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ENIGMAS OF MEGALOBLASTOSIS:

CURRENT PROBLEMS IN CLINICAL AND LABORATORY MANAGEMENT

Perspectives from the "Cobalamin Window"

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I. INTRODUCTION.

A review of the clinical and laboratory expression of megaloblastosis displays the history of medicine in all of its glory. From the description of the initial case in 1924 (1), to the exposition of our understanding of the megaloblastic anemias by Addison in 1849 (2), to the incisive and complete description of a major expression of megaloblastosis, that entitled "Pernicious Anemia" by Biermer in 1872 (3), we can span an enormous and rich literature over the next 100 years that has provided the characterization of megaloblastosis that permits it to be comfortably inserted into the medical school curriculum and become a classic portion of the clinical armamentarium. Indeed, one might say "What new vistas of megaloblastosis merit a review?" Not only have the clinical and laboratory features of the megaloblastic states been well defined, but highly specific therapy has been available for the management of patients with megaloblastosis. Since it is the purpose of this discussion to focus upon the aspects of megaloblastosis through a "window provided by the cobalamins", we can emphasize that it was West in 1948 who clearly proved the clinical activity of crystalline vitamin B_{12}, Hodgkin in 1956 who provided the complete structural formula, and finally in 1972 the complete total synthesis of B_{12} was carried out (4). Thus, one might reasonably ask why, with the full knowledge of cause, the biology and all of the characteristics of the therapy, a review is needed. It is our plan to review selected problems - laboratory, clinical and therapeutic - relative to the megaloblastosis and to highlight areas of direct clinical concern. That these areas are not minute is attested to by virtue of the fact that this review will focus on aspects related to the cobalamins. As the folates participate in aspects of megaloblastosis, they will of necessity be considered; nonetheless, the recent advances in folate biology will require a separate and subsequent consideration.

II. THE LABORATORY FEATURES OF MEGALOBLASTOSIS.

The identifying features of megaloblastosis are quite characteristic. These include the specific recognizable morphologic changes upon which the general diagnosis of megaloblastosis is made, as well as a variety of related laboratory features that, though nonspecific, serve to characterize and define the megaloblastic states.

TABLE 1

LABORATORY FEATURES OF MEGALOBLASTOSIS

I. HEMATOLOGIC FEATURES:

A. PERIPHERAL BLOOD:

RBC: MACRO-OVALOCYTOSIS (MCV >105)
ANISOCYTOSIS AND POIKILOCYTOSIS
HOWELL-JOLLY BODIES
RETICULOCYTOPENIA

WBC: SHIFT (RIGHT) PMN's - INCREASE IN MEAN LOBE AVERAGE (>3.35)
HYPERSEGMENTED PMN's
NUCLEAR TWINNING

B. BONE MARROW:

MEGALOBLASTIC DYSPOIESIS
(ERYTHROID, GRANULOCYTIC AND MEGAKARYOCYTIC)

TABLE 2

LABORATORY FEATURES OF MEGALOBLASTOSIS

II. OTHER LABORATORY FEATURES:

A. INEFFECTIVE ERYTHROPOIESIS:

INCREASED SERUM IRON (↑% SATURATION)
RINGED SIDEROBLASTS (DACIE "TYPE II")
INCREASED MARROW IRON

B. INCREASED SERUM LACTIC DEHYDROGENASE

C. INCREASED URINE MURAMIDASE

D. INCREASED SERUM URIC ACID

E. "DEFECTIVE" DEOXYURIDINE SUPPRESSION

III. THE ETIOLOGIC BASIS OF MEGALOBLASTOSIS.

Unlike many diseases, the diagnosis of megaloblastosis represents the simplest aspect of the clinical problem. The immediate consideration faced by the clinician following the diagnosis of megaloblastosis is the definition of the etiologic basis for that lesion (10).

TABLE 3

ETIOLOGIC BASIS OF MEGALOBLASTOSIS

1. DEFICIENCY OR DISTURBANCE OF COBALAMIN (B_{12}) METABOLISM
   - TRANSCOBALAMIN II DEFICIENCY

2. DEFICIENCY OR DISTURBANCE OF FOLATE METABOLISM

3. ALTERATIONS IN DNA SYNTHESIS:
   - SECONDARY TO DRUGS (PRIMARILY ANTINEOPLASTIC AGENTS)
   - INTERFERENCE WITH SYNTHESIS

4. COPPER DEFICIENCY

5. Di GUGLIELMO SYNDROME

6. (?) IDIOPATHIC MEGALOBLASTOSIS

IV. CURRENT ISSUES IN THE COBALAMIN ASSAY.

The differential diagnosis of the etiologic basis for megaloblastosis was significantly aided by the development of radioisotopic assay technology. By the mid-1960's it became possible to assay for B_{12} in serum and tissues (11), and evidence quickly accrued that serum levels provided a rather accurate assessment of the tissue concentration of B_{12}. Parenthetically, a similar correlation of serum folate to tissue folate is far less direct and the pitfalls in interpretation even more extensive than those to be described for vitamin B_{12}. As in any radioassay, a variety of binders for B_{12} were employed in quantitation of the cobalamins. Three general types of binders were exploited. Intrinsic factor represents the most specific binder for B_{12} and therefore represents the "gold standard" for binding characteristics. Because of problems of stability of intrinsic factor, two other general approaches have been exploited. In our laboratory, transcobalamin II, the physiologic binder of B_{12} was utilized (11, 12). The clinical potential of B_{12} assays was not missed by the radiopharmaceutical industry, and the result was the development of a large number of kits for B_{12} assay. Although these generally described intrinsic factor as their binder, most acknowledged significant concentrations of "R" binders admixed with the I.F.

The "R" binders were so named because of their rapid electrophoretic mobility, thereby separating them from intrinsic factor (13). Studies demonstrated that these "R" binders existed in virtually every biologic fluid (e.g. tears, saliva, milk, etc.). Renewed interest in these "R" binders was generated by recent evidence that they contaminate most of the commercially available vitamin B_{12} assay kits. Such contamination would be of little concern except that non-physiologic analogues were shown to be bound preferentially by "R" binders. These analogues have a low affinity for intrinsic factor and, in fact, are bound preferentially over intrinsic factor by "R" binders.

Until recently that was of interest but did not seem to provide a significant problem until Cooper and Whitehead (14) identified a patient in whom serial vitamin B_{12} deficiency existed with advancing neurologic deficit. Subsequent assay of sera by the microbiologic technique (*Euglena gracilis)* provided evidence that the neurologic lesion was due to vitamin B_{12} deficiency and subsequent clinical course corroborated this view. These studies were further expanded and other laboratories also demonstrated that cobalamin analogues were recognized by the current commercially available radioisotopic dilution assays because of their preferential binding to "R" binders. The result of this activity was the failure to identify cobalamin deficiency when it was actually present (15-17).

These observations led to a variety of studies and attempts to improve the commercially available kits. In general, these attempts focused in three general areas. First, evidence was provided that cobalamin analogues existed in animal tissues (18). Second, attempts to provide blocking binders for the existent contamination of the "R" protein were generated based upon the nonspecific binding of such "R" proteins to analogues (15, 16, 18). Finally, the most current attempts have involved improved technology to provide stable intrinsic factor to serve as the binding material in the competitive inhibition assay. The alternative to that is as mentioned above, the use of transcobalamin II (10-12, 19). As a result of these and clinical correlation studies (17, 20-22), it is now evident that the assay of cobalamins requires an appropriate clinical interpretation by the physician. In addition, the precision of the various kits commercially used is now known to vary quite appreciably (20), and their performance with various kinds of specimens from different hematologic disorders does show variation. These studies have therefore emphasized the requirement for appropriate reference sera, the defined range of normal not based on statistical analysis but rather on the recognition of patients who are B_{12}-deprived, and an appropriate index of suspicion for the application of other analytic techniques when borderline values are obtained. Finally, studies which served to identify patients whose serum cobalamin levels were normal by radioisotopic assay but in whom a deficiency state was recognized helped focus attention upon the clinical circumstances in which false positive and false negative diagnostic data occur with B_{12} technology.

TABLE 4

COBALAMINS

BIOLOGICALLY FALSE HIGH VALUES

1. CONCURRENCE OF CHRONIC GRANULOCYTIC LEUKEMIA AND B_{12} DEFICIENCY
   - DUE TO ↑ SERUM BINDERS FROM GRANULOCYTES

2. TRANSCOBALAMIN II DEFICIENCY
   - INHERITED

3. "R" BINDER CONTAMINATION IN ASSAY KITS
   - PRESUMES POSSIBLE PRESENCE OF ANALOGUES

4. CONGENITAL ENZYMATIC DEFECTS IN COBALAMIN METABOLISM

TABLE 5

COBALAMINS

BIOLOGICALLY FALSE LOW VALUES

1. POST RADIONUCLIDE IMAGING
   $\bar{p}$ ^{67}GALLIUM : 7-14 d
   $\bar{p}$ ^{99}TECHNETIUM: 1-2 d

2. FOLATE DEFICIENCY
   ≃10%

3. PREGNANCY
   3RD TRIMESTER NADIR (10%)

4. NEOPLASMS
   ESP. MYELOMA

5. TRANSCOBALAMIN I DEFICIENCY

6. DRUGS: ORAL CONTRACEPTIVES; HI DOSE VIT. C

Another feature of vitamin B_{12} metabolism is the long-recognized and recently confirmed (23) evidence that the measured serum value declines with age. In general, this decline is approximately 10 pg/ml for each decade beyond the sixth decade. Very limited population studies to date suggest that this decline is not associated with any other evidence of coenzyme deficiency.

The most critical aspect of the studies of B_{12} content is the presumption that vitamin B_{12} is a single moiety, and indeed the term vitamin B_{12} is no longer relevant. Classical vitamin B_{12}, more appropriately called cobalamin, consists of three main cobalamins in man:

1. Hydroxocobalamin
2. Adenosylcobalamin
3. Methylcobalamin

Although cyanocobalamin is present in some individuals in trace quantities, it is most often undetectable. Its biologic significance, if any, is uncertain (24). It is quite clear that the major biologic precursor moiety is hydroxocobalamin from which methylcobalamin and adenosylcobalamin are synthesized. The exact steps and mechanisms of this synthesis are not entirely clear.

Virtually all of the clinical data on aspects of B_{12} deficiency have related the state of B_{12} deprivation to the total B_{12} content identifiable either in plasma or tissues or both. In light of the fact that three identifiable coenzyme forms of B_{12} are physiologically important, it is not unreasonable to consider asymmetric decline of such coenzymes and the potential that one or another is the basis of the tissue expression of some of the manifestations of cobalamin deficiency. To date, the ability to quantitate the individual cobalamins in plasma or tissues in man has been severely limited by the requirement for large amounts of blood and complex thin-layer chromatographic and bio-autographic separation technology. The only meaningful information has been developed by Dr. John Linnell at Westminster Medical School in London and his studies have extended over the past decade (25-28). A representative compilation of this data is shown in Table 6. It is noteworthy that the resolution of this method, tedious and complex though it is, is such that it fails to separate hydroxocobalamin from adenosylcobalamin.

TABLE 6

COBALAMIN FRACTIONS IN MAN

PLASMA:	TOTAL B_{12} CONTENT pg/ml	Me Cbl pg/ml	[Ado and OH] Cbl pg/ml	CN-B_{12} pg/ml
NORMALS	385 ±31	251 ±22	126 ±12	8 ±1
PERNICIOUS ANEMIA	88 ±11	22 ±4	58 ±8	8 ±2
BONE MARROW:				
NORMALS	12,960 ±1440	1,700 ±300	10,940 ±1275	275 ±100
PERNICIOUS ANEMIA	6,450 ±1400	984 ±462	5,090 ±1100	375 ±140

Me Cbl : Methylcobalamin

Ado Cbl: Adenosylcobalamin

OH-Cbl : Hydroxocobalamin

CN-B_{12} : Cyanocobalamin

These studies have provided the following observations:

1.) Methylcobalamin has been noted to be the predominant cobalamin form in the plasma of normal individuals.

2.) In untreated pernicious anemia, methylcobalamin is disproportionately reduced in the plasma.

3.) In tissues (i.e. erythrocytes, leukocytes and bone marrow), a different cobalamin fractionation pattern is seen. Adenosylcobalamin (heretofore not clearly separable from hydroxocobalamin and therefore expressed as the combined content of hydroxy- and adenosylcobalamin) was the predominant form seen in tissues, both in the normal and in the B_{12}-deficient state.

Therefore, at least on the basis of these partial separation techniques, selective or fractional coenzyme deficiency could not be identified in tissues (25-29).

A final issue relevant to the development of cobalamin deficiency focuses on the biologic transport of the cobalamins. In humans, virtually all of the endogenous cobalamins are transported by transcobalamin I. Transcobalamin I, a glycoprotein, has the biologic and antigenic characteristics of an "R" binder similar to that present in other tissues or tissue fluids. In man, transcobalamin I is primarily a storage protein rather than a true, biologically relevant transport protein. By contrast, transcobalamin II is the important biologic transport protein, critical for the movement of cobalamins into the cell. In the search for a biologic model that can express the characteristics of cobalamin deficiency, it is noteworthy that all species but man have the bulk of endogenous plasma cobalamin attached to transcobalamin II (29). It is conceivable that the failure to have an explicit animal model of "pernicious anemia" may be based not only upon the differences to cobalamin partitions, but the different transport characteristics in other species.

Recent studies from this laboratory have demonstrated the applicability of high performance liquid chromatographic (HPLC) analysis as a means of fractionation of the cobalamins, to expand the search for a differential pattern of cobalamins during developing deficiency (30, 31). Application of these techniques to our studies in a murine model of B_{12} deprivation suggests that, in fact, an asymmetric deficiency of the cobalamin partition does occur, and it provides tantalizing data that some of the clinical expressions of cobalamin deficiency in man are the result of an asymmetric decline of specific cobalamin coenzyme forms.

V. ISSUES RELATIVE TO OTHER DIAGNOSTIC APPROACHES TO THE ETIOLOGIC BASIS OF MEGALOBLASTOSIS.

Other laboratory parameters have been exploited to help in the characterization of the etiologic basis for megaloblastosis.

TABLE 7

MEGALOBLASTOSIS

ETIOLOGIC MECHANISMS	-	LABORATORY STUDIES
- COBALAMIN DEFICIENCY		- SERUM "B_{12}" ASSAY (200-1000 pg/ml)
		- URINARY METHYLMALONIC ACID ASSAY (<5 mg/24 hr)
		- dUr SUPPRESSION: B_{12} CORRECTED
- FOLATE DEFICIENCY		- SERUM FOLATE (3-18 ng/ml)
		- RBC FOLATE (90-400 ng/ml)
		- dUr SUPPRESSION

Of the three physiological cobalamins, hydroxocobalamin is known to be a precursor to the synthesis of the two active coenzyme forms, methylcobalamin and adenosylcobalamin. The interesting feature of the biology of vitamin B_{12} is that these two coenzyme forms each participate in a single biologic reaction in man. Therefore, the complete biochemistry of the cobalamins can be characterized by the aspects of these two cobalamin-dependent biochemical pathways:

TABLE 8

VITAMIN B_{12} DEPENDENT REACTIONS

$$5\ Me\text{-}FH_4 + Homocysteine \xrightarrow[M\text{-}Cbl]{Methyltransferase} FH_4 + Methionine$$

$$Propionic\ Acid \longrightarrow Propionyl\ CoA \longrightarrow D\text{-}Methylmalonyl\ CoA$$

$$L\text{-}Methylmalonyl\ CoA \xrightarrow[Ado\ Cbl]{Mutase} Succinyl\ CoA$$

A. Methylmalonic and Propionic Aciduria:

Of the two cobalamin-dependent metabolic pathways, current evidence strongly suggests that methylcobalamin required in the methionine synthetase pathway is the cobalamin responsible for megaloblastosis. The exact biological role for the adenosylcobalamin-dependent pathway is not entirely certain. Its role in propionic oxidation appears critical and the source of material utilizing this pathway involves such diverse biologic materials as the aliphatic amino acids and cholesterol. The biochemical data suggest that this pathway is a "scavenger" or catabolic pathway and therefore does not normally have a role in synthesis. Biochemical deprivation of adenosylcobalamin to the level of enzymatic dysfunction has been shown to be associated with hepatic deacylization of methylmalonyl-CoA and the liberation of methylmalonic acid from the hepatocytes into the serum with subsequent renal clearance. The evidence of methylmalonic aciduria and propionic aciduria has become a viable marker for the recognition of B_{12} deficiency that is associated with functional impairment of the cobalamin-dependent pathway (32).

Three general methods for the assessment of methylmalonic aciduria and propionic aciduria have been applied at the clinical laboratory level:

1.) Thin-Layer Chromatography (10): Of the three general methods for identification and quantification of methylmalonic acid in urine, the least expensive is that of rapid thin-layer chromatography. Though rapid and inexpensive, its limit of sensitivity is approximately at the level of 50 mg/liter.

2.) Gas-Liquid Chromatography: The most sensitive assessment for methylmalonic acid in urine is by gas-liquid chromatographic analysis; this requires approximately 6 hours, but it can easily provide sensitivity capable of recognizing 1 mg/liter of urine (33).

3.) Enzymatic Analysis: Finally, a third method that is available exploits enzymatic technique and has a sensitivity at the level of approximately 4 mg/liter of urine (34).

These last two methods have provided for the first time clear evidence that methylmalonic aciduria is a normal event in humans (33, 34). The normal urinary excretion of methylmalonic acid is less than 5 mg/24 hours.

Methylmalonic aciduria and propionic aciduria have been identified by these assay techniques in the clinical circumstance of cobalamin deficiency. Scattered reports of the failure to demonstrate methylmalonic aciduria in classical vitamin B_{12} deficiency led to the use of an oral valine or isoleucine load in order to stress the metabolic pathway and develop a diagnostic test for B_{12} deficiency. The basic rationale for the use of methylmalonic aciduria as a criterion of cobalamin deficiency is based upon the reasoning that it actually reflects a product of the enzymatic deficiency state. Since such enzymatic deficiency should precede the structural features of megaloblastosis, methylmalonic aciduria should provide a highly sensitive test of "the minimal state of cobalamin deficiency". In earlier studies, Chanarin and coworkers (35) identified a small subset of patients (7 out of 23) who had classic evidence of a megaloblastic state but failed to demonstrate methylmalonic acid excretion even after a valine or isoleucine load. Their investigations recognized that significant difficulties existed at that time in the measurement of MMA, its wider application being precluded because of the technological problems plus the fact that it might fail to identify some patients with minimal B_{12} deficiency state. The recent methodologic advances (33, 34) have eliminated the initial consideration raised by Chanarin. Nonetheless, we have now recognized eight patients with the classic clinical picture of megaloblastosis characterized by low serum B_{12} levels and a response to physiologic doses of B_{12} who have failed to have evidence of increased methylmalonic acid excretion in spite of the application of sensitive assay technology (33, 34). These findings suggest several possible explanations. It is conceivable that methylmalonic aciduria reflects only the potential asymmetric deprivation of the propionate catabolic pathway, and in those individuals with evidence of megaloblastosis and the absence of methylmalonic aciduria the actual pathophysiologic event is one of an asymmetric decline in tissue cobalamins. Alternatively, the regulation of the deacylation of methylmalonyl-CoA is not known and the absence of methylmalonic aciduria may simply reflect the level at which that event occurs in hepatocytes. In any event, one can conclude that methylmalonic aciduria recognizes the presence of vitamin B_{12} deficiency but its absence does not preclude that clinical state.

B. Deoxyuridine Suppression Test:

In 1964, Sven Killman described an experiment that led to a clinical laboratory test now labeled the deoxyuridine suppression test (36). This study demonstrated that exogenous deoxyuridine strongly inhibited or suppressed the uptake of labeled thymidine into normal bone marrow cells but did not so inhibit the uptake of labeled thymidine from bone marrow cells obtained from cobalamin-deficient subjects. These observations strongly supported the concept of the "methylfolate trap" as the basis whereby cobalamin deficiency expressed megaloblastosis.

This observation has subsequently been applied to develop a clinical test termed deoxyuridine suppression test.

The basis of this test is that normal bone marrow cells when incubated in the presence of radioactive thymidine incorporate less thymidine in the presence of cold deoxyuridine than in the absence of the cold substrate. Thus, to aliquots of the marrow, nonradioactive deoxyuridine is also added. Deoxyuridine does not reduce the incorporation of the labeled thymidine into DNA as effectively in megaloblastic cells as it does in normal cells. This failure of deoxyuridine to suppress the incorporation of radiolabeled thymidine is theoretically related to the inability of megaloblastic cells to utilize the pathway that converts deoxyuridylate to thymidylate (37-39). Although the actual biochemical mechanisms that are related to deoxyuridine suppression are clearly far more complex than posed by this simplistic scheme (40), the evidence is quite clear that the deoxyuridine suppression test can identify the presence of cells with deficient content of vitamin B_{12} and/or folate. Attempts to increase the sensitivity of the test and specificity of the test in order to differentiate B_{12} from folate have been developed (39), and the deoxyuridine suppression test is a reasonable addition to the clinical armamentarium in the differential diagnosis of megaloblastic state.

VI. RECENT CONSIDERATIONS IN THE APPROACH TO THE PATHOPHYSIOLOGIC MECHANISMS OF COBALAMIN DEFICIENCY.

Once the diagnosis of megaloblastosis is made and the etiologic basis defined, the most critical clinical consideration is the characterization of the pathophysiologic mechanism that produced the deficient state. Of the potential pathophysiologic mechanisms responsible for cobalamin deficiency, clearly those circumstances of deficient absorption are the most critical considerations in the differential diagnosis.

TABLE 9

PATHOPHYSIOLOGIC MECHANISMS
RESULTING IN COBALAMIN DEFICIENCY

1. DEFICIENT INTAKE

2. DEFICIENT ABSORPTION:
   - A. INTRINSIC FACTOR DEFICIENCY:
     - 1.) PERNICIOUS ANEMIA
     - 2.) GASTRIC ATROPHY
     - 3.) GASTRIC RESECTION
     - 4.) DEFECTIVE I.F. MOLECULE
   - B. PANCREATIC INSUFFICIENCY
   - C. CLEAVAGE OF I.F.-B_{12} COMPLEX
     - 1.) BACTERIAL OVERGROWTH
     - 2.) FISH TAPEWORM
   - D. ILEAL MALABSORPTION
     - 1.) MALABSORPTION SYNDROMES
     - 2.) ILEAL RESECTION
     - 3.) DRUG INTERFERENCE AT RECEPTOR SITES

3. INCREASED UTILIZATION:

   (?) HYPERTHYROIDISM; FETAL USE; LIVER DAMAGE

Four recent clinical observations have interesting bearing on some of the classic concepts of B_{12} absorption and represent important issues in our understanding of patients with cobalamin-deprived states.

A. The Role of "R" Binders in the Absorption of B_{12}:

Over 50 years ago Dr. William Castle postulated that a factor present in the human gastric juice was essential for normal hematopoiesis (41-43). His subsequent studies suggested that this factor, termed "intrinsic factor", was the critical moiety that was absent in patients with pernicious anemia. Intrinsic factor was extensively studied over the past 50 years and was clearly demonstrated to be secreted by gastric parietal cells. It has been isolated, purified and characterized in terms of its molecular weight and many of its biologic properties. Indeed, the entire character of vitamin B_{12} requires the facilitated mechanism of intrinsic factor (44, 45).

A second look at this classical concept of B_{12} absorption was stimulated by a tangential observation by Patricia MacIntyre and her coworkers at Johns Hopkins in 1956 (46). They demonstrated that pancreatic insufficiency was associated with a malabsorption of crystalline cobalamin. Although most of the patients with pancreatic insufficiency do not develop vitamin B_{12} deficiency, a few cases of true cobalamin deficiency in this circumstance have now been seen. Extensive studies since these initial observations have confirmed this evidence of "malabsorption" of vitamin B_{12}. A related study, of interest, showed that the cobalamin malabsorption in humans was not significantly altered by prior in vitro binding of the cobalamin either to human gastric juice or (crude) hog intrinsic factor. Indeed, such attempts to correct the malabsorption failed even when the complex was administered orally or directly into the distal ileum (47). However, cobalamin was absorbed normally in these patients (with pancreatic insufficiency and evidence of an abnormal Schilling test) when the administered cobalamin was first bound to human gastric juice, and then the intrinsic factor-B_{12} complex was incubated with solubilized trypsin or chymotrypsin (47). These data suggested that the pancreatic proteases affected in some critical way the structure of intrinsic factor, and that such an alteration was necessary for the known facilitated absorptive mechanisms required for cobalamin. In spite of this attractive hypothesis, extensive studies in a variety of laboratories failed to demonstrate a direct effect of the pancreatic proteases on the intrinsic factor or on the small bowel. Recent studies by Bob Allen and coworkers (48, 49) and Kapadia (50) have provided critical evidence suggesting that the time-honored conceptual mechanism for the absorption of vitamin B_{12} may in fact be incomplete.

Their studies have shown that the binding affinity of the "R" protein derived from saliva is greater than that of gastric intrinsic factor at the pH range (of approximately 2) found in the stomach. This increased binding affinity in the low pH milieu of the stomach suggests that salivary "R" proteins may have a physiologic binding role in the absorption of B_{12}. As we mentioned before, the ubiquitous nature of the "R"-type binders have long suggested a biologic or physiologic role (51). Several features of the "R" binders found in saliva are of interest. First, "R"-type binding protein is clearly identifiable in gastric juice. Second, the potential of the salivary "R" binders can best be appreciated in terms of the quantitative magnitude of these binders identifiable in saliva. For instance, 25 nanomoles of "R" binder protein are produced into the saliva each 24 hours, and it is noteworthy that this content of "R" binder is capable of binding all the cobalamin present in the normal diet (ranging from 4-14 nanomoles per 24 hours) plus that quantity of B_{12} which turns over via the enterohepatic circulation, i.e. the total bile content of cobalamin (which ranges between 2 and 8 nanomoles per 24 hours). Thus, adequate quantities of "R" binders appear present in gastric juice to have a significant physiologic role (51, 52). Finally, these studies have demonstrated that in the circumstance of a high pH (pH 8) this selectively high affinity of salivary "R" protein for binding to cobalamin is no longer evident and that at that pH there is preferential binding of intrinsic factor to the B_{12} (48, 49)

These studies and the previous data concerning pancreatic insufficiency have suggested an important physiologic role for the "R" binders found in tissue fluids and have posed potential solutions to the enigma of the malabsorption identified with pancreatic insufficiency.

In brief, the proposed model suggests that cobalamin is bound to the "R" protein generated primarily from saliva or from gastric secretions. This binding occurs during the initial exposure of the cobalamin-containing food in the acid milieu of the stomach. The cobalamin binding to the "R" proteins continues as the gastric contents are expelled into the alkaline environment of the duodenum. Contact of the "R" binder protein-vitamin B_{12} complex with pancreatic proteases in the duodenum results in a partial degradation of the "R" protein, enabling the cobalamin to be released and then for it to become bound to intrinsic factor. The intrinsic factor-cobalamin complex, then, can be appropriately transported to the terminal ileum with a configuration of the complexes critically related to the receptor sites on the intestinal mucosal cells with subsequent absorption of the B_{12}.

This model of the absorption of vitamin B_{12} helps provide some important understanding of the physiologic role of the "R" binders in absorption and explains some of the mystique of the measurable malabsorption of B_{12} in pancreatic insufficiency. It further helps to explain some of the growing number of drugs that appear to interfere with the absorption of vitamin B_{12}.

B. Intrinsic Factor Secretory Failure in Folate Deficiency:

One of the simplest adjuvants to the clinical diagnosis of both etiology and pathophysiology is that of gastric analysis with assessment of the pH of the gastric juice and the determination of the intrinsic factor content. This simple procedure, often overlooked, has the significant advantage of providing informative data concerning the etiology and some of the most important helpful data in terms of the pathophysiology in circumstances of cobalamin deficiency.

In order to obtain adequate data, the examination of gastric juice should be done with tube adequately placed into the stomach and under the circumstances of maximal stimulation. In general, the presence of a high pH in the basal secretory aliquot (pH >6) and, more importantly, the evidence that following gastric secretory stimulation the pH fails to fall (and indeed classically rises slightly) have provided the supportive evidence that the megaloblastosis is based upon the classical pernicious anemia. Although this was a satisfactory conclusion in earlier days, simple, available technology permits the clinician to derive a great deal more information from the gastric analysis and increase the specificity of this assay technique. Thus, the availability of gastric juice provides the opportunity for a direct assay of intrinsic factor secretion as well as the determination of antibodies to intrinsic factor. Decreased intrinsic factor secretion provides evidence that at least at that point in time intrinsic factor secretory activity has a potential role in the clinical event. When one adds the finding of positive antibodies to intrinsic factor, there is substantiation that the secretory deficit of intrinsic factor is a biologically and clinically significant one.

This distinction has had recent emphasis in the clinical arena. It has long been recognized that folate deficiency has the capacity to induce significant mucosal changes that can range even to atrophy of gastrointestinal villus structure. Three patients recently studied at this institution have been demonstrated to have all of the clinical and laboratory characteristics of significant folate deprivation with adequate cobalamin stores and adequate cobalamin function. During the evaluation of their megaloblastosis, gastric achlorhydria and absent intrinsic factor secretion were identified. The availability of tissue folate analysis (red cell folate) helped substantiate folate deprivation as the basis for the atrophic gastric mucosa and secretory activity. This event, previously well recognized in terms of clinical problems and interpretation of the Schilling test, seemed a reasonable extension of that observation. Administration of folate and repair of the deprived state resulted in documented return of gastric secretory activity and intrinsic factor secretion. The interval required for return of function was approximately four months. (E. Frenkel, unpublished data).

That such an event exists is not surprising, and the admonition concerning the use of simply gastric acid content as a criterion of intrinsic factor secretion is further emphasized by the long acknowledged evidence of a dichotomy between intrinsic factor and acid secretion. Thus, though uncommon, cases of clinically evident cobalamin deprivation secondary to intrinsic factor secretory insufficiency are well recognized in patients who have adequate gastric acid secretion. Thus, it is worthy of stress that the intrinsic factor assay provides evidence of such cases of selective intrinsic factor failure as the potential mechanism for megaloblastosis.

C. Cimetidine and Intrinsic Factor Secretion:

The mechanisms of intrinsic factor secretion are unknown. Evidence from physiologic observations suggests that a small pool of intrinsic factor collects in the parietal cells and represents the first wave of secretory activity of those cells when they are stimulated by those physiologic or pharmacologic agents that stimulate acid secretion.

The advent of inhibitors of the H_2 receptor site provided the opportunity to further dissect the difference between acid secretion and intrinsic factor production. Early studies were carried out in this institution in conjunction with Drs. John Fordtran and Charles Richardson in the era of meteimide, and these studies have been repeated with cimetidine. These observations showed that H_2 receptor inhibitors do, in fact, decrease intrinsic factor secretion (53, 54). In light of our newer knowledge of the role of intrinsic factor in gastric juice and the fact that the degree of suppression generated by the current use of cimetidine and its relatively short (weeks to months) duration of administration, clinically relevant suppression of intrinsic factor secretion does not appear to be an important sequela of cimetidine use. Troublesome is the failure of this model to further elucidate the important mechanisms of intrinsic factor secretion.

Finally, the one major advance achieved by these studies in this institution was the development of methodology permitting the assay of intrinsic factor under the stimulus of a test meal, an assay technique which heretofore had not been possible (E. Frenkel, J. Fordtran and C. Richardson, in preparation).

D. Drug Effects on Cobalamin Absorption:

Recent evidence from a variety of countries has provided ample demonstration that an ever increasing list of drugs chronically administered to individuals have an important role in the production of cobalamin deficiency. Of the therapeutic agents commonly utilized, the important ones are neomycin, colchicine, PAS, slow-release potassium, metformin, ethanol and methotrexate.

A different kind of pharmacologic interaction which limits cobalamin absorption has been documented in individuals utilizing megadoses of vitamin C for a variety of reasons. Evidence has been provided that such megadose therapy destroyed cobalamins during their transport through the gastrointestinal tract and possibly during transport and storage in tissues (55). In addition, ascorbate may further cause spuriously low cobalamin levels in the serum

if the vitamin B_{12} assays are performed without the addition of sufficient cyanide (56). Although this may be a simplistic view of the interrelationships (57, 58), there is evidence to suggest that a risk does exist with a long term ingestion of megadoses of vitamin C.

VII. CLINICAL FEATURES OF COBALAMIN DEFICIENCY.

The clinical findings in cobalamin deficiency have been extensively reviewed and encompass the items in Table 10:

TABLE 10

CLINICAL MANIFESTATIONS OF COBALAMIN DEFICIENCY

ANEMIA - MEGALOBLASTIC TYPE	
NEUROLOGIC DEFICIT	- PERIPHERAL NEUROPATHY
	- SUBACUTE COMBINED DEGENERATION - CORD
	- "MEGALOBLASTIC MADNESS" - CEREBRAL
	- BLUE-YELLOW COLOR VISION DEFECT
GLOSSITIS	
HYPERPIGMENTATION	
SPLENOMEGALY	
C_3 HYPOCOMPLEMENTEMIA	
PSEUDOTUMOR CEREBRI	
INFERTILITY	

An appropriate understanding of these clinical findings requires characterization of the mechanism whereby the deficiency state is translated into an actual tissue lesion.

VIII. ENIGMAS IN THE MECHANISM OF MEGALOBLASTOSIS.

An explanation of megaloblastosis in cobalamin deficiency demands an understanding whereby the well defined clinical and laboratory findings occur:

TABLE 11

MOLECULAR MECHANISMS OF MEGALOBLASTOSIS

?HOW DOES DNA SYNTHETIC DEFECT PRODUCE:

- MEGALOBLASTOSIS
- CHROMOSOMAL CHANGES
- INEFFECTIVE HEMATOPOIESIS

All of the biochemical data accrued to date has focused the fundamental basis of megaloblastosis as a defect in DNA synthesis in the bone marrow. It of course needs to be stressed that this cellular alteration affects all replicating cells, not just the marrow. The hematologic expression only reflects the high renewal rates of hematopoietic cells. Evidence that the megaloblastosis is due to defective DNA synthesis has its origin in a variety of observations.

TABLE 12

MOLECULAR MECHANISMS OF MEGALOBLASTOSIS

PRIME FAULT:

DEFECT IN DNA SYNTHESIS:

1. IN B_{12} OR FOLATE DEFICIENCY:
   - DEFECTIVE dTMP SYNTHESIS
   - ↑ Tdr (SALVAGE) UPTAKE
   - ANEMIA RESPONDS TO THYMIDINE.
2. INHERITED FORMS OF MEGALOBLASTOSIS (e.g. OROTIC ACIDURIA) HAVE DEFECT IN DNA SYNTHESIS.
3. DRUGS WHICH CAUSE MEGALOBLASTOSIS HAVE THEIR EFFECT ON DNA SYNTHESIS.

Since a critical rate-limiting step in DNA synthesis is that related to the conversion of deoxyuridylic acid to thymidylic acid in the pre-polymerization step of DNA synthesis, this site became the primary "suspect" when it became evident that a specific folate coenzyme form was required for this critical conversion. This coenzyme form of folate, 5,10 methylene tetrahydrofolate (probably in the pentaglutamate state), has helped focus the interrelationships between vitamin B_{12} and folic acid. The generation of this folate coenzyme form permits us to appropriately focus upon the biochemical pathway that appears to be singular in the genesis of megaloblastosis. This pathway involves the so-called homocysteine to methionine shuttle in which 5 methyl tetrahydrofolic acid (a tissue form of folate) is involved in the conversion of homocysteine to methionine to provide the biochemical machinery for a variety of methylation steps. In so doing, the storage form of folate is converted to an active folate coenzyme form.

This reaction is catalyzed by an enzyme, methyltransferase, which has an absolute co-requirement for the vitamin B_{12} coenzyme form, methylcobalamin. It is this pathway, then, that provides the unique interaction of both folate and vitamin B_{12}. The evidence to date supports this site as the critical mechanism in the genesis of megaloblastosis.

In the early 1960's it was suggested that in vitamin B_{12} deprivation the megaloblastosis was actually the result of a "methyl folate" entrapment at this metabolic site and not due to a direct B_{12} effect. This "methyl folate trap" concept, though generating considerable controversy in the past two decades, does appear true and, in fact, identifies in an important way the singular site of the interaction of the two moieties. The pivotal event in this reaction is the conversion of the storage form of folate to a polyglutamate tetrahydrofolate active coenzyme pool. Although not understood, methylcobalamin is critical in the genesis of the polyglutamate coenzyme folate forms.

How the defect in DNA synthesis generated by this biochemical interaction is actually translated into an identifiable histologic change in the tissues that characterizes megaloblastosis is the fundamental question that we face. In an attempt to dissect the specific molecular mechanism for megaloblastosis, considerable interest has focused upon those drugs known to induce megaloblastosis.

TABLE 13

BIOCHEMICAL EFFECTS OF DRUGS KNOWN TO INDUCE MEGALOBLASTOSIS

DRUG	SITE OF ACTION	POTENTIAL SEQUELAE ON DNA PRECURSORS
5-FLUOROURACIL METHOTREXATE	THYMIDYLATE SYNTHETASE THYMIDYLATE SYNTHETASE (INDIRECTLY)	↓ dTTP
HYDROXYUREA 6-MERCAPTOPURINE AZASERINE	RIBONUCLEOTIDE REDUCTASE	↓ dATP ↓ dGTP
AZAURIDINE	OROTIDYLATE DECARBOXYLASE	↓ dTTP ↓ dCTP
CYTOSINE ARABINOSIDE	DNA POLYMERASE (ESPECIALLY α AND β)	↓ gap

From our understanding of these agents, we can not only identify the biochemical site of their effect but also some of the potential sequelae on DNA precursors. Any explanation, then, of the molecular mechanisms for megaloblastosis must take into account the multiple sites at which these drugs are capable of inducing a defect and generating megaloblastosis. A variety of possible molecular mechanisms have been generated to attempt to explain the translation of the biochemical defect into the tissue lesion recognized as megaloblastosis (59-62).

TABLE 14

MOLECULAR MECHANISMS OF MEGALOBLASTOSIS

I. ALTERED DNA BASE COMPOSITION:

- ADENINE, GUANINE, THYMINE AND CYTOSINE BASE CONTENT NORMAL IN MEGALOBLASTIC MARROWS.

II. DEFECTIVE DNA POLYMERASE FUNCTION.

HIGH K_m FOR α (AND β) POLYMERASE REQUIRES MORE DEOXYRIBONUCLEOTIDE TRIPHOSPHATES (THAN γ).

RESULTANT EXCESS INITIATION OVER ELONGATION: DEFECTIVE GAP FILLING OF NEWLY INITIATED FRAGMENTS.

III. DEFECTIVE REPLICATION FORK UNION:

- OKAZAKI FRAGMENT SYNTHESIS DEFECT WITH PERSISTENT SINGLE STRANDED REGIONS.

IV. REDUCED RATE OF DNA REPLICATION FORK MOVEMENT.

IX. THE MECHANISM OF THE NEURAL LESION IN COBALAMIN DEFICIENCY.

Of the variety of non-hematologic clinical events that occur in cobalamin deficiency, the most classical is that of the development of neurological abnormalities which, as noted above, can affect the spinal cord, peripheral nerves or central neural tissue. The structural defect, known for nearly a hundred years, is best characterized as a dysmyelinization in which myelin alteration is the primary and initial change with subsequent injury to the axon with its deterioration and final loss. This phenomenon is seen in those megaloblastic states due to deficiency of vitamin B_{12} or to those circumstances in which there is an inhibition of its function. The basis of the neurologic defect in vitamin B_{12} deficiency has been a problem of importance equal to that of the mechanism of the

megaloblastosis. Although a variety of theories have been generated (63-65), the number of actual observations made on the lesion are very modest:

TABLE 15

OBSERVATIONS RELATIVE TO NEURAL LESIONS IN B_{12} DEFICIENCY

THESIS:

1. NEURAL LESIONS MAY OCCUR WITH MODEST (OR ABSENT) ANEMIA.
2. ADMINISTRATION OF FOLATE TO B_{12}-DEFICIENT PATIENT:
   - CORRECTS MEGALOBLASTIC ANEMIA.
   - RESULTS IN DEVELOPMENT OR WORSENING OF NEUROLOGIC DEFICIT.
3. MECHANISMS MAY BE MULTIPLE:
   a. DYSMYELINIZATION:
      - SUBACUTE COMBINED - CORD
      - PERIPHERAL NEUROPATHY
   b. ?:
      - CORTICAL ("MEGALOBLASTIC MADNESS")
      - COLOR BLINDNESS

Studies in this institution have focused upon the fact that vitamin B_{12} participates in but the two biochemical reactions described earlier. The well known dichotomy between the megaloblastosis and the neurologic lesion, the evidence of improvement in the megaloblastosis with folate administration, yet worsening of the neurologic lesion with that therapy, and the recognition of cases of neurologic deficit in the absence of megaloblastosis have all served to focus attention on the second metabolic pathway, that of the propionate catabolic cobalamin dependent reactions. The observations to date can be summarized:

TABLE 16

OBSERVATIONS RELATIVE TO NEURAL LESIONS IN B_{12} DEFICIENCY

OBSERVATIONS:

1. HUMAN PERIPHERAL NERVES:
   a. IDENTIFICATION OF ODD CHAIN FATTY ACIDS (66)
   b. CONC. OF ODD CHAIN FATTY ACIDS CORRELATES WITH DEGREE OF PERIPHERAL NEUROPATHY. (66, 79)
2. INCREASED FATTY ACID SYNTHESIS (68, 69)
3. INCREASE IN HEPATIC AND NEURAL ENZYMES OF FATTY ACID SYNTHESIS (↑ CONTENT AND ↑ ACTIVITY):
   ↑ a) FATTY ACID SYNTHETASE (70-73)
   ↑ b) ACETYL CoA CARBOXYLASE (70, 73)
   ↑ c) CLEAVAGE ENZYME (74, 75)
4. PROPIONATE (PROPIONYL-CoA) IS EFFECTIVE SUBSTRATE FOR FATTY ACID SYNTHETIC ENZYMES. (72)
5. INCREASE IN METABOLIC INTERMEDIATES (SUBSTRATES):
   ↑ PROPIONYL CoA (72)
   ↑ METHYLMALONYL CoA (76-78)

In addition to these findings, a variety of interrelated metabolic abnormalities have now been characterized in the circumstance of cobalamin deficiency.

TABLE 17

OTHER BIOCHEMICAL ISSUES IN B_{12} DEFICIENCY

1.	INCREASED KREBS CYCLE ENZYME ACTIVITY (ESPECIALLY CITRATE SYNTHASE)	(74, 75)
2.	INCREASED HEPATIC MITOCHONDRIAL BIOGENESIS (IN MAN AND ANIMAL)	(74, 76)
3.	INCREASED HEPATIC GLYCOGEN	(74)
4.	INCREASED CARNITINE (FOR) MITOCHONDRIAL SHUTTLE - PROPIONYL CARNITINE	(79)

As with the circumstances of megaloblastosis, the exact cause and effect and molecular translation of the neurologic lesion are still not clear. The temporal relationships of the development of the neurological lesion are quite in keeping with an altered myelin secondary to abnormal fatty acid synthesis. The rate of repair would also fit in a sequential way with these same findings.

Until recently, two parallel observations provided some concern relative to this thesis. The first was the early observations on patients with congenital disorders of propionate and methylmalonate metabolism, so-called "ketotic hyperglycinemia". Infants with severe metabolic acidosis were shown to have disorders of propionate and methylmalonate metabolism as their basis, and these so-called congenital forms of methylmalonic aciduria have been characterized as circumstances of defective enzyme structure and function in the propionate catabolic pathway. Recent evidence, summarized by Rosenberg (80), has provided significant substantiation to a clinical parallel between cobalamin deficiency and circumstances of some of the selected forms of congenital methylmalonic aciduria. These differences disappear when the children have the opportunity for neural maturation and for significant duration of the existent metabolic defect. Thus, this initial thorn has now been displaced.

A second problem in the thesis as herein defined is the recent evidence that nitrous oxide has the capacity to induce an abrupt megaloblastic state. The evidence from the initial observations from England, where nitrous oxide is used extensively, provided evidence of a highly specific interference with methionine synthetase activity as the basis for the megaloblastosis (81 and 82). These initial studies were supplemented by clinical evidence that a toxic effect of nitrous oxide included a neuropathy following self-medication over relatively long periods. This neuropathy was characterized by numbness and tingling of the extremities, loss of dexterity, poor balance, impotence and interference with sphincter control (83-85). The finding of neurologic involvement with this highly specific lesion shifts the focus from the metabolic pathway discussed above to the possibility that the neurologic deficit was in part or totally based upon altered methylation of myelin basic protein and that the neurologic deficit, then, focused on the same metabolic pathway as that of megaloblastosis (72, 73).

Recent and yet unpublished data from two laboratories has provided an important broadening of the lessons of nitrous oxide toxicity. First, it is now apparent that methionine synthetase is not the only enzyme affected by nitrous oxide but that, in fact, there is good evidence that methylmalonyl mutase is also affected, although at a somewhat lesser level. These recent observations, then, continue to support the basic thesis defined above. Indeed, the uncommon neurologic deficit and its sequence, following nitrous oxide exposure (81-85) mimic the events described above.

X. FUTURE VISTAS OF THE COBALAMINS.

Thus, in spite of our remarkable knowledge of biochemistry and physiology relative to cobalamin biology, many of the basic questions expressed nearly a century ago still remain unexplained.

A variety of important questions presently under study include:

1.) The definition of the "minimal criteria of the deficient state." These studies involve the recognition and quantification of the metabolic markers of the minimally-deprived state.

2.) The significance of the decreased cobalamin content with age.

3.) The physiologic, and possibly pathophysiologic, role of analogues of the cobalamins. Their biologic existence in man is established, but their biologic significance is uncertain. Some analogues may serve to interfere with the action of individual cobalamin fractions. The role of these analogues in cell metabolism and their potential for use as growth regulators are not known.

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