocobalamin probably does not occur normally *in vivo*, and in the coenzymatically active forms of the vitamin the sixth Co coordinate site is occupied by a methyl group or by 5'-deoxyadenosyl ribonucleoside. It appears that *in vivo* synthesis of the coenzymes involves progressive reduction of Co^{III} to Co^{I} before addition of the active substituent at the sixth position. The structure of one of the B_{12} coenzymes, 5'-deoxyadenosyl cobalamin ($dA-B_{12}$) is illustrated in Figure 1.

Nutritional Aspects[9]

Vitamin B_{12} is synthesized by bacteria through essentially the same metabolic pathway as are the pyrroles in heme. It does not occur in the plant kingdom, and in the higher animals is derived solely

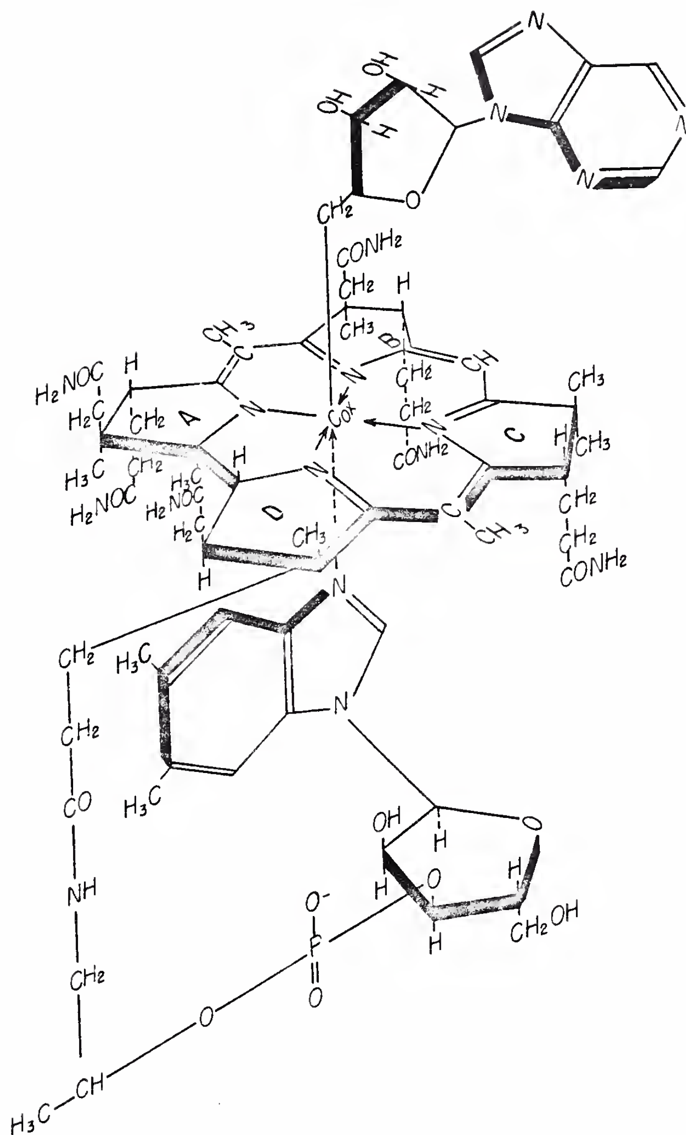


Figure 1. Structure of 5'-deoxyadenosylcobalamin. From Harris JW, Kellermeyer RW: The Red Cell. Cambridge: Harvard University Press, 1970, p 355.

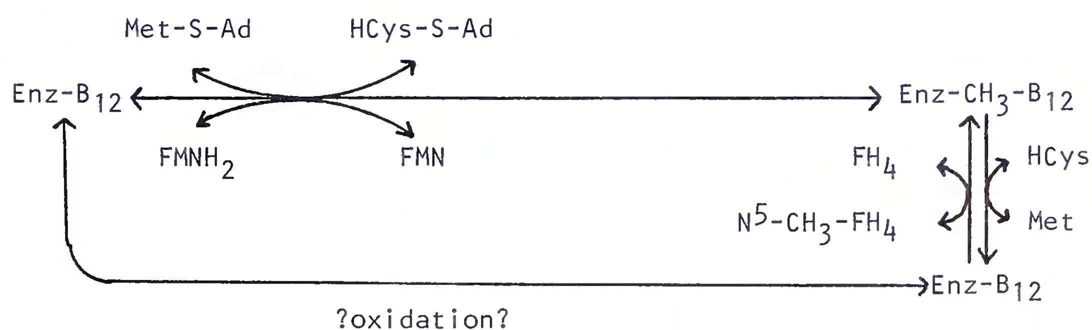
from dietary sources. It is so ubiquitous among many soil organisms that even domestic water supplies may serve as dietary sources, and bacterial contamination of the exclusively vegetarian diets of some mammals may be sufficient to prevent deficiency. Animal food sources contain significant amounts of the vitamin, the highest concentration occurring in liver. It has been estimated that essentially all dietary B_{12} is available for absorption, limited only by the 2-3 mcg maximum daily capacity of the intrinsic factor-ileal receptor absorption system.

Biochemistry

The two coenzymes forms of vitamin B_{12} are involved in reactions which have been grouped by Silber and Moldow[8] into two classes. Those requiring dA-B_{12} involve hydrogen transfer along a carbon chain, with reciprocal movement of a second group in the opposite direction:

Only one such pathway, the isomerization of L-methylmalonyl CoA to succinyl CoA, has been convincingly demonstrated in man. Decreased activity of methylmalonyl CoA mutase, as measured either by increased urinary methylmalonic acid excretion[10] or by decreased $^{14}CO_2$ production from leukocyte homogenates incubated with ^{14}C(carboxy)-D-methylmalonyl CoA[11] has been reported as an index of the B_{12} deficiency state in man.

An appreciation of the role played by the other B_{12} coenzyme, methylcobalamin (CH_3-B_{12}) in mammalian systems arose from studies in rats demonstrating capability for *de novo* synthesis of the methyl group of methionine from ^{14}C-formate.[12] In 1951, Bennet *et al.* reported that the liver-derived factor, permitting growth of rats on a diet free of labile methyl groups but containing folic acid and homocysteine was vitamin B_{12}.[13] Additional evidence linking B_{12} with methionine biosynthesis was derived from a strain of *Escherichia coli* requiring either B_{12} or methionine for growth. It was demonstrated that in the presence of cobalamin, the organism synthesized methionine *de novo* provided that the medium also supplied homocysteine, folate, and a source of single-carbon units.[14,15] Further investigations on the same organism revealed that the immediate methyl donor for the reaction was N^5-methyltetrahydrofolate (N^5-CH_3-FH_4).[16] In 1964 Buchanan and co-workers reported a partially purified enzyme preparation from pig liver, with similar properties and cofactor requirements.[17,18] Enzyme activity was found to directly parallel B_{12} content of the preparation. Requirements for $FADH_2$ and S-adenosyl methionine (Met-S-Ad) were also demonstrated, and the latter was shown to serve a catalytic rather than stoichiometric function in the methylation process. On the basis of these studies the following sequence of reactions has been proposed to take place:



The precise catalytic role of S-adenosyl methionine remains unclear. Taylor and Weissbach[19] have suggested that a "priming" methylation of the cobamide prosthetic group of the holoenzyme is involved, since methyl iodide can replace Met-S-Ad in their assay (*E. coli*) system. They postulate that subsequent regeneration of CH_3-B_{12} with each methyl group transfer to homocysteine results from stoichiometric amounts of substrate N^5-CH_3-FH_4.

A cobalamin-independent methyltransferase active only for the triglutamate form of N^5-CH_3-FH_4 has been identified in other strains of *E. coli*.[20] Evidence suggesting its presence in human liver has also been presented,[21] but its quantitative significance in mammalian systems is unknown. The well-established actions of B_{12} coenzymes are summarized in Table 1. The implications of searches for other metabolic roles for the cobalamins in man will be discussed later.

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reaction (mechanism [illegible]

the cysteine-containing [illegible]

methyl iodide can replace the [illegible]

Those products that subsequently [illegible]

group transfer [illegible] homocysteine [illegible]

substrate [illegible].

A coherent [illegible]

the thiolate [illegible] of [illegible]

of *E. coli* [illegible] suggesting [illegible]

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system is [illegible]

summarized in [illegible]

roles [illegible]

TABLE 1

B_{12}-DEPENDENT REACTIONS

REACTION	PATHWAY	ORGANISM
	1. 5'-DEOXYADENOSYL-B_{12}-DEPENDENT	
Methylmalonyl CoA mutase	Propionate oxidation	Propionibacterium shermanii, animals, man
Glutamate mutase	Glutamic acid fermentation	Clostridium tetanomorphum
Dioldehydrase	Glycol metabolism Glycerol metabolism	Aerobacter aerogenes Lactobacillus (unidentified)
Ribonucleotide reductase	Deoxyribonucleotide formation	Lactobacillus leichmanii
Ethanolamine deaminase	Ethanolamine fermentation	Choline-forming Clostridium
β-Lysine isomerase	Lysine fermentation	Clostridium sticklandii
	2. METHYL-B_{12}-DEPENDENT	
N^5-methyltetrahydrofolate-homocysteine transmethylase	Methionine synthesis	E. coli, pig, man
Methane synthesis	Methanol fermentation	Methanosarcina barkerii
Acetate synthesis	Carbon dioxide metabolism	Clostridium thermoaceticum

FOLIC ACID

The relationship of an essential nutrient other than vitamin B_{12} to the development of megaloblastic anemia began to be elucidated in 1931, with the recognition of a "pernicious anemia of pregnancy" occurring in Hindu women.[22] In contrast to Addisonian anemia, achlorhydria and neurological signs of posterolateral column degeneration were consistently absent.[23] In addition, these patients responded with hematologic remissions both to crude - but not purified - liver extract, and to "marmite", an autolyzed yeast preparation,[22] again separating them from patients with classical pernicious anemia.[24] The initial hypothesis that chronic dietary deprivation of vitamins A and C was responsible for this form of megaloblastic anemia[24] was subsequently shown to be erroneous, but studies of an experimental megaloblastic anemia in monkeys and many bacteriologic growth studies were required before folic acid was shown to be the active principle in the therapy.[25]

Biochemistry of the Folates

This subject and most other aspects of these compounds have been exhaustively reviewed recently,[26] and only a summary emphasizing pertinent points will be attempted here.

The parent compound of the folates is pteroylmonoglutamic acid (PGA). Although it functions effectively in supporting growth

of organisms dependent on folates, it, like cyanocobalamin, is merely a stable isolatable form not coenzymatically active *in vivo*. PGA can be considered to consist of three portions: the pteridine, *para*-aminobenzoic acid, and L-glutamic acid. Naturally occurring folates differ from PGA in three respects. First, the pteridine nucleus is reduced at positions 5,6,7, and 8. Second, single-carbon units at different oxidation states are bound covalently to the nitrogens at positions 5 or 10 or bridged between them. Third, additional L-glutamyl residues may be linked to the parent compound through unique peptide linkages; i.e., α-amino to the γ-carboxyl group of the proximal glutamate. Folates coenzymatically active in man are probably all monoglutamates; food folates are primarily hexa- and heptaglutamates and are acted on by an enzyme termed "conjugase" to liberate the monoglutamates prior to absorption from the gastrointestinal tract.[27] Other details of the absorptive process will be discussed later. The structures of PGA, monoglutamate coenzymes, and the triglutamate are illustrated in Figure 2.

Folates function in a great many one-carbon transfer reactions in mammalian systems. Their principal roles are summarized in Figure 3. In a general sense, this schema simply suggests what has been confirmed experimentally, that labelled carbon is readily interconvertible *in vivo* among formate, formaldehyde, serine (β -carbon), purine (2- or 8-carbon), histidine (imidazole-2-carbon), or methionine (methyl carbon). Of key importance to the present discussion is that, in mammalian systems, one of these reactions, the methylation of homocysteine, is also dependent upon the presence of cobalamin, as discussed earlier.

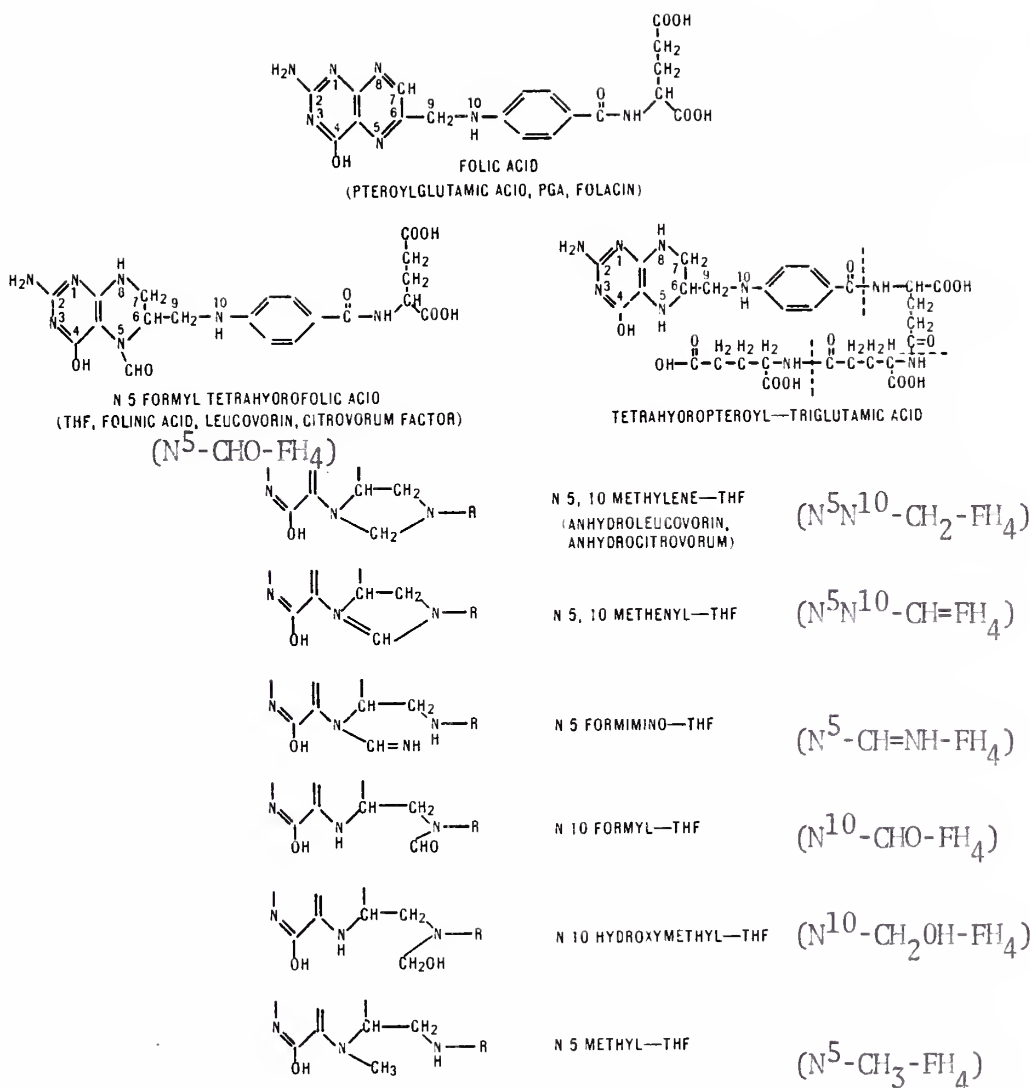

Figure 2. Structures of the Folates. Adapted from Harris JW, Kellermeyer RW: The Red Cell. Cambridge: Harvard University Press, 1970, p 395.

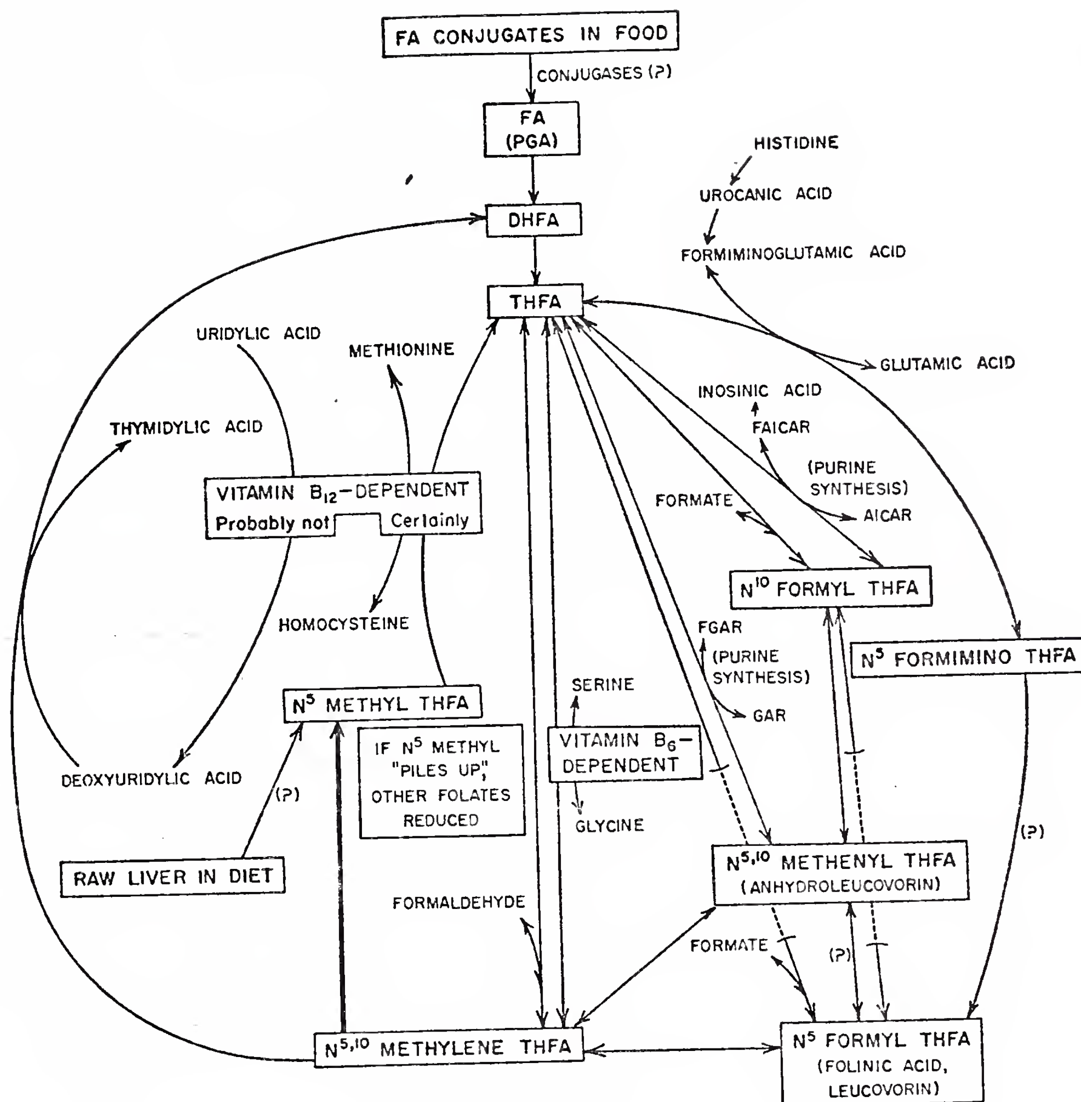

Figure 3. Folate-dependent Reactions. The central role of vitamin B_{12} in regeneration of free tetrahydrofolate is illustrated. From Herbert V: Drugs effective in the megaloblastic anemias. The Phamacological Basis of Therapeutics, Goodman LS, Gilman A eds. New York: The MacMillan Company, 1970.

THE ASSESSMENT OF FOLATE DEFICIENCY

Although more than four decades have elapsed since publication of Wills' initial studies of folate deficiency, significant unsettled problems remain to confront the clinician attempting to assess folate nutritional status. Indeed, until Herbert documented the progressive development of the deficiency syndrome in a normal subject maintained on a folate-poor diet,[28] there was not general agreement that megaloblastic anemia could arise solely as a consequence of dietary folate deprivation. While this study resolved the above issue, it implicitly revealed another; if megaloblastic anemia, hematologically indistinguishable from that resulting from gastric atrophy, could arise secondary to folate deprivation, how were the two syndromes to be distinguished clinically? Prior to 1960, the distinction was made on the basis of the non-hematologic accompaniments of B_{12} deficiency. Absence of neuropathy was obviously less than reliable in this regard since it was not a universal accompaniment of anemia caused by B_{12} deficiency. The sensory neuropathy commonly observed in alcoholic patients further diminished the reliability of the neurologic features as a discriminatory tool. Achlorhydria, consistently present in Addisonian anemia, was also found to have limited usefulness in the differential diagnosis, since it occurred in a significant percentage of older normal individuals. The demonstration of "histamine-fast" achlorhydria and achylia gastrica were found to have greater diagnostic value, but at the expense of considerable discomfort to the

patient. In summary, "folate deficiency" was considered a diagnosis made primarily by exclusion.

Attempts to refine the clinician's ability to differentiate B_{12} from folate deficiency have been directed along five major avenues. Each of these will be discussed below.

Morphologic Distinctions between B_{12} and Folate Deficiencies

From time to time, various aspects of the peripheral or marrow morphology have been touted as characteristic more of one anemia than the other, but these assertions have uniformly been found to be without merit. For example, it has been the "clinical impression" of many hematologists that extreme macrocytosis is seen principally in B_{12} deficiency.[29] McPhedran *et al.*'s recent study of 100 consecutive patients with macrocytosis demonstrates the fallacious nature of this belief;[30] the highest MCV's were actually observed in cirrhotic patients with folate deficiency but normal plasma B_{12} levels.

Therapeutic Trials

After Wills' initial studies it was felt by some that the hematinic in "marmite" was the same active principle, present in fresh liver, which was effective in pernicious anemia. Even after this was shown not to be the case,[24] many physicians persisted in the treatment of Addisonian anemia with folate, a practice which led to the demonstration

by Vilter *et al*. in 1950 that while folate could regularly induce hematologic remissions in pernicious anemia, it seemed to exacerbate or even precipitate neuropathy.[31] Vilter *et al*. proposed that folate exerted a "mass action" effect on hematopoeisis in B_{12} deficiency, forcing some biosynthetic reactions in the nucleoprotein pathway which were also dependent on cobalamins. He suggested that this effect could be deleterious in terms of the separate needs of non-hematologic tissues for B_{12}. These studies led eventually to the formulation of a concept distinguishing the physiologic from the pharmacologic effects of folate. In 1960 Marshall and Jandl published their studies demonstrating the utility of this concept.[32] Patients classified by other criteria as folate-deficient were found to respond with brisk reticulocytoses to daily parenteral administration of 400 mcg of PGA. No additional reticulocytosis was observed when the dose was increased to 15 mg daily. Just the opposite was found in patients with Addisonian anemia; no responses occurred with the smaller dose, but all patients had maximal hematologic responses to 15 mg of parenteral folic acid. Great enthusiasm was expressed for the clinical trial as a diagnostic tool, but this approach has not received general acceptance for several practical reasons. The financial cost of the twenty-odd days of observation required for an adequate trial is often prohibitive. Complications such as congestive heart failure precipitated by prolonged profound anemia may render such an extended undertaking unjustifiable. Deficiency not yet manifested by anemia[28] might be undetectable by this approach. Finally, deficiencies of the two vitamins may coexist, which further complicates the design and interpretation of such studies.

Assay of Folate Activity in Human Tissues

Greater success has been experienced with attempts to assess folate status by measurement of the quantity of vitamin present in body fluids. Although early reports predicted the futility of this approach,[33] a bioassay of "folic acid activity" in human serum which appeared to be of remarkable clinical utility was published in 1959.[34] Subsequently, it was shown that early workers' inability to detect folates in the serum of fasting normal subjects was the consequence of two unappreciated factors; i.e., the specific auxotrophisms of various assay organisms[35] and the nature of the folate coenzymes present in human serum.[36] Almost all serum folate was found to be N^5-CH_3-FH_4, which would support the growth of *Lactobacillus casei* but not *Streptococcus faecalis*, which had been used as the assay organism by most earlier investigators.

Although minor modifications of the original assay technique have been introduced, the estimation of serum folate by bioassay has become a valued clinical tool. It is not without its shortcomings. In practical terms the assay is tedious, time-consuming, and therefore not generally available. It is subject to the same types of distortions as are other bioassays; e.g., artifactually low values resulting from the presence of antibiotics or antimetabolites in the serum. Of greater clinical import, it is perhaps too sensitive to the fluctuations of normal diets. While Herbert was able to define a "normal range" between 7 and 24 ng/ml of serum folic acid activity, the statistical basis of

this definition was never stated, and he was forced to include groups classed "suggestive of deficiency" and "indeterminate" to describe subjects with values between 3.0 and 6.9 ng/ml.[37] His own study of experimental folate deficiency demonstrated that only three weeks of negative folate balance sufficed to produce a fall in serum folate to less than 3.0 ng/ml (indicative of "unequivocal deficiency"). A full 18 weeks elapsed before macrocytosis appeared, and only after 19 weeks of essentially no folate intake did megaloblastic anemia develop.[28] In summarizing the experience of many observers, Chanarin noted that approximately one-third of all hospitalized patients had serum folates below the normal range.[38] Finally, it is possible that deceptively normal values might occur in individuals depleted of folate stores, if the specimen assayed were obtained within six hours of a folate-rich meal.[39] For these reasons, there is now a general consensus that quite apart from the technical difficulties of the assay, the sensitivity of the serum test renders it useless as a screening procedure and relegates it to a confirmatory role in suspected folate deficiency.

Since the information actually sought in measurement of folate levels is an estimation of the status of intracellular active coenzyme pools, it might be predicted that direct tissue assays might be of greater diagnostic value. This is also suggested by Herbert's folate deprivation study, in which erythrocyte folates were measured by bio-assay.[28] Abnormally low values of this parameter did not appear until 17 weeks, much closer temporally to the appearance of clinical deficiency. In an attempt to correlate the morphologic manifestations of deficiency

with biochemical changes, Cooper and Lowenstein studied 63 patients who had serum B_{12}, serum folate, and red cell folate determinations made within 48 hours of bone marrow aspiration.[40] None of these patients was receiving antimetabolites or antibiotics, and none was presumed to have pernicious anemia. Low serum folates (<4.1 ng/ml) were found in 48 of the 63. Only 21 of these 48 had any megaloblastic marrow changes. Every megaloblastic marrow was accompanied by either a depressed RBC folate (17 patients) or low serum B_{12} (4 patients). In contrast, among the 27 patients with normoblastic maturation but depressed serum folates, RBC folates were normal in all except 4 or 5. The authors concluded that the serum folate was the more valuable screening test since it identified all megaloblastic patients. An alternative reading of these results is that measurement of both red cell folate and serum B_{12} will identify all megaloblastic patients with far fewer "false positive" tests. This is the more widely accepted position, and the erythrocyte folate determination has gradually replaced the serum folate estimation as the most widely used clinical indicator of tissue folate depletion.[41]

Although the red cell folate has been shown to be an extremely valuable clinical tool, some theoretical arguments can be raised against its use as an index of "tissue" folate stores. The tissue being assayed is highly atypical; the very biosynthetic processes believed to be deranged in megaloblastosis are inoperative in the mature erythrocyte. Moreover, red cell folate is not freely exchangeable with other tissue pools. Intraerythrocytic folate seems to be primarily in the form of

high polyglutamates[41] trapped intracellularly for the life of the erythrocyte,[28,42] and the cell's entire folate pool is acquired before it reaches maturity.[43] Of more practical significance, the test cannot be relied on to assess folate status in the presence of B_{12} deficiency. This intriguing problem will be discussed further (vide infra p. 45).

Minimal data is available on the folate content of liver. Total hepatic folate content seems to correlate poorly with the fasting serum folate.[44] The "normal range" is unknown, but what values are available suggest that liver folate is depleted in nutritional folate deficiency severe enough to produce anemia. It is perhaps normal in human B_{12} deficiency,[45] but reduced in B_{12}-depleted rats.[46,47] The necessity of resorting to invasive techniques in the acquisition of this type of information obviously diminishes its clinical usefulness.

Recently a non-competitive binding radioassay of folate sufficiently sensitive to measure accurately the range of concentrations present in human serum has been published.[48] Although the peculiarities of bioassay are obviated by this approach, the objections to any measurement of serum folates as the index of body stores remain pertinent. Application of the test to measurement of red cell folate would seem to be an appropriate modification.

Folate Loading Studies

While intravenously administered folic acid has been found to disappear more rapidly from plasma in folate deficient subjects than

in normal controls, clearance of some forms of folate may actually be retarded in B_{12} deficient patients.[37] Such studies have had widespread investigative application (<u>vide</u> <u>infra</u> p. 30), but they are too complex to be of routine assistance in the differential diagnosis of megaloblastic anemia.

<u>Studies of Metabolic Derangements Produced by Folate Deficiency</u>

As might be apparent from inspection of Figure 3, the metabolic disturbances to be anticipated as a consequence of the depletion of folate coenzymes are numerous. Many attempts to assess effective folate stores have been based upon searches for abnormal accumulation or depletion of metabolites involved in folate-dependent single-carbon transfers. Perhaps the most widely employed of these is the so-called FIGlu test. In 1953 a metabolite of histidine was found to be present in increased amounts in the urine of folate deficient rats.[49] Subsequently, this substance was identified as formiminoglutamate (FIGlu). In the presence of a formiminotransferase enzyme and FH_4 as acceptor, free L-glutamate was liberated with the formimino group passing into the "single-carbon pool". In the absence of folate, catabolism of histidine was interrupted at FIGlu, which spilled into the urine. In 1959 Broquist and Luhby found increased amounts of FIGlu in the urine of folate deficient patients.[50] Although no actual data were given, he claimed that the finding was specific for folate deficiency; i.e., normal or absent excretion was supposedly found in

persons with pernicious anemia. This test has been widely adopted clinically in slightly modified form. Most investigators have administered two to twenty gram doses of histidine orally, then measured urine FIGlu. Excellent concordance has been found between the results of this assay and the red cell folate in individual patients.[51,41] As experience has accumulated, the test has been shown to be less specific than originally hoped. Abnormally elevated urine FIGlu's in pernicious anemia have been a frequent finding, even in the absence of a low serum or red cell folate.[37,40,41] Increased amounts of the compound also appear in the urine of patients with liver disease, Hodgkin's disease, and myelosclerosis, in the absence of either vitamin deficiency; false negatives are often observed in the megaloblastic anemia of pregnancy or in the presence of severe protein deficiency.[51] The FIGlu test is no longer performed routinely in most institutions.

Employing far more "physiologic" doses (0.5 mg) of ^{14}C-2(imidazole)-L-histidine, injected intravenously, Fish and coworkers were able to differentiate folate deficient from B_{12} deficient subjects by monitoring $^{14}CO_2$ expired over a two hour period.[52] Cumulative $^{14}CO_2$ excretion was reduced by an order of magnitude, and the time to maximal minute excretion (T_{max}) was delayed severalfold among folate deficient patients as compared with controls or B_{12} deficient subjects. The latter two groups were indistinguishable. Although these data are impressive, the number of patients studied is small and the test is not widely available. Moreover, a recent attempt to demonstrate a similar defect _in vitro_ has been unsuccessful.

Erythrocytes of rats which had been rendered folate deficient or B_{12} deficient by means of dietary restriction were incubated with minute amounts of ^{14}C-2(imidazole)-L-histidine. No differences were observed in either T_{max} or cumulative evolution of $^{14}CO_2$ between the two deficient groups and control animals.[53] The interpretation of this finding in a highly specialized system such as the red cell is of course problematic.

Since the biosynthesis of the purine nucleus is completed by transformylation from a folate coenzyme to 5-amino-4-imidazole carboxamide (AICAR), yielding inosine monophosphate (IMP), abnormal accumulations of AICAR have been sought as an index of significant folate depletion. Surprisingly, the first such effort indicated that increased urinary excretion of AICAR was not observed in folate deficient patients but was universally detected in untreated pernicious anemia.[54] Another report soon followed, however, in which this discriminatory capacity was refuted,[55] and this test has fallen into disuse.

Recently a study purporting to demonstrate a more generalized derangement of single-carbon metabolism specific for folate deficiency has appeared. Tran _et al_. incubated erythrocytes of rats made deficient in B_{12} or folate with ^{14}C-formate and measured the $^{14}CO_2$ evolved over three hours. B_{12} deficient animals yielded results indistinguishable from controls, but $^{14}CO_2$ production by red cells of folate deficient rats was markedly reduced.[53] The study is unfortunately virtually uninterpretable for several reasons. No parameters were reported which assured that the animals were indeed depleted of one or

the other vitamin; in fact, the "folate deficient" group may have been choline deficient. In addition, reproducibility between experiments seems to have been poor. Finally, an earlier in vivo study in rats had demonstrated abnormalities of endogenous and exogenous formate metabolism which were caused by either vitamin deficiency.[56]

The interconversion of glycine and serine is yet another folate-dependent biotransformation,[57] N^5N^{10}-CH_2-FH_4 being the required cofactor. In rats, folate deficiency was shown to abolish the incorporation of ^{14}C-formic acid into the β-carbon of serine of hepatic and visceral protein,[58] but no abnormality of glycine-serine interconversion was observed when this was first studied in human folate deficiency.[59,60] The techniques employed were insensitive (measurement of plasma serine before and after 25 gram glycine infusion) and the patients studied less than ideal (all had tropical sprue and megaloblastic marrows; all responded to subsequent folate treatment). In 1970, a preliminary report appeared in which defective glycine-serine interconversion, assessed by ^{14}C-formate incorporation into the β-carbon of serine of lymphocytes in vitro, was found in 8 patients with folate deficiency.[1] A "relative" defect was also observed among 12 patients characterized only as having gastrointestinal malabsorption and normal indices of folate stores. In the folate deficient group the defect was not entirely abolished even after four to six weeks of daily oral PGA therapy. Unfortunately, no patients with B_{12} deficiency were reported,

which renders the specificity of the assay problematic.[a] Some of the experimental work to be presented here will be directed towards questions raised by this report; e.g., whether the results can be duplicated, what specificity can be claimed for the assay if it is indeed a reliable index of folate deficiency, and what significance can be ascribed to the presence or absence of such abnormalities in vitamin B_{12} deficiency.

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[a]While the present studies were in progress, a more detailed report of this work appeared.[1a] The authors stated that they had observed impaired serine formation in lymphocytes from pernicious anemia patients, but no data were given.

THE BIOCHEMICAL BASIS OF MEGALOBLASTOSIS

Many of the cytomorphologic features of the megaloblastic anemias suggested a profound derangement of metabolic events occurring within the cell nucleus, but the nature of these changes could not have been appreciated prior to the elaboration of our present concepts of the structure and function of the genetic apparatus. Elucidation of the several roles of folates in the synthesis of both purines and pyrimidines rendered attractive the concept of impaired nucleic acid biosynthesis as the central lesion in megaloblastic erythropoeisis. The long-recognized "maturation arrest" and "nucleocytoplasmic dyssynchrony" so characteristic of the megaloblastic marrow were interpreted as morphologic expressions of this biochemical abnormality. This thesis was supported by the observations that uracil or thymine given orally were capable of inducing hematologic remissions.[31] Rundles and Brewer reported that oral orotic acid therapy of pernicious anemia resulted in a limited remission in 1958.[61] Morphology had more specific information to reveal about underlying biochemical mechanisms, however; hemoglobinization (protein synthesis) and hence messenger RNA synthesis seemed to proceed relatively unimpaired in the megaloblast. In retrospect, Thorell's earlier demonstration in 1947 of the presence of normal to increased amounts of RNA in megaloblasts[62] was supportive of this concept. In 1963, Williams and coworkers[63] added the information that while incorporation of purines and pyrimidines into RNA of pernicious anemia megaloblasts was normal, there was an impaired

incorporation of adenine, guanine, and cytosine into DNA. This was found to be ameliorated by addition of exogenous B_{12} to the cultures.

Role of Ribonucleotide Reductase

In the light of the above evidence, attention was quite naturally directed towards the characteristics distinguishing the two polynucleotides from one another; i.e., the presence of deoxyribose and the replacement of uracil by thymine in DNA. An early hypothesis advanced by Beck[64] in 1961, and restated more emphatically in 1965,[65] focused on the B_{12}-dependent ribonucleotide reductase present in *Lactobacillus leichmanii*. Beck developed an analogy between the morphologic and biochemical abnormalities produced in this organism by growth on a B_{12}-depleted medium (long, filamentous organisms unable to divide, with impaired DNA synthesis in the face of normal RNA and protein synthesis) and the morphologic aberrations of megaloblasts. He suggested that the pathogenetic lesion in pernicious anemia might be an inability to reduce ribonucleotides to their deoxy- analogues. One obvious conceptual flaw in this hypothesis was that it failed to account for the intimate nutritional relationships between folates and B_{12}[13] and for their interdependent roles in the development and amelioration of megaloblastosis.[66,67] Furthermore, as experimental evidence accumulated, it became clear that the reductase of mammals differed markedly from that of *L. leichmanii* in terms of substrate requirements and control mechanisms, resembling far more the B_{12}-independent reductase isolated from *E. coli*.[8,68] Nevertheless, Beck

continued to advocate his position, citing unpublished studies from his own laboratories supposedly demonstrating the B_{12}-dependence of marrow reductase.[69] In 1969, Fujioka and Silber reported their inability to stimulate marrow reductase with exogenous B_{12}.[70] Sufficient purification was not possible, however, to exclude the presence of enzyme-bound cobalamin in their preparations. An additional feature reminiscent of the *E. coli* reductase, inhibition by hydroxyurea, was also found by Fujioka and Silber. Beck's studies apparently remain unpublished, and his hypothesis appears untenable at the present time. The properties of the different ribonucleotide reductases are summarized in Table 2, modified from Silber and Moldow.[8]

The Methylfolate Trap Hypothesis

Following the elucidation of the role of vitamin B_{12} in the methylation of homocysteine, Noronha and Silverman noted that methionine administered to B_{12}-depleted rats could eliminate their excessive FIGlu excretion, and also alter the distribution of folate coenzymes in liver from predominantly N^5-CH_3-FH_4 to N^5- and N^{10}-CHO-FH_4. They concluded that the folates had been trapped, by B_{12} deficiency, as N^5-CH_3-FH_4, and that administered methionine had in some way acted as an acceptor for the folate methyl group, thus circumventing the trap and regenerating free FH_4, which became an effective receptor for the formimino group of FIGlu.[71] Herbert and Zalusky applied this concept to the clinical setting. They noted that 17 of 100 consecutive patients with pernicious

TABLE 2

COMPARISON OF RIBONUCLEOTIDE REDUCTASES

SOURCE	*L. leichmanii*	*Escherichia coli*	*Mammalian*
SUBSTRATE	triphosphate	diphosphate	diphosphate
H DONOR	Thioredoxin-like protein	Thioredoxin	Thioredoxin
CATION REQUIREMENT	none	Mg	Mg
STIMULATION BY METAL	none	Fe	Fe
ALLOSTERIC REGULATION	yes; dATP less important	yes; dATP negative effector	yes; dATP negative effector
DEOXYADENOSYL-B_{12} REQUIREMENT	yes	no	probably no
INHIBITION BY HYDROXYUREA	?	yes	yes

anemia presented with serum folate levels elevated to a degree seen otherwise only in subjects ingesting large amounts of folic acid. They proposed that this may have represented a "piling up" of methylfolate in the serum,[17] since most serum folate was N^5-CH_3-FH_4.[36] To test this hypothesis, they infused 15 mcg/kg of folic acid intravenously into normal control subjects and patients with folate, B_{12}, of combined folate and B_{12} deficiencies. Folates other than N^5-CH_3-FH_4 were cleared rapidly from the serum of all deficient subjects, but in the patients with untreated pernicious anemia N^5-CH_3-FH_4 tended to be retained for several hours. This plateau was abolished after treatment with vitamin B_{12}. Moreover, the initiation of B_{12} treatment in pernicious anemia regularly induced a fall in plasma folate. On the basis of this evidence, they concluded that the underlying defect in megaloblastosis was a generalized slowing of folate-mediated single-carbon transfers, and that B_{12} deficiency could bring this about indirectly through its role in the regeneration of FH_4 from N^5-CH_3-FH_4.[17] They also suggested a re-interpretation of the findings of Noronha and Silverman more in keeping with the known roles of the individual metabolites; i.e., that methionine abolished FIGlu excretion by acting as an end-product inhibitor of the enzymes leading to the formation of N^5-CH_3-FH_4. Herbert and Zalusky's "methylfolate trap hypothesis" has become the most widely accepted formulation attempting to account for the obviously intimate interrelationship of vitamin B_{12} and folic acid.

Several studies tending to support this proposal shortly followed. In the most comprehensive of these, Waters and Mollin found the mean serum folate of 100 consecutive patients with untreated

pernicious anemia to be significantly higher than that of healty controls. Perhaps more revealing, the highest serum folates were found among patients with the lowest serum B_{12} levels, provided that the degree of anemia was minimal. The authors suggested that the lower serum folate seen inconsistently in more anemic subjects were a consequence of anorexia leading to true nutritional folate depletion in these patients. A fall in serum folate apparently precipitated by B_{12} treatment was again observed.[72]

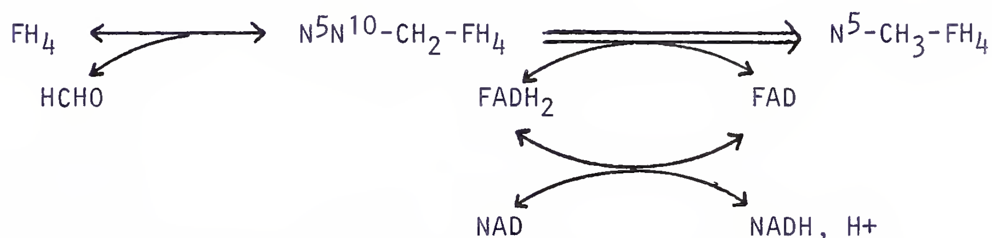
That B_{12} deficiency impairs utilization of plasma methylfolate is by no means established with certainty. The results of some clearance studies have been distinctly at variance with Herbert and Zalusky's findings. Chanarin and Perry were unable to distinguish the clearance of infused synthetic N^5-CH_3-FH_4 from the plasma of pernicious anemia patients as compared to control subjects; clearance was accelerated in folate-deficient patients.[73] All such studies have been criticized as being highly nonphysiologic. Herbert's infusions produced peak serum folate levels of greater than 300 ng/ml in some subjects; in Chanarin's study the maximal concentrations were near 50 ng/ml. The only parameter assessed in many patients was disappearance from plasma, which may be a very poor index of changes occurring in intracellular pools. Recently a clearance study in which some of these factors were better controlled has appeared.[74,75] Two patients with pernicious anemia were given infusions of 5 mcg/kg of N^5-$^{14}CH_3$-FH_4-^3H before treatment and again after establishment of remission with B_{12} therapy. Three others were given only 0.05 mcg/kg of N^5-CH_3-FH_4-^3H. In the first group, serum folates were raised to greater than 50 ng/ml

five minutes after infusion. No rise in the serum folate was produced by the 100-fold smaller dose used in the second group. In the group given the much larger dose, there was insignificant delay of plasma clearance of N^5-CH_3-FH_4, as compared with post-treatment clearance rates. In the three patients to whom only tracer amounts were administered, clearance of methylfolate was definitely retarded, and was significantly accelerated by 1000 mcg of CN-B_{12} injected 24 hours after the infusion. No such enhancement of clearance by an injection of B_{12} was seen when the infusions were repeated with the patients in remission. Urinary excretion of the infused materials was found to be two- to four-fold greater before treatment. After therapy, greater utilization presumably accounted for less appearing in the urine. Moreover, while less than ten per cent of the lower dose of infused folate-^{3}H appeared in the urine within 6 hours, forty-four per cent of the ^{14}C administered in the 100-fold larger dose was excreted in this same time period. In summary, the most "physiologic" studies reported to date strongly suggest that B_{12} deficiency does result in reduced transfer of N^5-CH_3-FH_4 from the plasma to body tissues.

Based upon the assumption that increased endogenous methylfolate pools would dilute $^{14}CO_2$ production from exogenous N^5-CH_3-FH_4, it has been suggested that such increased pools were not present in B_{12} deficient rats.[76] Since the pathways by which the methyl group of methionine (and hence N^5-CH_3-FH_4) is converted to CO_2 are unknown, the actual meaning of this finding is unclear and may have little to do with methylfolate pool sizes.

The methylfolate trap hypothesis requires that, once formed, N^5-CH_3-FH_4 can be metabolized only in the presence of the cobalamin-dependent methyltransferase. No role other than the methylation of homocysteine has been found for N^5-CH_3-FH_4 in man. If, as suggested by Foster,[21] a cobalamin-independent transferase is also present, the trap would be ineffective, but there are no studies yet available which support Foster's contention. It would be anticipated that decreased activity of the methyltransferase would result in decreased *de novo* methionine synthesis. This is supported by the finding of low serum methionine levels in pernicious anemia which return to normal following B_{12} treatment.[77] Accumulation of homocysteine would be expected also, and has been observed.[78]

Methylfolate might also be released from the trap by re-oxidation to its precursor, N^5N^{10}-CH_2-FH_4. The enzyme involved, N^5N^{10}-methylenetetrahydrofolate reductase, has been studied extensively from both mammalian liver and *E. coli*. Although usually assayed in the "reverse" direction, by trapping of formaldehyde released nonenzymatically from N^5N^{10}-CH_2-FH_4[79] as the dimedone adduct,



the presence of an artificial electron acceptor, menadione, is necessary to drive the reaction in this direction. The enzyme from *E. coli*, assayed in this manner, was found to have a K_{eq} of 2400 favoring

reduction of methylenefolate. Assayed in the "forward" direction by monitoring $FADH_2$ oxidation, the K_{eq} was calculated at 3400. Only one of the methyl group hydrogens becomes labelled when N^5N^{10}-CH_2-FH_4 is reduced enzymatically in tritiated water, also suggesting functional irreversibility.[80] Although no thermodynamic data are available on the mammalian enzyme, they at least share similar pH optima. A somewhat capricious attempt to circumvent the "trap" clinically was reported by Herbert _et al._,[81] who injected menadione, both intravenously and directly into the marrow. No reticulocytosis, fall in serum folate, or reversion of the marrow morphology towards the normoblastic state was observed. The authors admit that the study is uninterpretable.

The regulatory role of methionine in the biosynthesis of methylfolate has also received considerable attention. Herbert and Sullivan demonstrated that in patients with megaloblastic anemia methionine suppressed FIGlu excretion. They suggested that inhibition of methylenefolate reductase could be responsible.[82] Dietary methionine supplementation did reduce the activity of the reductase from rat liver, but only by 25-50%.[46] In _E. coli_, it was found that the presence of methionine in the growth medium resulted in low activities of the reductase in homogenates. The mechanism was not allosteric modification of the apoenzyme, since addition of methionine directly to homogenates did not reduce activity.[80] In summary, these studies, if applicable to the human reductase system, would suggest that FH_4 cannot be regenerated by oxidation, and that methionine may exert its effect on

FIGlu excretion by repressing synthesis of the reductase. Such a sparing effect of methionine on folate trapping is at odds with Rundle and Brewer's single case report of a patient with pernicious anemia in whom methionine feeding seemed to accelerate the development of relapse.[61]

It has also been suggested that the effect of methionine in reducing FIGlu excretion is not a manifestation of liberation of "trapped" folates, but simply a result of competition for a common membrane transport mechanism for methionine and histidine, reducing delivery of the latter to catabolic pathways.[82] This seems unlikely in view of the observation that dietary methionine supplementation permitted normal $^{14}CO_2$ excretion from B_{12} deficient rats given injections of ^{14}C-2(imidazole)-histidine.[76] No such facilitation of histidine catabolism was observed in a single patient with untreated pernicious anemia,[83] but $^{14}CO_2$ excretion was normal in this subject prior to methionine supplementation.

The trap hypothesis obviously requires that N^5-CH_3-FH_4 be totally ineffective in inducing remission in B_{12} deficiency. It has been impossible to obtain large enough quantities of methylfolate to test this directly. The ability of oral folic acid to elicit maximal hematologic responses raises questions about the metabolism involved in absorption of folates. Some studies have indicated that oral PGA is totally reduced and methylated by the intestinal mucosa,[84] other studies that some oral PGA is absorbed unchanged.[85,86] When both portal and systemic folates were analyzed after administration of 500 mcg

of L-PGA, apparently the compound was absorbed unchanged, followed by extensive hepatic reduction and methylation (or displacement of liver methylfolate).[87] While much remains unknown about the absorption of folic acid, present evidence suggests that at least some does enter the systemic circulation in reduced forms other than N^5-CH_3-FH_4. This "untrapped" fraction might be sufficient to support hematopoeisis in pernicious anemia patients treated with oral PGA.

Portal venous folates have also been characterized following ingestion of folinic acid (N^5-CHO-FH_4). This compound appeared to have been almost completely reduced to N^5-CH_3-FH_4 by the intestinal mucosa.[88] On the basis of this information, it was assumed that "for tissues other than the intestine, the feeding of folinic acid is equivalent to the feeding of 5-methyltetrahydrofolate itself".[89] Accordingly, a patient with untreated pernicious anemia was given 6 mg of folinic acid daily for 6 days. The brisk reticulocytosis and reversion of the marrow to normoblastic maturation which followed were considered to have disproved the trap hypothesis.[89] Several arguments can be raised against this conclusion. In the experiments upon which this study was based,[86] approximately 15% (150 mcg) of the active stereoisomer of each 2 mg dose was absorbed into the systemic circulation unchanged. While the authors felt that the 450 mcg/day of "untrapped" coenzymatic folate thus introduced would have been insufficient to produce the changes observed, this is inconsistent with abundant evidence that the daily folate requirement of man is only about 50 mcg.[90] Moreover, it is well established that 400 mcg/day of PGA is sufficient to produce a hema-

tologic response in folate deficiency.[32] Finally, a recent study demonstrated that the 1-C unit of orally administered N^5-^{14}CHO-FH_4-^{3}H is rapidly cleaved from its carrier, not simply reduced to methylfolate.[91] This indicates that feeding of N^5-CHO-FH_4 is quite different from supplying pure methylfolate.

An observation which may suggest a role for cobalamins in hematopoeisis, independent of effects on folate metabolism, is that B_{12} seems to promote normal marrow maturation even in the "absence" of folate. Three folate deficient patients with megaloblastic anemias and normal serum B_{12} levels who responded to B_{12} therapy with near-maximal hematologic responses have been reported.[67] Others have been unable to elicit such responses,[92] and it can be postulated that the effect of B_{12} was to promote optimal redistribution of remaining folate coenzymes.[67]

Studies of Megaloblast Proliferation in vitro

The methyl trap hypothesis as originally conceived proposed only a generalized impairment of nucleic acid biosynthesis brought about by interference with folate-dependent single-carbon transfer reactions. For reasons discussed earlier, attention over the past decade has been focused on biochemical events peculiar to DNA synthesis. In 1964, Schmid *et al*. demonstrated that 54% of polychromatophilic megaloblasts, as opposed to only 23% of polychromatophilic normoblasts, were still actively synthesizing DNA. They suggested that megaloblastic

maturation was characterized by an increased percentage of "homoplastic" (non-differentiating) mitoses in the marrow.[93] Elaborate studies of marrow cytokinetics published in 1967 and 1968 better characterized the aberrations of DNA biosynthesis in pernicious anemia megaloblasts. A greater percentage of megaloblastic than normoblastic cells were found to be in the G_2 phase of the cell cycle, as demonstrated by their tetraploid (4C) DNA content and failure to take up ^3H-thymidine. While polychromatophilic normoblasts could easily be assigned to either the G_1,S, or G_2 phase of the cell cycle, megaloblastic marrows contained many polychromatophilic cells which were not engaged in DNA synthesis but yet had DNA contents scattered between the 2C and 4C amounts. These data were considered indicative of abortive DNA replication in megaloblasts. The increased frequency of cells in the G_2 phase was thought to suggest an inability of megaloblasts to enter mitosis following completion of DNA replication.[94,95] These observations were confirmed independently by Yoshida _et al._,[96] who found that the cell cycle abnormalities were eliminated within 24 hours of starting treatment with 1000 mcg CN-B_{12}. Yoshida's study also indicated that RNA and protein synthesis progressed at accelerated rates in megaloblasts from untreated patients.

In 1968, a biochemical lesion in DNA synthesis consistent with the methyl trap hypothesis was demonstrated in cultures of marrow megaloblasts by Metz and coworkers.[97] The methylation of deoxyuridine (dU) had earlier been shown to be impaired in B_{12}-deficient animals, and some workers had suggested that CH_3-B_{12} functioned coenzymatically

in this reaction. Although others were unable to demonstrate such a requirement for B_{12}, the role of N^5N^{10}-CH_2-FH_4 as C-1 donor was established.[98] Metz *et al.* incubated marrow cells *in vitro* with unlabelled dU and then added ^{3}H-thymidine (^{3}H-dTR) to the cultures. In normal marrows, the dU preincubation was able to suppress incorporation of subsequently added ^{3}H-dTR into DNA to less than 10% of control (non-dU-preincubated) values. This effect was considered to indicate methylation of the dU and subsequent competition with the added ^{3}H-dTR. In marrows from B_{12}-deficient patients, dU preincubation could not suppress ^{3}H-dTR incorporation, indicating a "block" in methylation of dU. Addition of 1 mcg of B_{12} to the culture medium partially re-established the dU suppression, but 50 mcg of PGA completely restored it. Even more striking was the finding that 50 mcg of N^5-CH_3-FH_4 was unable to restore dU suppression in B_{12}-deficient marrows, while it did so nearly as well as PGA in the marrow of a folate deficient patient. That the role of B_{12} was an indirect one was demonstrated by the ability of methotrexate to cancel its effect completely. These findings offered impressive support for the methylfolate trap hypothesis. It could now be postulated that the principal effect of the trapping of folates as N^5-CH_3-FH_4 was the depletion of its immediate precursor, N^5N^{10}-CH_2-FH_4, the substrate for the methylation of dU.[97] The inability of pernicious anemia megaloblasts to incorporate dU into DNA also argued against Beck's localization of the lesion at ribonucleotide reduction.

Metz's experiments were extended to study the effects of methionine and homocysteine on the defective dU suppression of megaloblastic marrows.[99] As discussed earlier, methionine had been shown to reduce FIGlu excretion and somehow to release "trapped" folates in B_{12} deficiency. Surprisingly, methionine was found to block the corrective effect of B_{12} in pernicious anemia marrows.[99] End-product inhibition of homocysteine transmethylase, as suggested in rat liver,[46] was invoked as a possible mechanism of this effect. Homocysteine partially restored dU suppression of ^{3}H-dTR incorporation in pernicious anemia megaloblasts in the absence of B_{12} or the presence of a B_{12} antagonist. Several interpretations of these findings were considered by the authors, including the presence of a B_{12}-independent transmethylase which could regenerate FH_4. Alternatively, homocysteine could have accepted some of the methyl groups of N^5-CH_3-FH_4 nonenzymatically by a mass action effect because of the large quantities of the amino acid present; 1 mg of homocysteine was added to the cultures, while only 50 mcg quantities of folate coenzymes were sufficient to restore dU suppression. An alternative interpretation of the methionine effect, not suggested by the authors, would require that methionine suppressed not only N^5N^{10}-CH_2-FH_4 reductase, but also pathways leading to the synthesis of methylenefolate. If this were the case, the available pool of free FH_4 for histidine catabolism would be increased by methionine, thus enhancing FIGlu breakdown; lack of N^5N^{10}-CH_2-FH_4 would then lead to impaired dU methylation. No data in support of this hypothesis are available, and its plausibility is questionable in view

of the presence of a functioning non-enzymatic pathway *in vivo* for the production of N^5N^{10}-CH_2-FH_4 from FH_4 and formaldehyde.[79]

On the basis of the dU suppression studies, the cytogenetic abnormalities observed in megaloblasts have been interpreted as the result of thymidine deficiency, leading to single-strand breaks in the DNA helix.[100] No differences in base composition of normal as opposed to megaloblastic marrow cell DNA have been demonstrated, however.[101]

The Role of Cobalamins in Pernicious Anemia Neuropathy

An attractive feature of the methylfolate trap hypothesis is that it seeks to explain the hematologic effects of B_{12} in terms of a folate-dependent action, and the neurologic effects of B_{12} in terms of a folate-independent function. It has been suggested that reduced activity of methylmalonyl CoA mutase, the only other enzyme known in man to require a B_{12} cofactor, could in some way produce the sensory neuropathy of pernicious anemia. Recently some experimental support for this speculation has been developed. Sural nerve biopsy slices from pernicious anemia patients and control subjects, when incubated with ^{14}C-propionate, both incorporated the label into fatty acids, but in the B_{12} deficient slices, label appeared extensively in anteiso-C_{15} and C_{17} saturated acids, whereas in control subjects the labelling was of C_{14} and C_{16} acids.[102] In rat glial cell cultures, cells grown in B_{12} deficient medium elaborated C_{15} and C_{17} acids, and propionate accumulated in the medium. These effects were reversible by B_{12}

supplementation.[103] The inference made from these findings is that the abnormalities of fatty acid biosynthesis observed *in vitro* may result *in vivo* in deranged metabolism of myelin surrounding peripheral nerves, and thus bring about subacute combined degeneration of the white matter of the spinal cord. A tremendous amount of speculation is required to postulate a causal relationship between the biochemical observations and the clinical lesions. At present, it seems prudent to regard the association as interesting but of undertermined significance.

Implications of Inborn Errors of Folate and B_{12} Metabolism

That impaired nucleic acid biosynthesis is the primary lesion in megaloblastosis is supported by the appearance of megaloblastic anemia in orotic acidura, an inherited disorder characterized by one or more blocks in the pyrimidine biosynthetic pathway.[104] The particular consequences of cobalamin-folate interdependence specified by the methyl trap hypothesis are supported by two recently described inborn errors of folate metabolism. Arakawa *et al*. studied an infant with megaloblastic anemia, increased urinary FIGlu, and elevated serum *L. casei* activity (N^5-CH_3-FH_4).[105] They suggested that a deficiency of N^5-CH_3-FH_4-homocysteine transmethylase was responsible for the clinical findings. Activity of the enzyme from liver was reduced to 30-50% of control values, but the range of activities of other enzymes measured was so great among "controls" that the reduction of transmethylase

activity in the patient could be considered to be within the limits of reproducibility of the assays. Also, no mention of homocystinuria, a possible consequence of such an enzymatic block, was made.

Methylenetetrahydrofolate reductase deficiency has been reported in two sibships.[106] This block would not be expected to produce megaloblastic anemia and apparently did not. Homocystinuria, presumably consequent to N^5-CH_3-FH_4 deficiency, was present in these patients. Surprisingly, neither serum nor tissue N^5-CH_3-FH_4 levels were reported. Methyltransferase activities were normal.

Inherited disorders of B_{12}-dependent metabolism have also been described in recent years.[107] Several children have now been reported with metabolic ketoacidosis and methylmalonic acidemia secondary to methylmalonyl-CoA mutase deficiency. The methyl trap hypothesis would predict no impairment of hematopoeisis resulting from such a block, and megaloblastic anemia has not been observed. The methylmalonic acidemia phenotype is now recognized to be genetically heterogenious, some patients apparently having a mutant apoenzyme, others being unable to accumulate the coenzyme dA-B_{12} intracellularly.[108] More profound genetic defects of cobalamin metabolism have also been described. An infant reported by Levy _et al_.[109] died at 7½ weeks of age, ending a clinical course characterized biochemically by persistent hypomethioninemia, homocystinemia, and methylmalonic aciduria. Megaloblastic changes were not observed in marrow aspirates, but frequent transfusions were required to maintain the infant's hematocrit. Tissue enzyme analyses revealed depressions of the B_{12}-dependent methyl-

transferase and methylmalonyl-CoA mutase, which could be rerestored towards normal by high concentrations of the appropriate cofactors.[111] The postulated enzymatic block in this child must be at a point common to the biosynthesis of both cobalamin coenzymes; i.e., transport into the cell or reduction of the central cobalt atom,[107] or some subsequent step required for formation of each coenzyme. A biochemically similar, though clinically less severe, lesion has been observed in two siblings by Goodman et al..[112,113] Both of these children had methylmalonic aciduria and homocystinuria, but plasma methionine levels were normal. Neither had megaloblastic or macrocytic changes, and on this basis Goodman raised arguments against the plausibility of the methyl trap hypothesis. The diminution of methyltransferase activity was asserted to be at least as great in his patients as occurs in pernicious anemia, because of the magnitude of homocystine excretion in the two conditions. Methyltransferase activities have not actually been measured in human B_{12} deficiency, but the reduced plasma methionine levels usually found would suggest a more profound reduction of methyltransferase in untreated pernicious anemia. At present, the issue remains unsettled. The lack of megaloblastosis in the infant dying with a far greater reduction of methyltransferase activity is more difficult to explain, but in the presence of such a profoundly disturbed biochemical milieu, the marked erythroid hypoplasia observed might have obscured any tendency to megaloblastic changes.

The possible role of the propionate — methylmalonate — succinate pathway in the pathogenesis of the neurologic manifestations

of pernicious anemia has been mentioned earlier. Although clinical signs of dorsal column dysfunction have not been reported in either methylmalonyl-CoA mutase deficiency or the "combined defect" syndromes, mental retardation and hypotonia[114] have been prominent features. Autopsy has either not included the neuropathology[115] or demonstrated diffuse and focal softening of cerebral white matter.[116] There is no justification at present for attributing the neurologic manifestations of these syndromes to the abnormalities of fatty acid biosynthesis observed _in vitro_ in B_{12} deficiency.

Other Postulated Roles for B_{12} in the Development of Megaloblastosis

An unconfirmed report has appeared suggesting a role for CH_3-B_{12} in methylation of tRNA.[117] It was suggested that in B_{12} deficiency, non-methylated tRNA would accumulate which would be less effective in amino acid transfer to ribosomes. The considerable evidence presented earlier suggesting normal protein synthesis in the face of impaired nucleic acid metabolism renders this hypothesis untenable.

A "geographic" rather than metabolic folate trap has been postulated by Chanarin[118] to be the basis of megaloblastosis in B_{12} deficiency. Erythrocyte folates are reduced in approximately two-thirds of B_{12} deficient patients and return to normal following institution of therapy.[119] This may simply be a reflection of an older peripheral red cell population rather than of impaired folate uptake by red cell

precursors.[118] The increased plasma/red cell folate "gradient" regularly observed in B_{12} deficiency[72] and its rapid disappearance following initiation of therapy suggest that folate uptake is indeed impaired. Analysis of this proposition is complicated by the fact that intracellular and extracellular folates are not freely exchangeable, the former being largely polyglutamates.[41] Kutzbach *et al.* have speculated that the impaired folate polyglutamate accumulation of rat hepatocytes in B_{12} deficiency may actually represent another manifestation of methyl trapping, if, as in *E. coli*, FH_4 but not N^5-CH_3-FH_4 is the substrate for polyglutamate synthesis.[46] In the absence of any understanding of the functions of polyglutamates in mammalian systems,[120] it is difficult to formulate any hypotheses explaining the known biochemical consequences of cobalamin deficiency on the basis of polyglutamate depletion.

PURPOSES OF THE PRESENT STUDIES

Sufficiently sensitive assay techniques have not yet been developed to render practicable the measurement of intracellular folate coenzyme pools in the clinical setting. Knowledge of the metabolic roles of each of these compounds does make possible prediction of the consequences of depletion of individual coenzymes. Since the methyl trap hypothesis requires a relative deficiency of intracellular N^5N^{10}-CH_2-FH_4, estimations of the effective pool of this form of folate would be illuminating. As glycine-serine interconversion requires this coenzyme, it would be reasonable to expect this reaction to be impaired consequent to B_{12} deficiency, if the methyl trap hypothesis were correct. Because impaired formate incorporation into serine by peripheral lymphocytes has been reported to be a sensitive indicator of folate deficiency,[1] the intact leukocyte would seem to be an ideal system in which to attempt confirmation of these expectations. It is also of importance to establish the specificity of the assay if it is to be used clinically as a diagnostic tool.

Although not believed to be involved in the development of megaloblastosis, methylmalonyl-CoA mutase activity is known to be reduced in B_{12} deficiency[11] and may be a more specific indicator of the deficiency state because of its independence of folate metabolism. Recent publications suggest that this independence may not be total. Low serum B_{12} levels which rose following initiation of specific therapy have been found in 40-60% of patients with folate deficiency.[121]

Intraerythrocytic cobalamin levels have been reported to be universally low in folate deficiency.[122] These observations suggest that an as yet unexplained B_{12}-folate interaction may influence the cellular uptake of both vitamins and raise the possibility that deficiency of either will bring about deficiency of the other. The fact that methylmalonic aciduria has been reported not to occur in pure folate deficiency[123] raises doubts about this hypothesis, but it is possible that a more sensitive assay would detect impaired B_{12}-dependent metabolism. Impaired methylmalonate oxidation by peripheral leukocyte extracts has been reported to be a reliable test for B_{12} deficiency, even in the absence of significant methylmalonic aciduria,[11] but the status of the propionate pathway has not been evaluated in uncomplicated nutritional folate deficiency.

MATERIALS AND METHODS

At the time of study, all subjects but one were inpatients on the wards of the Yale-New Haven Hospital. Control subjects were healthy laboratory personnel. Prospective patients were selected by means of erythrocyte folate bioassay using *Lactobacillus casei* as the test organism;[41] serum folate bioassay using the same organism; or plasma vitamin B_{12} isotope dilution assay.[124] These tests are routinely performed by the hospital's clinical microscopy laboratory on all patients found by Coulter Counter Model S determination to have erythrocyte MCV's greater than or equal to 115 cubic microns.[30] This screening program proved to be an effective tool for detecting unsuspected, and therefore untreated, folate and B_{12} deficiences. A few patients thus identified were admitted to the Clinical Research Center for study and to investigate the etiology of their deficiencies. Patients receiving antimetabolite therapy were excluded from the study.

Oral or written informed consent was obtained from all patients being studied.

Preparation of Leukocyte Suspensions

Cells were prepared as described by Rosenberg *et al*..[2] Following an overnight fast, 15-30 ml of venous blood was drawn into a plastic syringe containing 90 units of sodium heparin (Sigma Chemical Co.). The blood was then mixed with an equal volume of 1% (wt/vol)

polyvinyl pyrrollidone (PVP-360, Sigma Chemical Co., average MW 360,000) in 0.9 g/100 ml NaCl containing 45 mg/100 ml D-glucose. The erythrocytes were allowed to sediment for 45 minutes at 4°C at 140 x g for 15 minutes. The supernatants were decanted and the pink cell pellets gently resuspended with pasteur pipettes in isotonic (0.8 g/100 ml) ammonium chloride containing 45 mg/100 ml D-glucose at 20°C. Ten minutes sufficed to lyse remaining erythrocytes; the cell suspensions were then centrifuged at 100 x g for 15 minutes at 4°C. The cell pellets were washed in ice-cold Kreb's bicarbonate buffer at pH 7.4 (containing 45 mg/100 ml D-glucose), centrifuged again at 100 x g for 15 minutes, and finally suspended in a volume of buffer sufficient for the assays. All procedures were carried out in plastic or nonwettable glass apparatus. Components of the buffer were as follows:

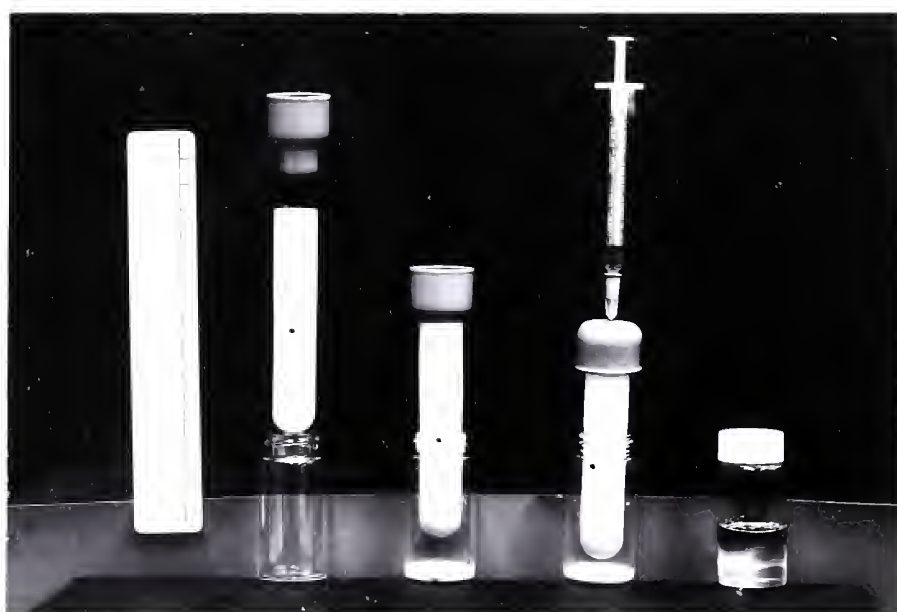
Na^+	144.1 mEq/l	Cl^-	130.6 mEq/l
K^+	6.0 mEq/l	$H_2PO_4^=$	1.2 mEq/l
Ca^{++}	6.8 mEq/l	$SO_4^=$	2.4 mEq/l
Mg^{++}	2.4 mEq/l	HCO_3^-	25.1 mEq/l

0.1 ml aliquots of cell suspensions were diluted in 1.9 ml of 4% acetic acid for enumeration by hemocytometer under phase microscopy. Final concentrations of leukocytes were between 6×10^6/ml and 30×10^6/ml.

Oxidation of ^{14}C-3-Propionate and ^{14}C-1,4-Succinate

Incubations were carried out in an apparatus developed by Hsia,[2] shown in Figure 4. Teflon tubes which fit snuggly into the mouths of scintillation vials were modified for the assay by drilling four holes of approximately 2 mm diameter about the perimeter of the tube (A, Figure 4). The tubes were then inserted into scintillation vials containing 0.5 ml "Hyamine" alkali so that the holes were occluded by the vial necks. 1.65 mcmole (0.25-1.69 mcCi) Na ^{14}C-3-propionate or 1.65 mcmole (0.34-1.50 mcCi) ^{14}C-1,4-succinic acid (both purchased from New England Nuclear Co.) in a total volume of 0.1 ml was placed in each tube. 0.9 ml of leukocyte suspension was added to each. The tubes were gassed for 1 minute with a 95% O_2: 5% CO_2 mixture, sealed with rubber stoppers (Figure 4B), and incubated for three hours at 37°C in a Dubnoff metabolic shaker.

At the conclusion of the incubation period, $^{14}CO_2$ formed from ^{14}C-labelled substrates was collected by placing the vials in ice with the tubes pushed about 0.5 cm farther into the vials, thus establishing continuity between the gas phases of the sealed tubes and the vials, and the reactions were terminated by injection of 1.0 ml of 6 N H_2SO_4 into the incubation mix (Figure 4C). The evolved CO_2 was collected in the Hyamine over 30 minutes, with a yield of at least 85%.[125] The apparatuses were disassembled, 10 ml of toluene - Liquifluor (New England Nuclear) added to each vial (Figure 4D), and the radioactive $^{14}CO_2$ counted in a Packard liquid scintillation spectrometer. Counts were corrected for quenching by the channels ratios method. Normal con-



A B C D

Figure 4. Apparatus used for leukocyte oxidation of propionate and succinate. Explanation of the legend can be found in the text.

Figure 4. Apparatus used for leukocyte oxidation of [illegible] and succinate. Explanation of the legend can be found in the text.

trol cells and cell-free blanks were run simultaneously with each assay. Results were calculated as nmoles of CO_2 evolved from the substrate over the 3 hour incubation per 10^8 leukocytes.

Formation of ^{14}C-L-Serine from L-Glycine and Na ^{14}C-Formate

Assay conditions were modified from those described by Ellegaard and Esmann.[1] 0.9 ml of leukocyte suspensions were pipetted into nonwettable glass scintillation vials containing 0.1 ml of "formate substrate" such that final incubation concentrations were calculated to be glucose 6.6 mM, L-glycine 20 mM, and Na ^{14}C-formate (New England Nuclear Co., s.a. 58.6 mC/mmole) 41 nmoles/ml. The vials were gassed for one minute with 95% O_2: 5% CO_2, capped, and agitated gently for four hours at 37°C in a Dubnoff shaker. Incubations were terminated by heating the vials for two minutes in a 95°C water bath. Normal control cells and cell-free blanks were run simultaneously with each assay.

After heating, the suspensions were transferred to glass centrifuge tubes, centrifuged at 250 x g for 10 minutes, and portions of the supernatants removed for separation of ^{14}C-L-serine.

Separation of ^{14}C-L-Serine from Na ^{14}C-Formate

Serine was separated by a modification of the isoionic exchange chromatographic technique described by Thauer *et al.*.[126] Columns (1 cm

inside diameter) were filled with 1.5 g of moist resin (Dowex 2-X8, 200-400 mesh), converted to formate from with 2 N ammonium formate.[a] Initially, several eluent solutions were used. It was found that when 2.0 ml samples, acidified to a final concentration of 0.01 N trichloroacetic acid (TCA) were applied to the columns and washed in with 1.0 ml H_2O and 1.0 ml of acid eluent, approximately 96% of applied ^{14}C-L-serine appeared in the initial 4.0 ml of eluate (Figure 5). Elutions were continued with increasing concentrations of formic acid or with 0.4 N sulphuric acid; with each of these it was consistently found that ^{14}C-formate was retarded beyond the volume containing serine (Figure 5). For routine assay, therefore, acidified 2.0 ml samples were applied, washed in with 1.0 ml of H_2O and 1.0 ml of 0.05 N HCOOH, and the corresponding 4.0 ml collected and its radioactivity counted (1.0 ml aliquots in 9.0 ml of "Aquasol", New England Nuclear Co.). Quench correction was by the channels ratios method. Radioactivity in the initial 4.0 ml eluate was assumed to be ^{14}C-3-L-serine derived from ^{14}C-formate, and results were calculated as nmoles ^{14}C-L-serine formed per 10^9 leukocytes over the four hour incubation period.

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[a]Thauer[125] prepared columns with 4 N ammonium formate. The concentration change was required to enable solubility of the initial column eluate in scintillation cocktail.

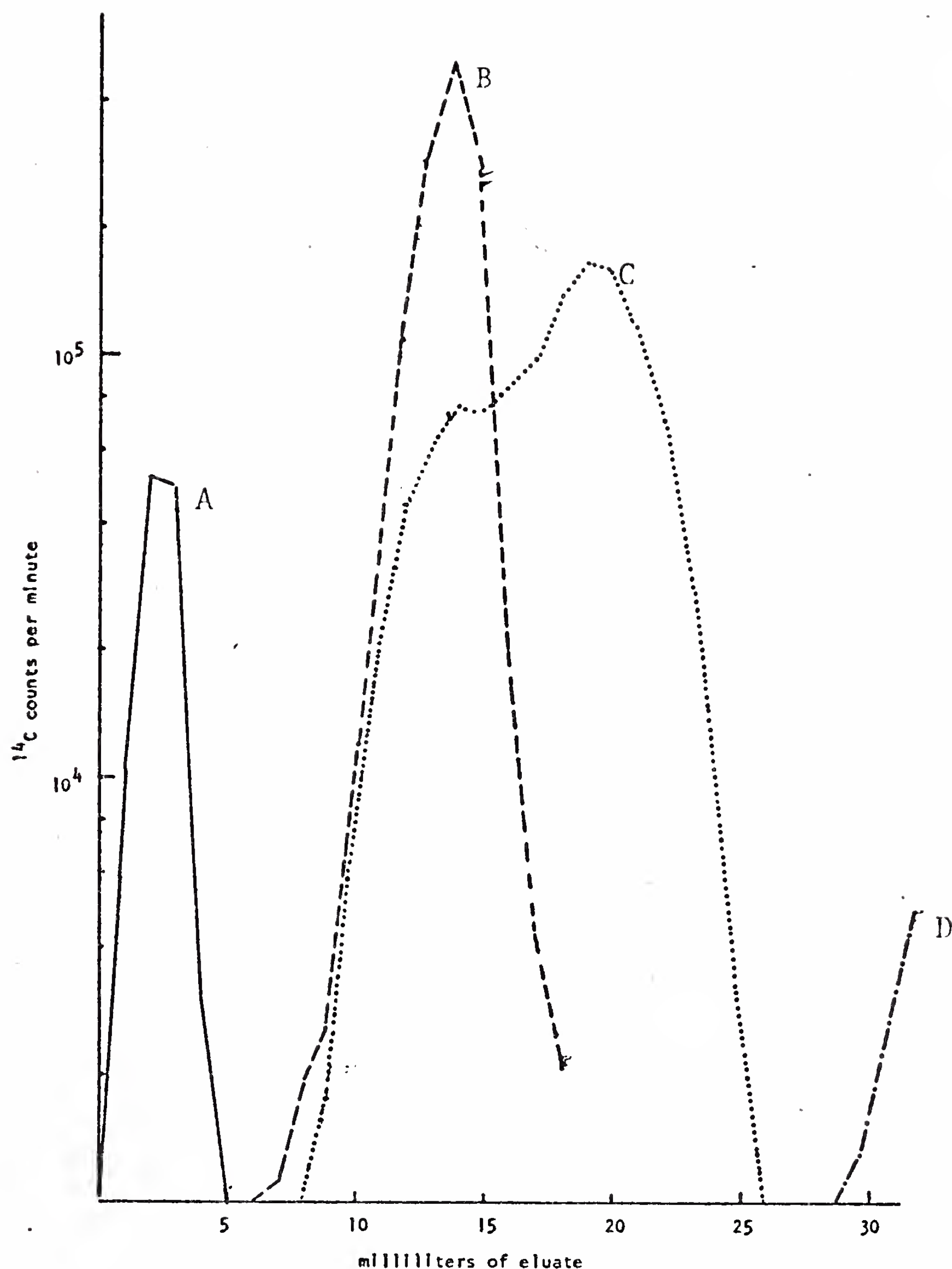

Figure 5. Isoionic Exchange Chromatography of ^{14}C-L-Serine and ^{14}C-Formate.

A: ^{14}C-L-Serine, eluted with formic acid (0.05μ Ci, 0.2 N HCOOH. 95.6 % of applied ^{14}C-L-serine appeared in the first 4.0 ml of eluate).

B: ^{14}C-Formate, eluted with sulfuric acid (0.6 μCi, eluted with 1.0 ml of 0.05 N HCOOH and 15.0 ml 0.4 N H_2SO_4. 96.7 % of applied ^{14}C-formate eluted as a discrete peak).

C: ^{14}C-Formate, eluted with formic acid (0.6 μCi, 0.2 N HCOOH. 93 % of applied ^{14}C-formate eluted as a discrete peak).

D: ^{14}C-Formate, eluted with dilute formic acid (0.6 μCi, 0.05 N HCOOH. 12.9 % of applied ^{14}C-formate had appeared prior to cessation of collection at 35 ml).

RESULTS

Studies of the Leukocyte Serine Hydroxymethylase Assay

In order to ascertain the effects of changes in assay ^{14}C-formate concentration on net incorporation into ^{14}C-serine, replicate assays were run with varying amounts of ^{14}C-formate in the incubation mixes, yielding the following results:

Na ^{14}C-formate in incubation mix	Cell Count	nmoles Ser / 10^9 WBC / 4 hrs
43 nmoles	17.9 x 10^6/ml	10.5, 10.2
91 nmoles	"	13.8, 12.6
143 nmoles	"	15.6, 15.5

Since a more than threefold increase in incubation ^{14}C-formate concentration produced no more than a 50% increase in formation of radioactive serine, it was concluded that at the concentrations tested, formate concentration was not the rate-limiting factor in total incorporation.[a]

Activity of serine hydroxymethylase was also sought in diploid human fibroblasts. These cells proved to incorporate ^{14}C-formate into serine at a rate approximately fifty times that observed in peripheral leukocytes.

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[a]This contrasts with the findings of Ellegaard and Esmann[1a] who observed a linear relationship between ^{14}C-formate concentration and ^{14}C-3-serine formation in lymphocyte suspensions.

In one preliminary experiment, replicate leukocyte suspensions were incubated with and without added glycine in the substrate solution. In the absence of exogenous glycine, ^{14}C-serine formation was reduced by approximately 50%.[a]

To confirm the identity of the eluate fraction collected and counted as ^{14}C-serine, 3.0 ml of the initial 4.0 ml eluate from one set of assays were taken to dryness in a vaccum dessicator, 0.02 ml (0.2 mcC) of ^{3}H-L-Serine (New England Nuclear Co.) and 0.18 ml of distilled water were added to each, and 0.1 ml of the resulting solutions were spotted on Whatman 3MM chromatography paper. This was subjected to electrophoresis at 2500 volts for six hours, using 6% formic acid as the mobile phase in a petroleum distillate (Varsol) bath. The electrophoretograms were cut into 1 x $1\frac{1}{4}$ inch strips and counted in "Liquifluor"-toluene in a Packard Tri-Carb liquid scintillation spectrometer with window widths and gains adjusted for maximum ^{14}C counting efficiency with minimal contamination of the low energy (^{3}H) channel by ^{14}C disintegrations. It was found that no ^{14}C peak appeared in the electrophoretograms of cell-free blanks, and that a single ^{14}C peak, migrating precisely with marker ^{3}H-L-serine over 23 inches, appeared in the eluates from live cell incubations. As no other peaks were detected, this confirmatory procedure was omitted from routine assays.

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[a]Similar data was also obtained by Ellegaard and Esmann.[1a]

In our preliminary experiments, Fe(II)[illegible] [illegible] were incubated with [illegible] added [illegible] solution. In the absence of [illegible] was reduced by approximately [illegible].

To confirm the identity of [illegible] counted as ^{14}C-serine, [illegible] set of assays were taken to dryness [illegible] (0.2 mCi) of ^{14}C-serine [illegible] distilled water were added to [illegible] solutions were spotted on [illegible] subjected to electrophoresis at [illegible] formic acid as the mobile phase [illegible] bath. The electrophoretograms [illegible] counted in "Liquifluor"-toluene [illegible] scintillation spectrometer [illegible] maximum ^{14}C-counting efficiency [illegible] mobility [illegible] been observed in the [illegible] a single ^{14}C-peak [illegible] [illegible] appeared [illegible] other peaks were detected [illegible] from routine assays.

[illegible]

Patients Studied

Pernicious anemia--

A1: A 59 year old caucasian male cook seen in the emergency room because of pleuritic chest pain and found incidentally to have a high MCV. Diagnosis based on demonstration of histamine-fast achlorhydria and Schilling tests (Stage I: 1.0% excretion; Stage II: 10% excretion).

A2: An 84 year old Swedish-American widow with an anemia unresponsive to iron therapy. Diagnosis made on the basis of histamine-fast achlorhydria and Schilling tests (0.8% excretion without intrinsic factor; 4.2% excretion with intrinsic factor).

A3: A 60 year old caucasian housewife, presumed to have pernicious anemia on the basis of a Stage I Schilling test yielding only 1.3% excretion of the ingested isotope over 24 hours.

Other B_{12} deficient patients--

B1: A 44 year old woman with heterozygous β-thalassemia admitted to the hospital for drainage of a chronic subdural hematoma following resection of an intracerebral meningioma. She had been taking oral diphenylhydantoin for 9 months at the time of study and had been transfused with 2 units of packed red cells one week prior to study. Stage I Schilling test revealed normal absorption of B_{12}. Her B_{12} deficiency was presumably a secondary phenomenon produced by profound folate deficiency.

B2: A 48 year old massively obese caucasian woman with an undiagnosed neurological disorder characterized by reduced motor function below C_5 with intact sensory modalities in all four extremities. At the time of study she was on a zero Calorie diet with daily oral 5 mg PGA supplements. Bone marrow aspiration, gastric secretion studies, and Schilling tests were not performed on this patient. The etiology of her B_{12} deficiency is not established.

Folate deficiency--

C1: A 25 year old caucasian woman with intractible temporal lobe epilepsy, taking oral diphenylhydantoin.

C2: A 42 year old housewife admitted for hysterectomy because of a myomatous uterus. Dietary history was consistent with folate deprivation.

Clinical laboratory data on all patients studied are summarized in Table 3.

Oxidation of ^{14}C-3-Propionate and ^{14}C-1,4-Succinate

As shown in Table 4, patients with vitamin B_{12} deficiency, whether or not resultant from pernicious anemia, uniformly demonstrated a substantially reduced capacity to oxidize ^{14}C-Propionate to $^{14}CO_2$, consistent with impairment of the methylmalonyl-CoA mutase reaction. Oxidation of succinate by pernicious anemia patients was slightly increased as compared to controls, but no significant difference in succinate oxidation was observed between control subjects and B_{12}-deficient patients without pernicious anemia, or between controls and all B_{12}-deficient subjects as a group.

Two folate deficient patients studied showed no measurable defects in oxidation of either propionate or succinate. Patient B1, listed as B_{12} deficient, was in addition profoundly folate deficient as well. Her serum B_{12} level rose spontaneously to 200 pg/ml within 48 hours of initiation of folate therapy.

Figure 6 contrasts the mean propionate oxidations of individual B_{12} deficient subjects with the values observed among normal controls. Comparison with laboratory data from Table 3 illustrates that the degree of impairment of the methylmalonyl-CoA mutase activity of leukocytes does not seem to correlate with either the degree of anemia or the severity of B_{12} deficiency as measured by plasma B_{12} assay.

TABLE 3

CLINICAL LABORATORY DATA OF PATIENTS STUDIED (prior to treatment)

Patient	Hematocrit %	MCV $microns^3$	Reticulocytes %	Marrow Morphology	Fe/IBC mcg/100 ml	RBC Folate ng/ml	Serum Folate ng/ml	Serum B_{12} pg/ml
PERNICIOUS ANEMIA PATIENTS								
A1	46	127	0.1	Megaloblastic	172/428	152	-	0
A2	36	121	2.0	Megaloblastic	155/362	146	-	0
A3	32	122	-	Megaloblastic	140/230	190	9.5	10
OTHER B_{12} DEFICIENT PATIENTS								
B1	31	81	0.7	Macrogranulocytic		73	2.2	155
B2	45	107		—		130*	-	100
FOLATE DEFICIENT PATIENTS								
C1	39	118		-		21	-	310
C2	37	116		-		40	3.1	510
Controls#						>150+	>3.0+	200-1200

*Determined 6 days prior to study. She received 5 mg PGA orally daily in the intervening 5 days.

#Normal values for the Yale-New Haven Hospital Clinical Laboratories.

+"Upper limit" of normal folate values is not established.

TABLE 4

LEUKOCYTE OXIDATION OF PROPIONATE AND SUCCINATE

Subjects	PROPIONATE			SUCCINATE		
	Number of Determinations	$\frac{\text{nmoles } ^{14}CO_2 / 10^8 \text{ WBC}}{3 \text{ hrs}}$ ($\bar{x} \pm 1$ S.D.)	Difference from Controls* (p)	Number of Determinations	$\frac{\text{nmoles } ^{14}CO_2 / 10^8 \text{ WBC}}{3 \text{ hrs}}$ ($\bar{x} \pm 1$ S.D.)	Difference from Controls* (p)
Controls	24	13.2 ± 4.8	-	22	40.0 ± 17.6	-
Pernicious anemia patients	12	4.4 ± 2.4	$< .001$	11	60.0 ± 30.0	$< .05$
Other B_{12}-deficient patients	6	5.6 ± 2.5	$<.001$	6	30.0 ± 13.6	NSD
Folate-deficient patients	4	10.5 ± 1.3	NSD	4	79.9 ± 19.5	$< .001$

*Determined by the unpaired t-test

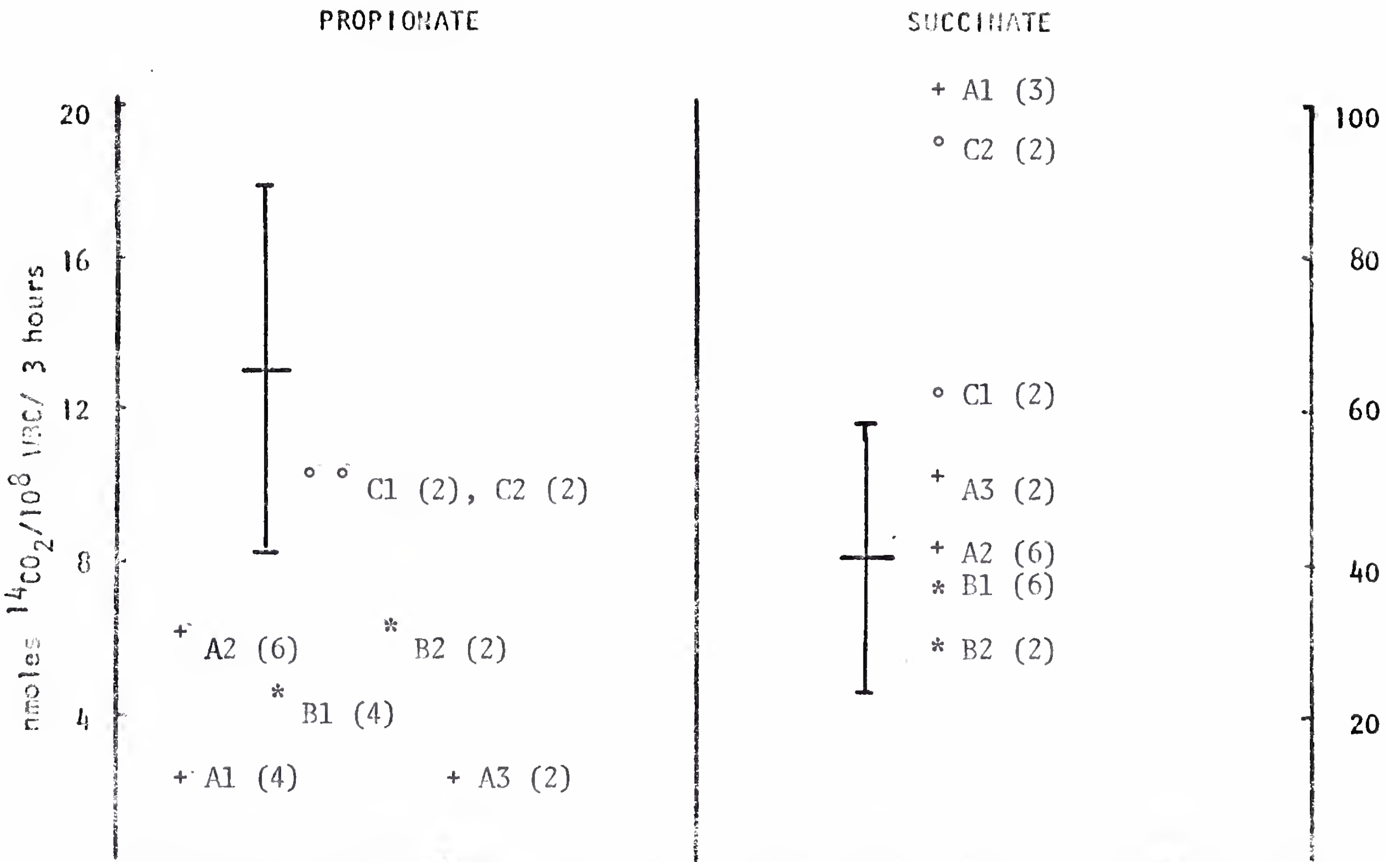

Figure 6. Oxidation of labelled propionate and succinate by intact leukocytes from controls and patients. Control data is presented as mean $\pm$ one standard deviation. Values for patients represent the mean of the number of observations in parentheses. Patients are identified as follows:

+: Pernicious anemia
*: Other B_{12} deficient patients
°: Folate deficient patients

Incorporation of ^{14}C-Formate into ^{14}C-L-Serine

B_{12} deficient patients, taken as a group, demonstrated a highly statistically significant reduction in the capacity of their leukocytes to incorporate ^{14}C-formate into labelled serine (Table 5). This was not simply a result of concomitant "quantitative" folate deficiency, since patient A3 had the greatest individual reduction in serine formation but the highest levels of red cell and plasma folates. Of interest is the fact that, judged by severity of anemia, her B_{12} deficiency was also the most profound among the pernicious anemia patients.

The mean pre-treatment values for ^{14}C-formate incorporation into serine by individual patients are illustrated in Figure 7.

An unanticipated finding was a rather striking correlation observed between the reduction of propionate oxidation and impairment of serine formation by leukocytes of the B_{12} deficient patients. This is illustrated in Figure 8.

Only two patients with folate deficiency unaccompanied by B_{12} deficiency were found for study. As seen in Table 5, no impairment of Glycine ⟶ Serine interconversion was detected. Both of these patients were studied several days after admission to the hospital, and the ingestion of a folate-rich diet may have restored this function to normal before the assays were performed. Neither of these patients had marrow aspirations or reticulocyte counts done prior to the day of assay, and neither was anemic. One additional patient not listed in

TABLE 5

LEUKOCYTE INCORPORATION OF ^{14}C-FORMATE INTO ^{14}C-L-SERINE

Subjects	Number of Determinations	$\frac{\text{nmoles } ^{14}\text{C-L-Ser} / 10^9 \text{ WBC}}{4 \text{ hrs}}$ ($\bar{x} \pm 1$ S.D.)	Difference from Controls* (p)
Controls	34	10.1 ± 3.3	-
Pernicious anemia patients	22	5.2 ± 1.8	$<.001$
Other B_{12}-deficient patients	6	5.9 ± 2.3	$<.001$
Folate-deficient patients	4	10.6 ± 1.2	NSD#

*Determined by the unpaired t-test

#NSD = not significantly different

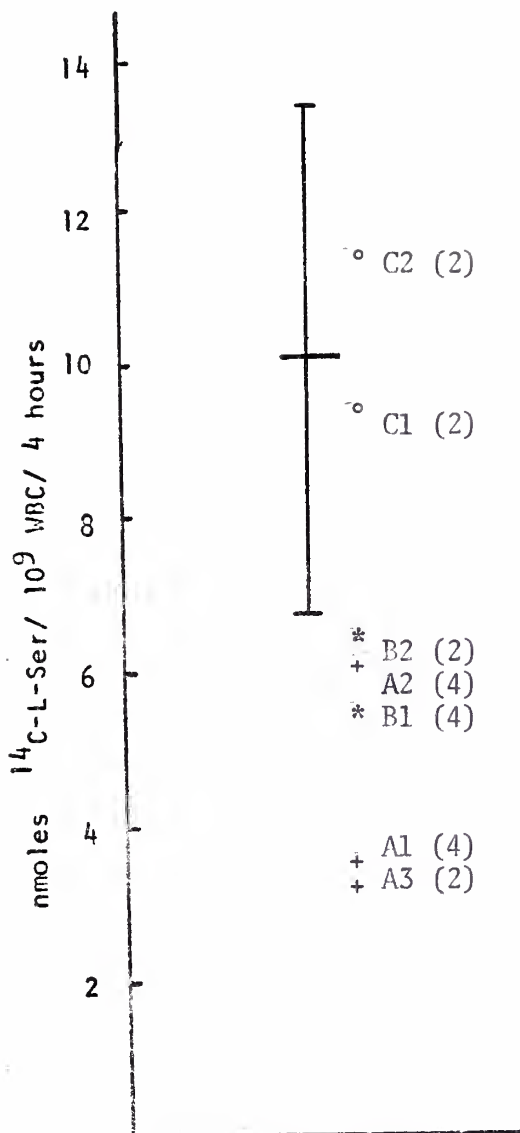

Figure 7. Incorporation of ^{14}C-formate into ^{14}C-L-serine by intact leukocytes from controls and patients. Control data are presented as mean $\pm$ one standard deviation. Values for patients represent the mean of the number of observations in parentheses. Subjects are identified as follows:

+: Patients with pernicious anemia
*: Other B_{12} deficient patients
o: Folate deficient patients

Figure 7. [illegible] concentration [illegible] in leukocytes from controls and patients. [illegible] as mean ± one standard deviation. Values [illegible] the number of the marker [illegible] are identified as follows:

+: Patients with [illegible]
a: Other [illegible]
c: [illegible]

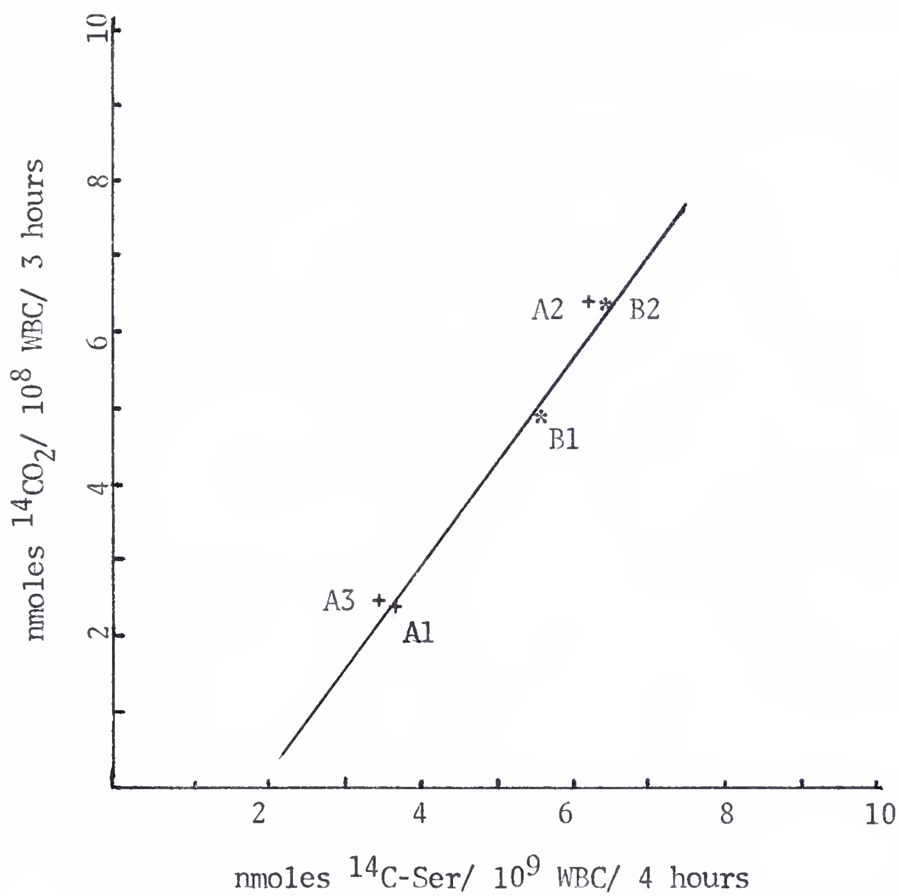

Figure 8. Correlation Between Serine Formation and Propionate Oxidation among B_{12} Deficient Patients.
+: Pernicious anemia patients
*: Other B_{12} deficient patients

Table 3 was also studied. This was a 26 year old woman with non-tropical sprue who had had a plasma folate level of 1.2 ng/ml one week prior to study. Serine hydroxymethylase activity of her leukocytes was assessed on the third day of PGA therapy, at which time her serum folate level was 24.6 ng/ml and her RBC folate 130 ng/ml. Reticulocyte count on the day of assay (5.4%) indicated active response to treatment, and incorporation of formate into serine was 9.8 nmoles/10^9 cells/4 hours, indistinguishable from normal subjects.

DISCUSSION

Oxidation of Propionate in B_{12} Deficiency

In ruminants, the propionate - methylmalonate - succinate pathway (Figure 9) plays a significant role in energy metabolism, but in other mammals it appears to function principally in the oxidative catabolism, *via* the tricarboxylic acid cycle, of several of the essential amino acids, odd-chain fatty acids, cholesterol, and thymine. Depletion of vitamin B_{12}, one of the cofactors in this series of reactions, has been shown to result in increased urinary excretion of methylmalonic acid.[10] Recently, Contreras and Giorgio have presented more direct *in vitro* evidence of a reduction in activity of the cobalamin-dependent methylmalonyl CoA mutase consequent to vitamin B_{12} deficiency. In their studies, leukocyte homogenates from patients with pernicious anemia demonstrated a decreased capacity to oxidize racemic ^{14}C (carboxy) methylmalonyl CoA to $^{14}CO_2$.[11] This defect could be partially overcome by addition of large amounts of the "missing" cofactor, dA-B_{12}, to the homogenates. The present studies confirm some of these findings. Intact leukocytes from patients with vitamin B_{12} deficiency uniformly demonstrated a decreased capacity to oxidize ^{14}C-3-propionate to $^{14}CO_2$. That this diminished $^{14}CO_2$ production was caused by depressed activity of methylmalonyl CoA mutase is strongly suggested by demonstration that oxidation of ^{14}C-1,4-succinate was normal or even increased in all patients studied. No apparent cor-

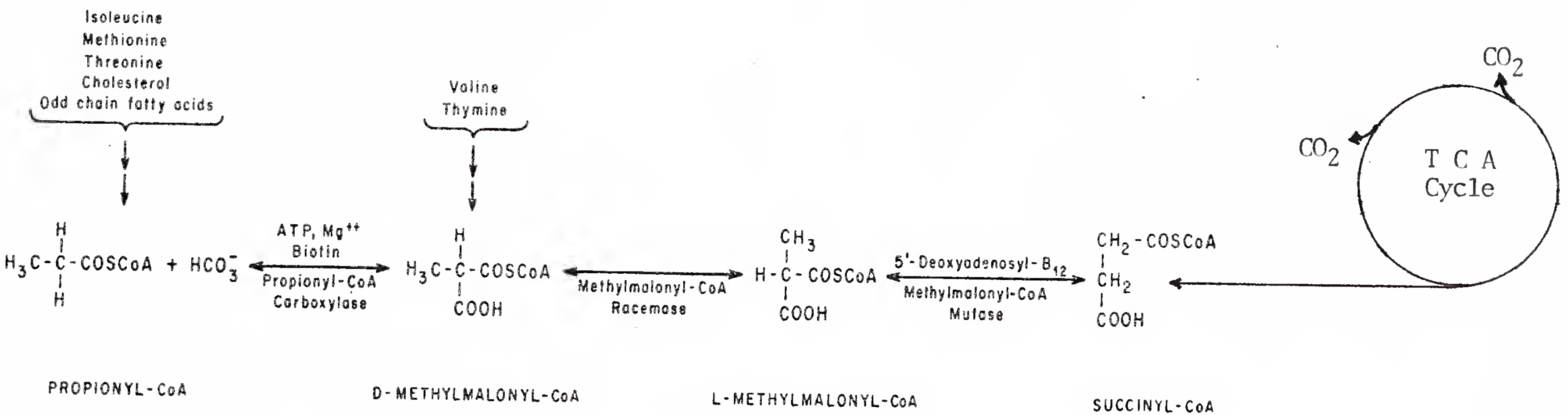

Figure 9. The Propionate — Methylmalonate — Succinate Pathway. The role of the pathway in the catabolism of amino acids, cholesterol, and odd-chain fatty acids through the tricarboxylic acid cycle is emphasized.

relation was observed between severity of B_{12} deficiency, as assessed by the usual indices of depletion (macrocytosis, serum B_{12} level, degree of anemia), and the magnitude of the reduction of propionate oxidation. Similar absence of such correlations were found by Contreras and Giorgio, who also failed to demonstrate any relationship between the magnitude of urinary methylamalonate excretion and the severity of impairment of mutase activity.

The extremely high values for ^{14}C-1,4-succinate oxidation seen in one B_{12} deficient patient in the present study may have represented a chance phenomenon, but they could also be indicative of the severity of the block in methylmalonate isomerization. If the endogenous production of succinyl CoA were diminished by reduced methylmalonyl CoA mutase activity, exogenous ^{14}C-succinate would constitute a greater fraction of the total pool, resulting in increased $^{14}CO_2$ production from this tracer. As seen in Figure 6, this hypothesis tends to be supported by the data from the patients studied; leukocytes demonstrating the least severe impairment of propionate oxidation also had the lowest values for $^{14}CO_2$ production from added ^{14}C-1,4-succinate. The number of patients studied is not large enough, however, to attribute definite significance to this observation.

Contreras and Giorgio also studied the oxidation of methylmalonyl CoA by homogenates of leukocytes from six patients with anemias not attributable to vitamin B_{12} deficiency (five with low serum folates, and one with iron deficiency resulting from blood loss). Four of the six demonstrated as severe a reduction in methylmalonyl CoA oxidation

as was observed in leukocytes from pernicious anemia patients. The data from this group of patients are extremely difficult to interpret, because of the absence of suitable controls demonstrating integrity of the remainder of the pathway leading to $^{14}CO_2$, and all of these patients appeared to be severely ill. Although five of the six were "folate deficient" none could be considered representative of pure nutritional folate deficiency. Nevertheless, the finding of reduced mutase activity is of interest in the light of the suggestion that folate deficiency does lead to reduction of intracellular cobalamin levels.[122] Measurement of urinary methylmalonate excretion would have been of interest in these patients, but this was not done. In the present studies, no impairment of propionate oxidation by intact leukocytes from two folate deficient patients was observed. Because neither of these patients was anemic, direct comparisons with Contreras and Giorgio's folate deficient subjects, all of whom had hemoglobin levels less than 10 g/100 ml, are perhaps unjustifiable. Succinate oxidation seemed also to be unimpaired in the two folate deficient patients studied here, which is at variance with the suggestion of Degrazia _et al_. that folate deficiency may for unknown reasons reduce capacity for succinate oxidation _in vivo_.[127]

Contreras and Giorgio demonstrated that, under the conditions of their assay, B_{12} deficient leukocyte homogenates were unable to utilize CN-B_{12}. That intact leukocytes can do so has recently been demonstrated _in vitro_ in cells derived from B_{12} deficient pigs.[128]

Several lines of evidence discussed earlier suggest that integrity of the propionate pathway is not essential to normal hematopoeisis. When pernicious anemia patients have been treated with B_{12}, methylmalonate excretion[123] and impaired in vivo oxidation of ^{14}C-2-propionate[129] have persisted for some time following establishment of hematologic remission. In the inborn error of metabolism, methylmalonicacidemia, in which only this pathway is impaired, administration of methionine aggravates methylmalonate excretion,[130] but such an effect is not observed in pernicious anemia.[131] Exogenous B_{12} deficiency is thought to interfere with both pathways requiring the vitamin. Since methionine is catabolized first by demethylation to homocysteine and then through the propionate pathway (Figure 8), no increase in methylmalonate excretion would be expected to be provoked by methionine administration to B_{12} deficient patients.

Formation of Serine in B_{12} Deficiency

The present studies demonstrate a decreased capacity of peripheral leukocytes from patients with B_{12} deficiency and megaloblastosis to incorporate ^{14}C-formate into serine. This strongly supports the "methylfolate trap" hypothesis, since the most plausible explanation of the finding is that B_{12} deficiency produced depletion of free FH_4 available to combine with the exogenous ^{14}C-formate. These findings are in agreement with the observations of Metz et al., who suggested that N^5N^{10}-CH_2-FH_4 pools were reduced in B_{12} deficiency

because of impaired deoxyuridine methylation by megaloblasts from patients with pernicious anemia.[97] The results of the present experiments also indicate that defective ^{14}C-formate incorporation into serine cannot be considered a sensitive and specific index of folate deficiency, as has been suggested by Ellegaard and Esmann.[1] Contrary to the claims of these authors, assessment of this function was not found to be of value in the detection of minimal folate deficiency. Several possible explanations for this discrepancy can be postulated. Ellegaard and Esmann assayed formate incorporation by lymphocytes, whereas the present studies were conducted on mixed leukocyte populations containing approximately 75% granulocytes. The latter are short-lived in the peripheral circulation. Since the two folate deficient patients studied here had been on hospital diets for at least 3 days prior to collection of blood for the assay, it is conceivable that a new, folate-repleted population of granulocytes may have appeared during that interval. Since little is known about the kinetics of folate uptake and storage by polymorphonuclear leukocytes,[132] it may be unjustifiable to attribute the differences observed to the cell types studied.

Objections can be raised against the choice of formate as the radioactive substrate in both of these studies. Formation of N^{10}-CHO-FH_4 requires ATP as well as FH_4 and formate. The equilibrium for dehydration of N^{10}CHO-FH_4 to N^5N^{10}-CH=FH_4 lies far in the direction of N^{10}-CHO-FH_4 at physiologic pH. NADPH is required to reduce the N^5N^{10}-CH=FH_4 to methylenefolate.[133] Formaldehyde, on the other hand,

reacts rapidly and non-enzymatically with FH_4 to generate methyl-enefolate. Formate was chosen as substrate solely because of the ease with which it could be separated from serine.

Implications and Unresolved Issues

Taken in concert, the studies of propionate oxidation and glycine-serine interconversion here reported are consistent with the currently accepted hypothesis of the metabolic effects of B_{12} deficiency. Folate-dependent metabolism was impaired in B_{12} deficiency, but B_{12} dependent metabolism was unaffected by folate deficiency. Recently, a new inborn error of metabolism has been recognized which raises doubts about the hematologic significance of this now well-established interaction of the two vitamins. Hakami *et al.* described two infants with megaloblastic anemia in the neonatal period which was shown to be caused by congenital deficiency of transcobalamin II, the β-globulin which seems to be important in facilitating cellular B_{12} uptake.[134] Both of these siblings responded to massive frequent B_{12} injections, but when B_{12} therapy was withdrawn in one, prompt relapse into a profound megaloblastic anemia ensued within six weeks. No abnormalities of either known B_{12}-dependent reaction could be detected well after megaloblastosis had developed.[135] It was suggested by the authors that an as yet unappreciated role of B_{12} must be involved in the production of megaloblastosis. It is difficult at present to assess the significance of these findings. The assertion that both the mutase

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Implications and Unresolved Issues

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and transmethylase enzymes were continuing to function normally in the face of unequivocal megaloblastosis was based on very gross indices (lack of methylmalonate excretion, homocystine excretion, or fall in plasma methionine). Nevertheless, the fact that pernicious anemia patients treated with folate eventually relapse hematologically even while on folic acid[31] suggests that more remains to be learned about cobalamin-folate interactions. In the light of the plasma/red cell folate gradient observed in B_{12} deficiency, the similar B_{12} gradient seen in folate deficiency, and the sequence of metabolic events occurring in development of anemia in transcobalamin II deficiency, a search for interactions of cobalamins and folates at the cell membrane might be productive. A possible mechanism for B_{12}-dependent cell folate uptake has just been suggested by Tisman and Herbert.[136] These investigators found that marrow cell uptake of ^{3}H-labelled $N^{5}CH_{3}$-FH_{4} was impaired in megaloblasts from pernicious anemia patients, and that uptake could be increased 10-30% by addition of CN-B_{12} to the cultures. Cell uptake of PGA was not significantly reduced in the pernicious anemia patients and was not stimulated by CN-B_{12}. The authors speculated that a membrane-bound, B_{12}-dependent transmethylase might play a role in uptake of serum methylfolate and that reduced activity of this enzyme in B_{12} deficiency could account for the serum/cell folate gradients seen in pernicious anemia.

The striking correlation observed in the present studies between the degree of impairment of folate-dependent serine formation and B_{12}-dependent propionate oxidation among B_{12} deficient patients

(Figure 8) is consistent with a role for B_{12} in cell folate uptake. Serine hydroxymethylase and methylmalonyl CoA mutase share no known cofactor requirements. The data therefore suggest that availability of coenzymes for both; i.e., N^5N^{10}-CH_2-FH_4 and dA-B_{12}, may be limited proportionately by a given degree of B_{12} deficiency. This correlation is not necessarily inconsistent with the methylfolate trap hypothesis. If the rate-limiting step in B_{12} coenzyme formation were an early one common to both CH_3-B_{12} and dA-B_{12}, the observed relationship might be expected. According to the hypothesis, serine formation is indirectly B_{12}-dependent, since the regeneration of FH_4 requires the presence of sufficient CH_3-B_{12} to catalyze the homocysteine transmethylase reaction. Proportionate depletion of both B_{12} coenzymes could in this manner result in similar reductions in the pool of free intracellular FH_4 and hence N^5N^{10}-CH_2-FH_4. Total $^{14}CO_2$ production and ^{14}C-serine formation are influenced by many factors in addition to coenzyme availability, such as the rates of apoenzyme synthesis and the affinities of the apoenzymes for their substrates and cofactors. The observed proportionate impairment of these two metabolic functions in a small number of B_{12} deficient patients thus raises the need for further studies to establish its metabolic basis.

One of the goals of the present undertaking was to achieve simplification of Ellegaard and Esmann's rather tedious assay. Although details were omitted from their brief report, they employed ion exchange chromatography followed by metaperiodate oxidation of the isolated serine, yielding $^{14}CH_2O$ specifically from the β-carbon of serine.[137]

The modifications introduced in the present studies, although simpler, may have diminished the specificity or sensitivity of the test. The results obtained here from study of only two folate deficient patients must be regarded as extremely preliminary, requiring confirmation in a larger series of patients. An alternative simplified metaperiodate assay specific for the β-carbon of serine[138] might be an appropriate tool to use in such future studies of glycine-serine interconversion in folate deficient patients. One of the goals of such studies would be to ascertain what, if any, correlation exists between integrity of the glycine-serine pathway and other measures of body folate stores, such as the red cell folate. It remains to be demonstrated whether or not the assay here developed can be adapted as a clinically useful test of folate deficiency. Studies of glycine-serine interconversion in intact leukocytes from folate deficient patients incubated in vitro with various folate compounds might reveal information about the largely unexplored area of folate transport in leukocytes. Future experiments might also be directed towards assessment of the effects on glycine-serine interconversion of anticonvulsants and antifolate antimetabolites.

The demonstration of high levels of serine hydroxymethylase activity in cultured fibroblasts suggests an alternative approach to the study of cellular metabolism in B_{12} and folate deficiencies. These cells are known to contain homocysteine transmethylase[111] and methylmalonyl CoA mutase,[108] and their uptake of vitamin B_{12} is stimulated by Transcobalamin II.[139] Fibroblasts cultured with dialyzed sera in selectively deficient media might yield a readily available, reproducible,

intact-cell system in which cobalamin-folate interrelationships could be further elucidated.

Although the present work strongly suggests that alterations in intracellular folate pools occur consequent to vitamin B_{12} deficiency, the roles played by such disturbances in the genesis of megaloblastosis will not be conclusively elucidated until the pools themselves can be more precisely measured.

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