

THE PHYSIOLOGICAL EFFECT OF VITAMIN B₁₂ DEFICIENCY IN HUMAN BLOOD

by

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of the requirements for the degree in

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature:

Date:

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ABSTRACT

The main aim of this workpiece was to establish the physiological parameters against which a vitamin B₁₂ deficiency could be measured. A comparison between the hematological values of healthy patients and those suffering from pernicious anemia due to vitamin B₁₂ deficiency was done. A specific case of pernicious anemia was used in the comparison of abnormal values to the values of normal healthy patients. The comparison consisted of blood analyses with the help of specified instruments, photomicrographs of bone marrow and blood smears and statistical data. A Coulter Counter Model ZF was used for the hematological analyses of blood, a radio-isotope assay for serum vitamin B₁₂ was done and photomicrographs were taken with a NIKON Microflex camera with photomicrographic attachments.

The importance of vitamin B₁₂ has been shown in this workpiece. With the use of techniques and certain instruments, the effects of a shortage of vitamin B₁₂ has been shown. Analyses of the blood from normal healthy patients was compared to that of patients suffering from pernicious anemia.

It was demonstrated that pernicious anemia is characterized by a low erythrocyte count, hematocrit (Hct), hemoglobin (Hb) and vitamin B₁₂ levels together with a higher mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV). In severe cases of pernicious anemia these levels are extremely high or low as the case may be. Together with these values, the investigation of pernicious anemic blood and bone marrow smears revealed abnormally large erythrocyte precursors and fewer leucocytes than normal.

Abnormally shaped cells, called macrocytes, were seen which was due to the disruption in deoxyribonucleic acid (DNA) synthesis caused by the vitamin B₁₂ deficiency.

This study produced a set of hematological reference values. The comparative study between healthy and pernicious anemic patients demonstrated a significant drop in serum vitamin B₁₂ values during pernicious anemia. The hematological effect was illustrated by the Coulter Counter blood analysis results and the microscopic examination revealed the presence of megaloblastic erythrocytes, oval erythrocytes, pear-shaped poikilocytes and polymorphonuclear neutrophils with hypersegmented nuclei in blood smears during vitamin B₁₂ deficiency. This diagnosis can be supported by the presence of megaloblasts and metamyelocytes in pernicious anemic bone marrow.

**DIE FISILOGIESE EFFEK VAN 'n VITAMIEN B₁₂ TEKORT
IN MENSLIKE BLOED**

deur

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UITTREKSEL

Die hoof doel van hierdie werkstuk was om fisiologiese grense te bepaal waarteen 'n vitamien B₁₂ tekort gemeet kan word. 'n Vergelyking tussen die hematologiese waardes van gesonde persone en dié van pasiënte met pernisiëuse anemie wat ontstaan het as gevolg van 'n vitamien B₁₂ tekort was uitgevoer. Die waardes verkry vanaf 'n spesifieke geval van pernisiëuse anemie was vergelyk met waardes vanaf normale gesonde persone. Hierdie vergelyking het bestaan uit bloed analyses, fotomikrograwe van bloed en beenmurg smere en statistiese data. Die hematologiese bloed analyses was met behulp van 'n Coulter Teller model ZF uitgevoer. 'n Radio-isotoop bepaling vir serum vitamien B₁₂ was gedoen en fotomikrograwe was met 'n NIKON Microflex kamera geneem.

Die belang van 'n vitamien B₁₂ tekort was in hierdie werkstuk gedemonstreer. Die effek van hierdie tekort is deur die gebruik van sekere instrumente en tegnieke aangedui en die resultate hiervan is vergelyk tussen gesonde persone en pasiënte met 'n vitamien B₁₂ tekort.

Hierdie studie het bevestig dat pernisiëuse anemie gekenmerk word deur verlaagde eritrosiet, hematokrit (Hct), hemoglobien (Hb) en vitamien B₁₂ vlakke tesame met verhoogde gemene korpuskulêre hemoglobien (GKH) en gemene korpuskulêre volume (GKV) vlakke. Gedurende ernstige gevalle van pernisiëuse anemie kan hierdie waardes uitermatig hoog of laag wees. Benewens hierdie waardes het die ondersoek van bloed en beenmurg gedurende vitamien B₁₂ tekort, abnormale groot eritrosiet voorgangers en 'n

verminderde hoeveelheid leukosiete getoon. Abnormale sel vorms was ook sigbaar a.g.v. die onderbreking in DNA sintese wat deur 'n vitamien B₁₂ tekort veroorsaak word.

Pernisieuse anemie word verkry wanneer daar 'n vitamien B₁₂ en/of folaat tekort in die dieët is of wanneer hierdie vitamieë nie geabsorbeer kan word nie. Die teenwoordigheid van makrosiete, ovaal eritrosiete, peervormige poikilosiete en polimorfonukleêre neutrofiele met hipergesegmenteerde kerne in bloedsmere dui op 'n megaloblastiese anemie. Hierdie diagnose kan ondersteun word deur die aanwesigheid van megaloblaste en reuse metamiëlosiete in die beenmurg. Die bepaling van vitamien B₁₂ en folaat vlakke in die bloed kan as addisionele bewysstukke vir 'n volledige diagnose dien.

Gedurende hierdie studie is daar 'n stel hematologiese verwysingswaardes vasgestel. Die vergelykende studie tussen gesonde persone en pasiënte met pernisieuse anemie het getoon dat daar 'n beduidende verlaging in serum vitamien B₁₂ waardes gedurende pernisieuse anemie is. Die hematologiese effek was ook duidelik waarneembaar in die Coulter teller se bloed analiese en mikroskopiese ondersoekes het die teenwoordigheid van makrosiete, ovaal eritrosiete, peervormige poikilosiete en polimorfenukleêre neutrofiele met hipersegmenteerde kerne in bloedsmere aangedui. Hierdie diagnose kan ondersteun word deur die aanwesigheid van megaloblaste en reuse metamiëlosiete in die beenmurg.

LIST OF ABBREVIATIONS

<	-	less than
>	-	greater than
1SD	-	one standard deviation
2SD	-	two standard deviation
a.g.v.	-	as gevolg van
Ca ²⁺	-	Calcium ion
cc	-	cubic centimeter
Ci	-	Curie
Co	-	Cobalt
CoA	-	co-enzyme A
d	-	dalton
dl	-	deciliter
DNA	-	Deoxyribonucleic Acid
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetra-acetic Acid
μ	-	micron (10 ⁻⁶ meter)
μg	-	microgram (10 ⁻⁶ gram)
fl	-	floruit

<i>g</i>	-	acceleration due to gravity
GKH	-	gemene korpuskulêre hemoglobien
GKHK	-	gemene korpuskulêre hemoglobien konsentrasie
GKV	-	gemene korpuskulêre volume
Hct	-	Hematocrit
HSA	-	Human Serum Albumin
I	-	Iodine
i.e.	-	<i>id est</i> , "that is"
ICB	-	Intracellular Cobalamin Binding Protein
IF	-	Intrinsic Factor of Castle
l	-	liter
M	-	Mole
MCH	-	Mean Corpuscular Hemoglobin
MCHC	-	Mean Corpuscular Hemoglobin Concentration
MCV	-	Mean Corpuscular Volume
mg	-	milligram (10^{-3} gram)
ml	-	milliliter (10^{-3} liter)
mm	-	millimeter (10^{-3} meter)
N ₂ O	-	Nitrous Oxide
NEG	-	Negative
ng	-	nanogram (10^{-9} gram)
no.	-	number
α	-	alpha

°C	-	degrees Celsius
pg	-	picogram (10^{-12} gram)
PGA	-	Pteroylmonoglutamic Acid
POS	-	Positive
RDA	-	Recommended Daily Allowance
RIA	-	Radio-Isotope Assay
RNA	-	Ribonucleic Acid
TC	-	Total Count
TC I	-	Transcobalamin I
TC II	-	Transcobalamin II
TC III	-	Transcobalamin III
THFA	-	Tetrahydrofolic Acid

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Chapter 1

INTRODUCTION

1.1. AIMS

The earliest reported case of pernicious anemia was in 1822 (Herbert, Colman and Jacob, 1980). Then in 1948 vitamin B₁₂ was isolated for the first time (Herbert, Colman and Jacob, 1980). Since those times the knowledge about vitamin B₁₂ has increased. This workpiece was put together with the aid of decades of research done by dedicated researchers. The techniques and instruments used in this workpiece are the culmination of decades of research into hematology and related fields. A short review is given to demonstrate the importance of vitamin B₁₂ and folic acid in the process of erythropoiesis. The process of erythropoiesis is also discussed to clarify terms in the following chapters.

The main aim of this workpiece was to establish the physiological parameters against which the condition of pernicious anemia can be measured. A comparison between the hematological values of healthy patients and those suffering from pernicious anemia will be attempted. A specific case of pernicious anemia will also be shown and compared to the values of normal patients. The comparison will consist of blood analyses with the help of certain instruments specified, photomicrographs of bone marrow and blood smears and with the aid of statistical measurements.

In this workpiece a short review of the discovery, importance and function of vitamin B₁₂ is given. The interrelationship with another important vitamin, namely folic acid, will also be described. The processes of the investigation will be described and the results achieved will be discussed. At the end a conclusion will be drawn as to the importance of the continuing investigation into this field of work.

Erythropoiesis is the process whereby erythrocytes are produced in bone marrow and released into the circulation. As vitamin B₁₂ is a vital link necessary for the normal production of erythrocytes, a brief outline of the erythropoiesis process will be discussed.

Chapter 2

LITERATURE

Part 1

2.1. INTRODUCTION: Vitamin B₁₂

A short background description of vitamin B₁₂ is given to illustrate the importance of this vitamin during homeostasis. This work will concentrate on the role of vitamin B₁₂ during erythropoiesis, as well as the interrelationship with folate in this aspect. The forms of anemia will also be discussed and a radio-isotope assay (RIA) for vitamin B₁₂ will be described.

2.1.2. History of Vitamin B₁₂:

As early as 1822 a case of anemia was reported which initiated the study of pernicious and megaloblastic anemia (Herbert, Colman and Jacob, 1980). The search for vitamin B₁₂ began in 1926 when Minot and Murphy discovered that when liver was given to patients suffering from pernicious anemia, the anemia was halted or cured (Pike and Brown, 1975). In 1929 Castle and associates demonstrated that an intrinsic factor found in human gastric juice was bound by another extrinsic factor found in animal protein (Doscherholmen, 1965). Castle found that normal gastric juice given with beef muscle caused a hematological remission in patients suffering from pernicious anemia (Doscherholmen, 1965). Vitamin B₁₂ was later identified as the extrinsic factor, found in protein sources, causing the hematological remission. In 1948 two groups of investigators independently isolated a compound from liver that imitated the results of Castle (Doscherholmen, 1965). It was discovered that this compound was identical to an animal protein factor which had been known for some time and was believed to be an unidentified vitamin. In this way the existence of vitamin B₁₂ came to light (Pike and Brown, 1975).

The next important development was the discovery of the biologically active forms/coenzymes of vitamin B₁₂ by Barker and associates (Stadtman, 1971).

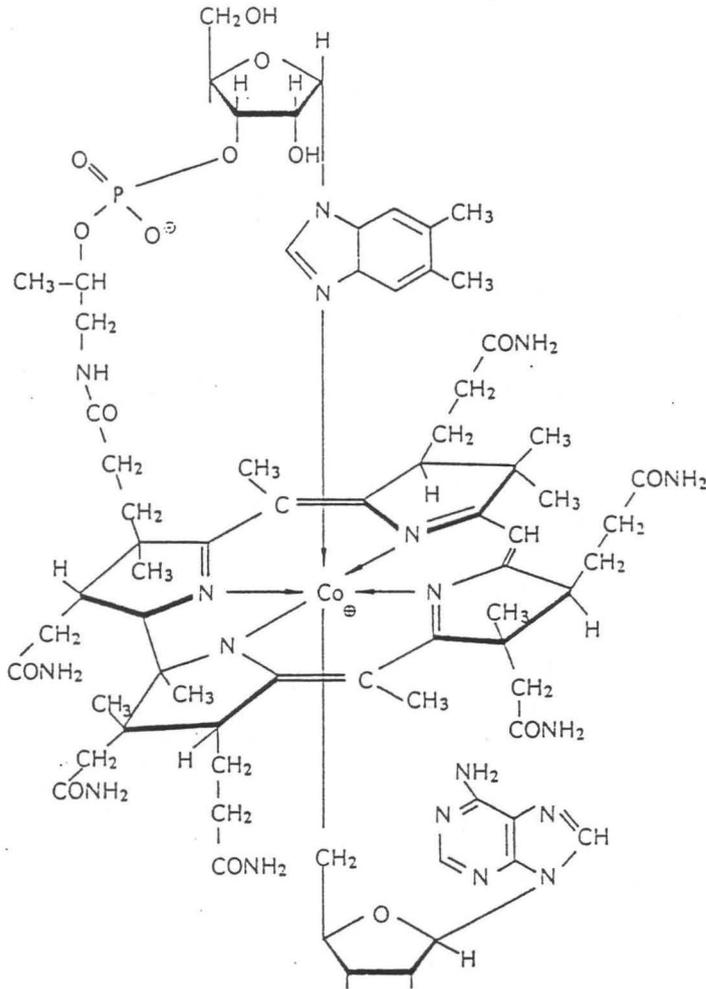


FIGURE 2-1. The structure of vitamin B₁₂. (Pike and Brown, 1975. *Digestion and Absorption*. In: *Nutrition: An Integrated Approach*. Second Edition John Wiley and Sons, Inc., New York.)

2-1-2-1. Structure:

The vitamin B₁₂ molecule is the largest and most complex of the vitamins. The structure was first described by Lenhert and associates in 1961 using an X-ray crystallographic analyses method (Lenhert and Hodgkin, 1961). Vitamin B₁₂ is the only vitamin containing an cobalt atom. The vitamin has a molecular

mass of 1355 dalton (d) and consists of two main parts, a corrin ring and a nucleotide. The corrin ring is composed of four pyrrole rings linked to a centrally located cobalt atom. The nucleotide consists of a base, 5,6-dimethylbenzimidazole. The nucleotide is attached to the corrin ring by ribose-3-phosphate and 1-amino-2-propanol. The nucleotide is also directly attached to the cobalt atom. The final bond of the cobalt atom can bind a variety of anions via a biologically unusual cobalt-carbon link and in so doing can form the various vitamin B₁₂ analogues (Herbert *et al.*, 1980). (see Figure 4-1). The various forms of vitamin B₁₂ are:

- a) cyanocobalamin
- b) hydroxycobalamin
- c) aquocobalamin
- d) nitritocobalamin
- e) methylcobalamin
- f) 5'-deoxyadenosylcobalamin/coenzyme B₁₂

In man, coenzyme B₁₂ and methylcobalamin represent the metabolically active forms of vitamin B₁₂ (Herbert *et al.*, 1980).

2.1.3. Vitamin B₁₂ Binding Proteins:

The binding proteins for vitamin B₁₂ have been classified into three classes by Allen (1975), namely:

- a) intrinsic factor (IF)
- b) transcobalamin II (TC II)
- c) cobalophilin

Briefly, the binding proteins have the following functions (Stenman, 1976):

- a) IF mediates the uptake of vitamin B₁₂ from the gastrointestinal tract (Stenman, 1976)
- b) TC II carries vitamin B₁₂ from the extracellular fluids into the cells (Stenman, 1976)
- c) the functions of cobalophilin is not yet known, but it seems to act as an antimicrobial agent by preventing the growth of micro-organisms by making the vitamin B₁₂ inaccessible to them (Stenman, 1976).

2.1.3.1. Intrinsic Factor (IF):

IF is secreted by the parietal cells of the stomach, binds to vitamin B₁₂ in the duodenum and transports the vitamin to the ileum where it is absorbed (Allen, 1975).

In 1929 Castle postulated the existence of a factor in human gastric juice essential for normal hematopoiesis and named it intrinsic factor (Castle, Townsend and Heath, 1930). More than thirty years later IF was finally isolated and characterized (Gräsbeck, Simons and Sinkkonen, 1966). Approximately five years later IF was found to originate in the parietal cells in the fundus of the stomach (Jacob and Glass, 1970; 1971).

IF is a glycoprotein with a molecular mass variously estimated as from 50 000 - 60 000 d to 100 000 - 120 000 d. The higher values are due to the formation of dimers (Jacob, Baker and Herbert, 1980). The amount of IF secreted per 24 hours is able to bind 50 - 200 µg of vitamin B₁₂. This is 10 - 50 times above the required amount of vitamin B₁₂ needed daily. Approximately 1% of the IF secreted is used daily to bind the vitamin (Allen, 1975).

2·1·3·2. Transcobalamin II (TC II):

TC II is a pure protein with a molecular mass of 38 000 d (Allen, 1975). The protein is present in small amounts (0,5 - 1,5 $\mu\text{g/l}$) in plasma, mostly in the free form, and is responsible for most (80%) of the unsaturated vitamin B₁₂-binding capacity (UBBC) of plasma (Jacob *et al.*, 1980).

The role of TC II is similar to that of IF in the sense that TC II chaperones vitamin B₁₂ through the bloodstream and enhances the movement of the vitamin across cell membranes (Toskes and Deren, 1973).

Since many plasma proteins are found in the liver, this was the first organ to be investigated as a possible source of TC II synthesis. Since then evidence has come to light indicating that TC II is also synthesized by many body cells, i.e. hepatocytes, fibroblasts and macrophages (Jacob *et al.*, 1980). TC II is found in plasma, spinal fluid, semen and extracellular fluid (Cooper and Rosenblatt, 1987).

The importance of TC II in vitamin B₁₂ absorption can be seen in patients with congenital absence of the protein who have malabsorption of the vitamin. These patients need large doses of vitamin B₁₂ to alleviate this condition (Stenman, 1975; Cooper and Rosenblatt, 1987).

2·1·3·3. Cobalophilin:

This group of vitamin B₁₂-binding proteins is known under a variety of names, namely cobalophilin, R-proteins and haptocorrin. The term "R-protein" was introduced by Gräsbeck to describe a non-IF vitamin B₁₂-binding protein in gastric juice with "rapid" mobility in electrophoresis (Gräsbeck, Simons and Sinkkonen, 1966). The name cobalophilin has been introduced to replace the name R-protein. This group of binders consists of several components, isoproteins, which have a slightly variable carbohydrate composition. Not all the isoproteins have the "rapid" electrophoresis characteristic and this caused the preference for renaming the group cobalophilin (Stenman, 1976).

Electrophoresis of the plasma yields two isoproteins with different mobilities, namely transcobalamin I (TC I) with an α 1-mobility and transcobalamin III (TC III) with an α 2-mobility. TC I is 80 - 100% saturated with vitamin B₁₂ in plasma and is present in large amounts, while TC III is largely unsaturated with the vitamin and is present in small amounts (Jacob *et al.*, 1980). Cobalophilin is present at concentrations of approximately 10 - 20 μ g/l (Allen, 1975).

The three groups of vitamin B₁₂-binders, IF, TC II and cobalophilin, all contain single polypeptide chains and a single vitamin B₁₂-binding site (Allen, 1975). Structural studies show that the binders differ in regards to:

- a) amino acid composition
- b) carbohydrate composition
- c) molecular mass
- d) interaction with vitamin B₁₂ as shown by their differences in affinity for vitamin B₁₂ and its analogues.

An intracellular vitamin B₁₂-binder has been found in cultured human fibroblasts (Rosenberg, Patel and Lilljeqvist, 1975). It has an apparent molecular mass of 120 000 d and has been named the intracellular cobalamin binding protein (ICB). This protein does not react with anti-cobalophilin or anti-TC II sera (Stenman, 1976). ICB is apparently necessary for the retention of vitamin B₁₂ in the cells. Lack of ICB is associated with defective conversion of cyanocobalamin to the metabolically active coenzyme forms methylcobalamin and 5'-deoxyadenosylcobalamin (Stenman, 1976).

2·1·4. Sources of Vitamin B₁₂:

The human body is unable to synthesize vitamin B₁₂ and it is therefore dependent on dietary sources, i.e. meat and dairy products, for the vitamin (Herbert *et al.*, 1980). This includes shellfish, fish and poultry.

The vitamin is synthesized by micro-organisms growing in the gastro-intestinal tract of animals (ruminants) or in the soil and water (Toskes and Deren, 1973).

The enzymatically active forms of vitamin B₁₂, coenzyme B₁₂ and methylcobalamin, are the dominant forms in foodstuffs (Herbert *et al.*, 1980).

2·1·5. Nutritional Requirement:

The human body does not need more than 1,0 µg of vitamin B₁₂ per day. This small amount would treat and return vitamin B₁₂ deficient patients with no stores of the vitamin to normal (Herbert, 1988). Studies showed that the minimal daily amount needed is less than 1,0 µg. At this level body stores are not rapidly replenished, but a hematological response does occur (Herbert, 1987; 1988). To minimize risks of too low an intake a recommended dietary allowance has been set at 3 µg per day (Herbert *et al.*, 1980). This amount allows for normal variation in usage and requirements. A higher intake of 4 µg for pregnant and lactating mothers is recommended to compensate for foetal drainage and breast feeding.

The average daily diet supplies 3 - 15 µg of vitamin B₁₂ per day (Herbert, 1987). The total body store has been variously estimated to range between 2,2 mg and 3 mg (Herbert, 1987).

2.1.6. Absorption and Transport:

Vitamin B₁₂ is the largest and most complex of vitamins and therefore its method of absorption and transport is unique and involved. Binding proteins of the vitamin play important roles in this process.

The absorption of vitamin B₁₂ takes place along two pathways (Herbert *et al.*, 1980; Herbert, 1987 and 1988). In the pharmacological path absorption is along the entire length of the small intestine and takes place by diffusion. This path accounts for approximately 1% of free vitamin B₁₂ absorption and is not dependent on IF. The physiological path is more complex in that binding proteins are used to transport the vitamin to a specific area of absorption, namely the ileum (Hagedorn and Alpers, 1977; Shaw, Meyers, Colman, Jayatilleke and Herbert, 1987). The ileal mucosal cells contain specific receptors to which the IF-vitamin B₁₂ complex binds (Herbert, Streiff and Sullivan, 1964; Katz and Cooper, 1974; Hagedorn *et al.*, 1977).

In the mouth, vitamin B₁₂ is split from ingested food by salivary enzymes and binds to cobalophilin present in the saliva (Herbert, 1988). The mouth contents is swallowed and the cobalophilin-B₁₂ complex ends up in the stomach. More vitamin B₁₂ is split from its bonds in food by gastric enzymes and acid and binds to cobalophilin. IF is secreted by gastric parietal cells, but cannot bind to the vitamin due to the acidic gastric medium (Herbert, 1988). Cobalophilin prevents the breakdown of vitamin B₁₂ by gastric enzymes and acid.

In the upper duodenum, the cobalophilin-B₁₂ complex comes into contact with an alkaline surrounding created by pancreatic secretion. Pancreatic enzymes split or breakdown cobalophilin. The released vitamin B₁₂ now binds with IF forming an IF-B₁₂ complex (Pike *et al.*, 1975). This complex is carried down to the ileum where absorption of vitamin B₁₂ can take place. The IF-B₁₂ complex binds to receptors on the ileal mucosal cells. An alkaline surrounding is again needed for this process. The vitamin is absorbed into the cell and later appears in the bloodstream bound to TC II (Cooper *et al.*, 1987). TC II delivers vitamin B₁₂ to the body cells either for storage or for use as an enzyme in certain reactions.



In addition, an enterohepatic circulation exists by which vitamin B₁₂ is recycled via bile secretion for absorption (Herbert, 1987). The vitamin is bound to cobalophilin in the bile, however, pancreatic enzymes digest this binder, setting vitamin B₁₂ free to bind with IF.

2.1.7. Storage and Excretion of Vitamin B₁₂:

Absorbed vitamin B₁₂ not immediately utilized, is stored in body tissues. The total body store ranges between 2,2 mg to 3 mg (Herbert, 1987). The liver is the main storage place of the vitamin, containing 50-90% of total body stores. A variety of tissues make up the remaining storage areas, i.e. muscle, skin, bone, lungs and spleen (Pike *et al.*, 1975). It appears that the main storage form of vitamin B₁₂ is coenzyme B₁₂ (Herbert *et al.*, 1980).

Vitamin B₁₂ has been found in urine as well as fecal excretion (Herbert, 1987). Excretion also takes place via bile, but the vitamin is reabsorbed through the enterohepatic circulation system.

Daily loss of the vitamin constitutes approximately 0,1% of the body pool (Herbert, 1987). This is equivalent to $\pm 3,6 \mu\text{g}$ per day, therefore to maintain the same total body pool size it would be necessary to replace $1,35 \mu\text{g}$ of vitamin B₁₂ daily (Herbert, 1987).

2.1.8. Functions of Vitamin B₁₂:

Vitamin B₁₂ functions in all the cells, especially those of the gastrointestinal tract, nervous system and bone marrow. A vitamin B₁₂ coenzyme participates in the synthesis of deoxyribonucleic acid (DNA) in bone marrow. The vitamin is needed for the maintenance of the myelin sheath around nerve structures and required for normal growth in children. In conjunction with folic acid, vitamin B₁₂ works towards the synthesis of red and white blood cells (Marshall, 1986). Vitamin B₁₂ is required for the synthesis and transfer of single-carbon units such as the methyl group in the synthesis of methionine and choline.

Coenzymes of the vitamin are needed for the conversion of methylmalonate to succinate (Robinson, Lawler, Chenoweth and Garwick, 1986).

The coenzyme form of vitamin B₁₂ was discovered by Barker and associates in 1958 (Pike *et al.*, 1975). This coenzyme contains an unusual binding of carbon covalently linked with cobalt. The chemical and biological activity of coenzyme B₁₂ depends on this carbon-cobalt link (Toraya, 1988).

Vitamin B₁₂ is known to participate in a number of enzymatic reactions in bacteria, but only two have been found to take place in mammalian tissue (Stadtman, 1971). These two intracellular reactions are (Cooper *et al.*, 1987):

- a) conversion of methylmalonyl coenzyme A (CoA) to succinyl CoA
- b) generation of methionine from homocysteine using 5-methyltetrahydrofolate as donor of the methyl group via the enzyme methyltransferase.

Evidence show that 5'-deoxyadenosylcobalamin also acts as a coenzyme for the interconversion of alpha and beta leucine (Poston, 1976).

Recently vitamin B₁₂ has been found in seminal plasma and is thought to be an important nutrient for the maintenance of normal fertility in both sexes (Carmel and Bernstein, 1984).

2.1.9. Deficiency of Vitamin B₁₂:

A deficiency of vitamin B₁₂ usually occurs due to a defect in absorption rather than an inadequate dietary intake. The symptoms of deficiency are (Pike *et al.*, 1975):

- a) weakness, tiredness
- b) sore tongue
- c) paresthesia
- d) constipation
- e) headache
- f) palpitation
- g) macrocytic anemia
- h) neurological damage

Deficiency prevents optimum erythrocyte development and this leads to anemia. Nerve damage can be found, especially to the myelin sheath that protects the nerve fibre, causing the loss of muscular coordination (Marshall, 1986). A lack of IF can cause the non-absorption of vitamin B₁₂ referred to as pernicious anemia. This causes the bone marrow to produce immature erythrocytes and results in the release of large erythrocytes into the circulation. These large erythrocytes are macrocytes and the condition is called macrocytic/megaloblastic anemia (Robinson *et al.*, 1986). Megaloblastic anemia can also occur after surgical removal of the stomach area that produces IF or the part of the ileum where the absorption sites are located (Robinson *et al.*, 1986). Vegetarians excluding eggs and/or milk products in their diet can also develop a deficiency (Herbert, 1988).

Megaloblastic anemia and the changes connected with pernicious anemia suggests that vitamin B₁₂ is essential for DNA synthesis. The functions of vitamin B₁₂ and folate are very much interrelated. A view on this interrelationship could shed some light on the causes of megaloblastic anemia. Both vitamin B₁₂ and folate are needed for the synthesis of thymidylate and therefore for DNA. A vitamin B₁₂ containing enzyme, vitamin B₁₂-transmethylase, removes a methyl group from methylfolate and delivers it to

homocysteine. In doing so methionine (methyl-homocysteine) is formed and tetrahydrofolic acid (THFA) is regenerated from which 5,10-methylene THFA is formed. The 5,10-methylene THFA is involved in thymidylate synthesis and thus formation of DNA (Herbert *et al.*, 1980).

In 1962, Noronha, Silverman, Herbert and Zalusky postulated how a deficiency in vitamin B₁₂ and the interrelationship with folate is a cause of megaloblastic anemia (Chanarin, 1987). If methionine synthesis is inhibited by a vitamin B₁₂ deficiency, then the regeneration of THFA from 5,10-methylene THFA is blocked. The above mentioned called this postulation the methylfolate block theory. This would lead to a decrease in purine synthesis necessary for DNA (Pike *et al.*, 1975; Herbert *et al.*, 1980).

From the above it is clear that a lack of either vitamin B₁₂ or folate will cause the methylfolate block. This may explain why the hematological damage of vitamin B₁₂ deficiency cannot be clinically distinguished from folate deficiency (Herbert *et al.*, 1980). In both cases the hematological damage is due to the lack of enough 5,10-methylene THFA which delivers a methyl group to deoxyuridylate converting it to thymidylate leading to DNA synthesis during the S-phase (Herbert *et al.*, 1980). The disruption in DNA synthesis causes many hematopoietic cells to die in the bone marrow. This situation could be called "ineffective erythropoiesis". The end product of DNA synthesis is megaloblastosis i.e. the formation of abnormally large blood cells due to a block in the S (synthesis) phase of cell replication. This is usually due to a vitamin B₁₂ or folate shortage with most of the body's replicating cells in the process of trying to double their DNA in order to divide, but not successfully (Pike *et al.*, 1975; Herbert *et al.*, 1980). The biochemical defect which causes poor thymidylate synthesis to form megaloblasts, may be the failure to lengthen DNA chains in the presence of a relatively normal ability to initiate DNA synthesis. This could be due to the lowered thymidylate concentrations remaining adequate to serve as a substrate for initiating DNA-polymerase, but not for lengthening DNA-polymerase (Herbert *et al.*, 1980).

Since then new evidence has been produced to challenge the validity of the methylfolate trap theory. In untreated pernicious anemia the serum folate, mostly methylfolate, is increased. Herbert and Zalusky deduced that in vitamin B₁₂ deficiency, methylfolate could not be oxidized back to methylenefolate (Chanarin, 1987). As more folate accumulated in the methyl form, a shortage of other forms of folate

analogues are formed. Herbert *et al.* (1964) proposed that there was an interruption in the transfer of formyl for purine synthesis and methylene transfer for thymidine synthesis. This then caused an interference in DNA synthesis and led to megaloblastosis. These deductions are difficult to test because patients with untreated pernicious anemia do not easily allow studies to be conducted on themselves. This situation was changed when it was discovered that nitrous oxide (N₂O) used during anaesthetic, is activated by organometallic complexes such as vitamin B₁₂ (Chanarin, 1987). The N₂O causes the oxidation of vitamin B₁₂ to an irreversibly inert form. Specifically, the N₂O inactivates the enzyme methionine synthetase, of which vitamin B₁₂ is a coenzyme, and this causes a "vitamin B₁₂ shortage".

Using this information practically, new facts have come to light disproving the methylfolate trap theory. Certain questions can now be put forward to challenge the validity of this theory (Chanarin, 1987).

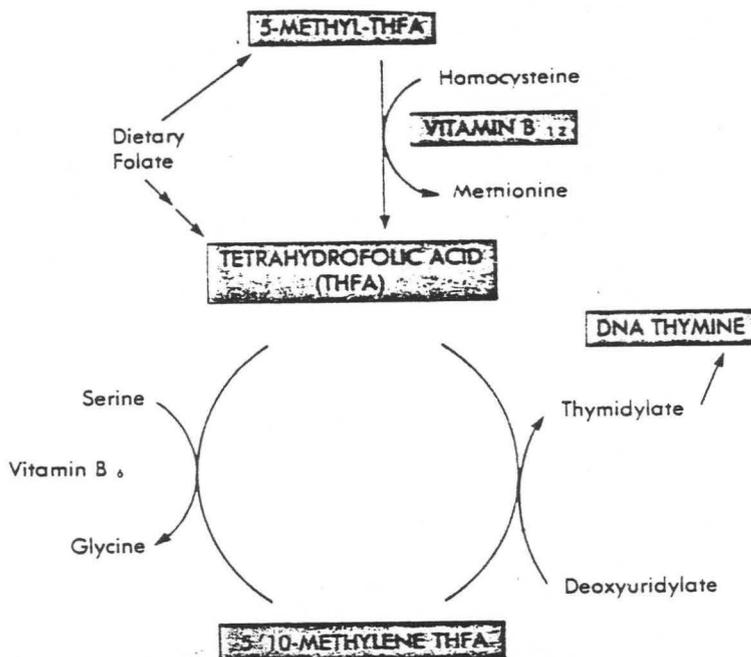


FIGURE 2.2. The interrelationship between vitamin B₁₂ and folic acid. (Robinson, Lawler, Chenoweth and Garwick. 1986. *The Water-soluble Vitamins*. In: *Normal and Therapeutic Nutrition*. Seventeenth Edition. MacMillan Publishing Company, New York.)

I. DOES METHYLFOLATE TRAPPING EXPLAIN THE KNOWN DEFECT IN VITAMIN B₁₂ DEFICIENCY?

The methyl group from methylfolate cannot be transferred to homocysteine in vitamin B₁₂ deficiency and therefore causes a blockage in methylfolate usage (Figure 2-2). Methylfolate does not correct this situation in vivo in bone marrow cells. Neither can tetrahydrofolate correct the defect in thymidine synthesis, but formylfolate is still active. The failure to use tetrahydrofolate and normal usage of formyltetrahydrofolate is not predicted by the methylfolate trap theory.

II. IS THE IN VITRO PREDICTION THAT METHYLFOLATE CANNOT BE OXIDIZED BACK TO METHYLENE AND FORMYLFOLATE CORRECT IN VIVO?

As early as 1961 Noronha and Silverman (Chanarin, 1987) found that rats supplemented with 1% methionine for 24 hours in their diet, contained decreased concentrations of methylfolate in the liver while the formylfolate and tetrahydrofolate concentration were higher. This took place in both control and vitamin B₁₂ deficient rats. This implied oxidation of the methyl group to formyl and carbon dioxide. This has been proven to be the case by Brody, Watson and Stokstad (1982). Thorndike and Beck (1977) found that the methyl group of methylfolate was oxidized similarly by lymphocytes from normoblastic controls and patients with pernicious anemia. This Once again the methylfolate theory was proven incorrect.

The most widely studied deficiency of vitamin B₁₂ is probably the effect on developing erythrocytes. This deficiency leads to megaloblastic anemia. Basically a disruption in DNA synthesis as well as in the number of cell divisions of erythrocytes during maturation is encountered. This causes the production of larger and fewer mature erythrocytes (Pike et al., 1975). There is also an increase in hemopoietic tissue in bone marrow. Total erythropoietic activity is increased, but effective erythropoiesis in terms of the delivery of normal functional erythrocytes into the circulation is reduced (Baker, 1967; Bell, Emslie-Smith and Paterson, 1980). These megaloblastic cells also have a shortened half-life.

Vitamin B₁₂ deficiency also effects the nervous system (Baker, 1967; Robinson *et al.*, 1986). Deficiency causes the demyelination of nerve fibres. A disruption in the normal functioning of the propionic pathway and methylmalonyl CoA reaction occurs which causes the formation of odd-chain and branched-chain fatty acids. The presence of these unusual fatty acids is implicated in the demyelination of nerve fibres (Van der Westhuyzen, Cantrill, Fernandes-Costa and Metz, 1983).

A pure deficiency in vitamin B₁₂ alone can actually only be noticed after two years. This is due to the fact that the body stores are only of vitamin B₁₂ approximately two years after the cause of deficiency (Herbert, 1987).

2.1.10. Treatment:

Vitamin B₁₂ deficiency in man is nearly always caused by inadequate absorption. This can usually be corrected by adding vitamin B₁₂ to the daily diet containing red meat, poultry and fish. An intramuscular injection of 1,0 µg daily is also sufficient (Herbert *et al.*, 1980). A single injection of 100 µg or more will produce complete therapeutic remission in any patient where vitamin B₁₂ deficiency is not complicated by other factors. This remission is then kept up by monthly injections of 100 µg (Herbert *et al.*, 1980; Herbert, 1987).

Part 2

2.2. INTRODUCTION: Folic Acid

A look at folic acid/folate is necessary to show the importance of its interrelationships with vitamin B₁₂, especially its role in erythropoiesis.

2·2·1. History:

Illnesses that might have been due to folic acid deficiencies were described as early as 1851. These deficiencies were mainly associated with anemia and malnutrition in pregnancy (Marshall, 1986). In 1937, Dr. L. Wills cured the anemia of pregnant women in India with a commercial preparation of autolyzed yeast called Marmite (Herbert *et al.*, 1980; Marshall, 1986). The missing substance was called "Wills Factor". During the 1930 s and 1940 s many water-soluble factors required by various animal species and micro-organisms had been described (Robinson *et al.*, 1986). Those factors were given names such as factor U (unknown factor needed for chick growth), vitamin B_C (anti-anemia factor for chicks), vitamin M (vitamin curing anemia, leukopenia, diarrhea and gingivitis in monkeys), L. casei factor (factor needed for growth of *Lactobacillus casei*), citrovorum factor and SLR factor needed for micro-organisms growth (Herbert *et al.*, 1980; Robinson *et al.*, 1986). It was later demonstrated that all these different factors were actually one, namely Wills Factor (Herbert *et al.*, 1980). In 1941 Mitchell and associates found Wills Factor in the leafy vegetable spinach. The Latin word for leaf is folium and the name folate was suggested for the above mentioned Wills Factor (Pike *et al.*, 1975). The structure of folic acid was identified by Angier and co-workers in 1945 (Robinson *et al.*, 1986). In the same year, Dr. T. Spies showed that folic acid was effective for the treatment of megaloblastic anemia in pregnancy (Robinson *et al.*, 1986).

2·2·2. Characteristics:

Folacin is the generic term for folic acid and other compounds having the activity of folic acid (Marshall, 1986; Robinson *et al.*, 1986). The complete chemical name for folic acid is pteroylmonoglutamic acid which is usually shortened to pteroylglutamic acid or PGA (Herbert *et al.*, 1980; Marshall, 1986; Robinson *et al.*, 1986). The folic acid molecule has a molecular mass of 454 d. The molecule consists of three linked components, namely a pteridine group, para-aminobenzoic acid and glutamic acid (Pike *et al.*, 1975). Pure crystalline folic acid is a bright yellow compound which is only slightly soluble in water (Robinson *et al.*, 1986).

2.2.3. Sources:

The folates are widely distributed in animal and plant foodstuffs. The highest concentrations are found in green vegetables, liver, yeast and nuts (Cronje, 1987). Other sources are grains, pork, eggs and dairy foods (Robinson *et al.*, 1986). Folic acid is very susceptible to oxidative destruction (Herbert *et al.*, 1980). As much as 50-95% of the folate content of food may be destroyed by extended cooking or other processing (Robinson *et al.*, 1986). All the folate is lost from refined food such as hard liquor and candies.

The naturally occurring folates are metabolically active and are usually in the polyglutamate form (Herbert *et al.*, 1980). Conjugases in mammalian tissue liberate pteryl-di- and pterylmonoglutamates from conjugates and make the folate available for absorption (Herbert *et al.*, 1980). Folacin is mainly stored in the liver. The active form is tetrahydrofolic acid. Ascorbic acid prevents the oxidation of this active form. In this way adequate levels of the folate needed for metabolism is maintained (Robinson *et al.*, 1986).

2.2.4. Requirements:

The Recommended Daily Allowance (RDA) for folic acid is 400 μg for adults. For pregnancy the allowance is 800 μg and for lactation it is 500 μg . The daily needs for infants are set at 30 - 45 μg and children aged 1 to 10 years old it is 100 - 300 μg (Herbert *et al.*, 1980; Marshall, 1986; Robinson *et al.*, 1986; Ganong, 1987). These amounts are more than the needs for normal metabolism. In cases of folic acid deficiency 50 - 250 μg of synthetic folic acid is enough to correct the condition (Marshall, 1986). The daily folate requirement is dependent on the daily metabolic and cell turnover rates, therefore it is increased by anything which increases metabolic rate i.e. infection and increases in cell turnover i.e. hemolytic anemia and tissue growth in the fetus (Herbert *et al.*, 1980).

2·2·5. Storage and Excretion:

Normal total-body folate stores are 5 - 10 mg of which approximately half is in the liver (Herbert *et al.*, 1980). Most of the stored folate is in the polyglutamate form. The folate stores are sufficient for only 3 - 5 months and therefore a folate deficiency can develop rapidly (Herbert *et al.*, 1980; Cronje, 1987).

Folic acid is lost via urine, sweat and faeces (Cronje, 1987). Folate is also found in bile secretion which contains approximately 100 μg of biologically active forms (Herbert *et al.*, 1980). The folate excreted via urine and faeces is inactive and is mainly in the acetamidobenzoylglutamate form (Herbert *et al.*, 1980).

2·2·6. Absorption and Transport:

The mechanism of intestinal absorption of folic acid is not yet clearly understood (Cronje, 1987).

About 25% of the folacin in food is in the free form and is easily absorbed. Before the polyglutamate forms can be absorbed, the extra glutamate groups must be removed by conjugases, an enzyme present in the mucosal cells of the proximal intestine, to form monoglutamate which is easily absorbed (Bell *et al.*, 1980; Robinson *et al.*, 1986). The site of absorption is the proximal intestine. In the mucosal cell the folate is converted to 5-methyltetrahydrofolic acid, the main form of folate in the blood (Bell *et al.*, 1980). The folate then enters the portal circulation.

The plasma transport of folate is also unclear. It appears that plasma folate is distributed in three fractions, namely free folate, folate bound to low and high affinity binders (Herbert *et al.*, 1980). The binders appear to have the following three functions:

- a) delivery of folate to the liver
- b) control of folate distribution, breakdown and excretion in deficient states
- c) transport of oxidized folates from cerebrospinal fluid to blood.

In the liver more folate is converted to 5-methyltetrahydrofolic acid and from there part of the folate is transported to body cells (Cronje, 1987). Once in the cell the methyltetrahydrofolic acid is converted to tetrahydrofolic acid with the help of methylcobalamin (Bell et al., 1980). The remaining 5-methyltetrahydrofolic acid is excreted in urine or by the gut via the gall bladder (Cronje, 1987).

2.2.7. Function:

The active form of folate is tetrahydrofolic acid (THFA). This is linked to several single carbon groupings:

- a) methyl
- b) hydroxymethyl
- c) formyl
- d) formimino

The ability to link up with and to donate these single carbon groups forms the basis for the biochemical function of folate (Robinson et al., 1986). THFA in conjunction with vitamin B₁₂ is essential for DNA synthesis and therefore essential for normal erythropoiesis. This interrelationship has been discussed previously. Folate is also necessary for (Robinson et al., 1986; Cronje, 1987):

- a) the formation of serine from glycine
- b) the formation of choline
- c) the conversion of homocysteine to methionine
- d) synthesis of thymidine from deoxyuridine
- e) synthesis of purine
- f) conversion of histidine to glutamate

Thymidines and purines are required for DNA synthesis and purines for RNA synthesis. These compounds are required by all cells that renew themselves i.e. erythrocytes, skin, mouth lining, gut, kidney tubules, bladder epithelium etc. (Cronje, 1987).

2.2.8. Deficiency:

Folic acid deficiency is usually a result of inadequate dietary intake or is secondary to disease. Deficiency produces an anemia in which the erythrocytes are abnormally large and their numbers are reduced (Marshall, 1986). Most of the symptoms of folate deficiency mimic those of vitamin B₁₂ deficiency. The exception is that folate deficiency does not cause neurological damage (Jarrett, 1979; Robinson *et al.*, 1986; Cronje, 1987).

With a deficiency, the serum folate level is reduced and there is a change in erythrocyte production in the bone marrow (Robinson *et al.*, 1986). The anemia that is produced is characterized by a reduction in erythrocyte levels, release of large blood cells in the circulation, low hemoglobin, leukocyte and platelet levels (Robinson *et al.*, 1986). The diagnosis for folate deficiency is usually based on serum and/or erythrocyte concentrations of folate. Values for serum folate equal to or greater than 6 ng/ml are generally regarded as normal. Normal values for red cell folate are 160 - 650 ng/ml (Robinson *et al.*, 1986) and for serum folate it is 1,5 - 27,3 ng/ml (Cronje, 1987).

Anemia due to folate deficiency has been observed in elderly people who have had poor diets and various organic diseases. Anemia has been noted in pregnant women, in some women using oral contraceptives and in infants where not sufficient folate was included in the diet (Pike *et al.*, 1975; Marshall, 1986; Robinson *et al.*, 1986). Folate deficiency often accompanies disease conditions in which the requirement for folate is increased as in leukemia (Robinson *et al.*, 1986).

The most common causes of folate deficiency are pregnancy and lactation (Pike *et al.*, 1975; Jarrett, 1979). This is due to the rapid cell growth by the foetus. Folic acid is drawn from the mother and if there is not

sufficient folate in the mother's diet to supplement the usage by foetal cell growth, anemia will occur. Higher foetus folate levels than maternal levels supports this argument (Pike et al., 1975).

2.2.9. Treatment:

A patient with folate deficiency is treated with 100 μg of folic acid orally or parenterally daily (Herbert et al., 1980). This dosage produces a maximal hematological response for folate deficiency, but not for patients with vitamin B₁₂ deficiency (Herbert et al., 1980; Robinson et al., 1986). Therapy of doses of folate greater than 0,1 mg daily is necessary when the deficiency condition is complicated by factors which may suppress hematopoiesis, factors that increase folate requirement and conditions that reduce folate absorption (Herbert et al., 1980). The therapy should then be 0,5 - 1,0 mg per day. Maintenance therapy is normally 0,1 mg of folate daily for 1 - 4 months. This should only be stopped when the daily diet includes at least one fresh fruit or vegetable (Herbert et al., 1980).

The administration of folic acid to patients with megaloblastic anemia causes dramatic reversal of the abnormal changes in bone marrow. The erythrocytes become normal in size, their number increases, the total hemoglobin increases and leukocyte levels return to normal (Robinson et al., 1986). Folate will cause remission of the anemia in vitamin B₁₂ deficiency, but is not effective in preventing or correcting the neurological disturbances (Pike et al., 1975; Jarrett, 1979; Marshall, 1986; Robinson et al., 1986). Therefore folic acid is not administered to a person with megaloblastic anemia until tests, i.e. determination of serum concentrations of the vitamins, are done to establish that the condition is due to folate deficiency rather than to vitamin B₁₂ deficiency. The amount of folate in vitamin supplements is therefore regulated to avoid masking the existence of vitamin B₁₂ deficiency and delaying its diagnosis by curing the anemia (Robinson et al., 1986).

Part 3

2.3. INTRODUCTION: Erythropoiesis

Erythropoiesis is the process whereby erythrocytes are produced in bone marrow and released into the circulation. As vitamin B₁₂ is a vital link necessary for the normal production of erythrocytes, a brief outline of the erythropoiesis process will be discussed

2.3.1. The Erythrocyte:

The human erythrocyte is a circular, elastic, non-nucleated, eosinophilic, biconcave disk that stains red with Romanowsky stain and whose primary function is the transport of oxygen bound to hemoglobin. The erythrocyte averages 7,2 μ in diameter and 2,1 μ in thickness. The MCV is 87 fl with a range of 76-96 fl. There are $4,8 \times 10^6$ erythrocytes /mm³ in the adult female and $5,4 \times 10^6$ /mm³ in the adult male with an average life-span of 120 days. The formation of erythrocytes takes place in the bone marrow of the adult human (Platt, 1979; Ganong, 1987).

2.3.2. Erythrocyte Development:

In general, developing erythroid cells show the following changes (Platt, 1979):

- a) the cells decrease in size and the cytoplasm dominates the cell interior
- b) nucleoli disappear and the chromatin becomes progressively coarser
- c) the cytoplasm becomes less basophilic due to a decrease in ribonucleic acid.

The developing erythroid cell goes through the following stages during its formation (Platt, 1979):

- a) primitive stem cell/hemohistioblast
- b) hemocytoblast
- c) pronormoblast/proerythroblast/rubriblast
- d) basophilic normoblast/early erythroblast/prorubricyte
- e) polychromatophilic normoblast/late erythroblast/rubricyte
- f) acidophilic normoblast/normoblast/metarubricyte
- g) reticulocyte
- h) erythrocyte

The maturation of the erythrocyte starts with its development from a multipotential cell, the hemohistioblast or stem cell. This is a large oval cell, 25-35 μ , with a large oval nucleus with fine chromatin. One-third of the cell volume contains a lilac-grey cytoplasm with minute polychromatic granules. The hemohistioblast matures into a hemocytoblast, 20-30 μ . The cytoplasm becomes smaller, bluish and nongranular. Under stimulus of erythropoietin the hemocytoblast divides, producing one differentiated and one undifferentiated daughter cells. The differentiated daughter cell is found in marrow, lymph nodes, spleen and the liver. This cell is 25-35 μ in diameter with an oval nucleus that almost fills the diameter. The undifferentiated cell remains so and therefore prevents the depletion of stem cells (Platt, 1979).

From the differentiated cell a pronormoblast is formed. It is approximately twice the size of an erythrocyte, 14 - 19 μ in diameter. The nucleus is large, vesicular, granular and light purple. The cytoplasm is a granular light blue, later becoming purplish blue. The nucleus contains several nucleoli.

By mitosis a basophilic normoblast is formed from the above mentioned cell. This cell is 12 - 17 μ in diameter. The nucleus is dark purple and nucleoli are absent. The cytoplasm is navy blue in colour. At this stage the formation of hemoglobin is started.

The next cell formed by mitosis is the polychromatic normoblast which is 12 - 15 μ in diameter. The nucleus is smaller with condensed chromatin. The cytoplasm is bluish-red (polychromatic) which is caused by the red staining of the formed hemoglobin.

The normoblast forms by maturation from the polychromatophil over a period of two days. The cell is 8 - 12 μ in diameter. The nucleus is dark and small with condensed chromatin forming a cartwheel appearance. Later the nucleus becomes picnotic i.e. the nucleus disappears by breaking up and disappears by lysis or expulsion. The cell cannot synthesize DNA anymore and therefore cannot divide. Due to the increase in hemoglobin synthesis, the cytoplasm stains eosinophilic. The nucleus is usually eccentrically placed.

A reticulocyte (7 - 10 μ) is formed next by the expulsion of the nucleus or its break up. The reticulocytes enter the circulation and after approximately two days mature into erythrocytes.

Maturation of the erythroblasts therefore involves a decrease in cell size, increased condensation, nucleus becomes picnotic, hemoglobin synthesis and a change in staining reaction of the cytoplasm from basophilic via polychromatophil to eosinophil (Keele, Neil and Joels, 1982).

2.3.3. Anemia and Erythropoiesis:

Erythropoiesis is normally regulated to maintain the erythrocyte number and hemoglobin content within a narrow range. In anemia there is a reduction in the number of circulating erythrocytes or a decrease in their hemoglobin content. Anemia occurs when erythropoietic tissue cannot supply enough normal erythrocytes to the circulation. The normal balance between formation and break down is maintained by a daily output of 2×10^{11} erythrocytes from the bone marrow, with the cells surviving for about 120 days (Keele, Neil and Joels, 1982). This balance is upset if there is an excessive loss of blood, i.e. by hemorrhage, or if there is a defect in the production of erythrocytes by the bone marrow.

Vitamin B₁₂ and folic acid are vitally important for the normal maturation of nucleated erythrocytes/normoblasts (Keele, Neil and Joels, 1982). When these substances are absent, the red bone marrow becomes hyperplastic and spreads through the shafts of the long bones, e.g. femur, tibia, fibula, humerus, radius and ulna (Keele, Neil and Joels, 1982). Due to the vitamin shortage, derangement of DNA synthesis occurs which leads to the proliferation of abnormal nucleated erythroid cells called early, intermediate and late megaloblasts in the bone marrow. These megaloblasts are larger than their corresponding normoblasts and the cytoplasm becomes prematurely filled with hemoglobin.

The late megaloblast loses its nucleus to become a macrocyte with a diameter of 8,2 μ , MCV of 95 - 160 fl and a MCH of 50 pg. The MCHC is normal because the increased amount of hemoglobin is distributed throughout a larger cell (Keele, Neil and Joels, 1982). There is a reduction in the number of circulating erythrocytes. Nucleated red cells are sometimes seen in blood smears.

Megaloblastic hemopoiesis typically occurs in pernicious anemia and in anemia due to folate deficiency. Megaloblastosis occurs because DNA production is limited, but not RNA production. Vitamin B₁₂ and folate are both needed for the synthesis of thymidylate, the nucleotide of thymine which is found in DNA but not RNA. Pernicious anemia is due to the reduced absorption of vitamin B₁₂ from the ileum. The primary lesion is atrophy of the gastric mucosa which leads to the reduced secretion of IF by the parietal cells. Normally IF forms a complex with vitamin B₁₂ and promotes its absorption (Keele, Neil and Joels, 1982).

2·3·4. Megaloblastic Erythropoiesis:

A deficiency in vitamin B₁₂ and folic acid causes the developing erythroid cells to be larger than normal in all stages of development. This is possibly due to the cells growing for longer periods without undergoing mitosis (Platt, 1979). Abnormal mitosis, multiple nuclei, more cytoplasm in relation to nucleus in early forms, less homogeneous cytoplasm and premature hemoglobin development with associated immature nuclei and eosinophilic cytoplasm are typically observed in these cells. The earliest form of these cells is the promegaloblast, 19 - 27 μ in diameter. The nucleus is a light purple with a stippled reticular chromatin

pattern without the clumping as found in the pronormoblast. Three to five nuclei are present. The cytoplasm is more in relation to the nucleus and there may be lighter staining areas around the nucleus.

The megaloblast (basophilic megaloblast) is 15 - 22 μ in diameter. It is larger than the basophilic normoblast. The nucleus has a more finely divided chromatin and is usually placed eccentrically. There are no nuclei and the cytoplasm is a darker royal blue. There is less cytoplasm than in the promegaloblast, but more than in its normal counterpart, the basophilic normoblast.

The megaloblastic polychromatophilic normoblast (polychromatophilic megaloblast) is 10 - 18 μ in diameter and is therefore bigger than its normal counterpart, the polychromatophilic normoblast. The nucleus is round and eccentric, has fine reticular chromatin which does not clump as in the polychromatophilic normoblast.

The megaloblastic orthochromic normoblast (orthochromic megaloblast) measures 8 - 15 μ and is often 3 - 4 times the size of its normal counterpart, the acidophilic normoblast. The nucleus is smaller than its predecessor, is purplish blue and shows clumping of coarse to dense chromatin material. Occasionally the cell contains 2 - 3 abnormal nuclei without nucleoli. The cytoplasm is darkly eosinophilic and more abundant than its normal counterpart.

The megaloblastic reticulocyte (macrocyte) and megalocyte (erythrocyte) measure 9 μ or more in diameter. They are a dark to, light bluish orange or orange and contain no nucleus.

A number of factors are needed for normal erythropoiesis to take place. They are:

- a) erythropoietin - this is a hormone stimulating the production of erythrocytes.
- b) amino acids
- c) copper - needed for the absorption of iron and maturation of the erythrocytes.
- d) vitamin C - necessary for iron absorption.
- e) vitamin B₆ - important for the synthesis of heem.
- f) vitamin B₁₂
- g) folic acid
- h) iron - needed in the formation of hemoglobin.
- i) cobalt - forms part of vitamin B₁₂.
- j) hormones - certain hormones i.e. thyroxine and testosterone increase the erythropoiesis process.

2.4. CONCLUSION OF LITERATURE

Vitamin B₁₂ and folic acid are vitally important for the normal development of the body and its cells. The most well known feature of a deficiency in these vitamins is the effect on the production and maturation on the erythrocytes in the bone marrow. Large or megaloblastic erythrocytes are formed and found in the blood. These cells have a shorter life span than healthy erythrocytes. Fewer erythrocytes are also produced and therefore the erythrocyte count in blood is lower than normal. In order to evaluate these conditions the abnormal values will be measured and discussed. However, in order to do this a sound physiological basis to which these values can be compared is needed. Therefore the physiological reference ranges will have to be determined.

Chapter 3

MATERIALS and METHODS

A variety of methods were used and various substances and solutions had to be mixed and prepared for use. What follows is a description of these processes together with their uses.

Part 1

3-1. BLOOD ANALYSIS AND INSTRUMENT DESCRIPTION

3-1-1. INTRODUCTION: The Electronic Blood Cell Counter

There are many semi-automated electronic blood cell counters available today varying considerably in their capabilities. The counters range from relatively simple counters able to count one type of particle to sophisticated counters capable of determining erythrocytes, leucocytes and thrombocyte counts as well as the hemoglobin level, packed cell volume/hematocrit, mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), thrombocrit, thrombocyte distribution width, erythrocyte distribution width, thrombocyte size and differential white cell count (Cronje, 1987).

The introduction of electronic counters has minimized human error and ensures accurate and reliable results. More blood specimens can also be determined and the lengthy and tedious manual operation is eliminated.

There are many various makes of electronic counters available today and results are obtained rapidly. This makes it possible to analyze more specimens than would be possible via the manual method. Thousands of cells can be counted in comparison to the hundreds or less using manual methods.

One of two basic principles is used by these counters, namely:

- a) the voltage impedance principle
- b) the flow cell-dark field principle

The Coulter Counter used is based on the voltage impedance principle.

3.1.2. The Voltage Impedance Principle:

Via a vacuum pump, a mercury manometer syphon creates a vacuum in a special aperture tube which has a small opening near the bottom. Platinum electrodes are situated in and outside this tube. These electrodes are sensors that start cause the start and stop of the analyses procedure. A sample vial containing the test substance diluted with Isoton is placed in the operating position and the aperture tube is immersed in the vial. An electric current flows between the two electrodes via the electrolyte solution through the aperture (Cronje, 1987).

The counter counts pulses in a standard electrolyte volume as interferences in the constant voltage flow occur. Mercury flows through the manometer which causes the same amount of electrolyte to be drawn from the sample vial through the aperture. From a specific dilution, a specific volume of blood is drawn through the aperture in a given time. The cells pass through the aperture, causing a voltage drop which is counted as an impulse. From this the counter can determine the actual size of the particle. The particles/cells can then be counted within a predetermined size range by setting a cut off threshold on the counter. This is done by setting the threshold dial controls. Thus cell fragments, dust particles and thrombocytes are not counted on an aperture set for counting erythrocytes. The count is then expressed as the amount of specified cell type per liter of whole blood (Platt, 1979; Cronje, 1987).

3-1-3. The Coulter Counter

The instrument used was the Coulter Counter Model ZF. This counter employs the basic Coulter Principle to simultaneously count and size cells or particles suspended in an electrolytic solution. Each count starts at zero and is taken from a specified volume of suspension. The polarity of the aperture current reverses for each count to maintain reproducibility. A numeric display provides the count of cells of the chosen size increment.

3-1-3-1. Description of Coulter Counter:

The model ZF can be divided into two parts. The Sample Stand is used for the initial counting of the cells in suspension. The Electronic Counter analyzes the count and transforms it for numeric display.

The Sample Stand contains the:

- a) beaker platform
- b) aperture tube
- c) the stopcocks
- d) manometer
- e) vacuum pump
- f) control panel

The Electronic Counter contain the following:

- a) sizing monitor
- b) debris monitor
- c) numeric readout
- d) amplification, aperture current and threshold dials
- e) aperture current polarity lamps (POS, NEG)

At the rear of the unit an Accessory Output Connector and Signal Output Connector is situated for the connection of accessory equipment. Accessories used were the MCV/Hct accessory for determination of the mean cell volume (MCV) and hematocrit (Hct). A Coulter Hemoglobinometer was used to determine the hemoglobin content.

The principle of operation for the Coulter Counter is as follows:

When the stopcock is opened, a vacuum is formed in the instrument. This causes a sample to be drawn through the aperture which unbalances the manometer mercury causing it to flow past a start contact resetting the Electronic Counter to zero. When the stopcock is closed, the mercury moves back to its balanced position. The mercury still acts as a siphon and draws the sample through the aperture. As the mercury passes the start position, the electronic counting is started and when it passes the stop position, the count stops. The distance between the start and stop positions is calibrated to produce a reproducible sample volume.

3-1-3-2. The Coulter Erythrocyte/MCV/Hct Computer:

The computer works in conjunction with the Coulter Counter to automatically compute and numerically display the MCV, Hct and corrected erythrocyte count. The correction for the erythrocyte count done by the computer automatically compensates for count loss in the Coulter Counter due to more than one cell passing through the aperture at a time.

A Coulter Dual Diluter III was used for the dilution of the samples.

3·1·3·3. Coulter Dual Diluter III:

The diluter is a semi-automated portable instrument used for fast and precise dilutions. The diluter makes use of an electro-mechanical system which controls two separate dilution channels. One channel mixes blood/Isoton II in the 1:500 ratio and the other in a 1:100 ratio (for 1:50 000 dilutions). Isoton II is an azide free filtered isotonic diluent based on saline. Isoton II prevents cell size change which can cause wrong results.

The dilution ratio requirements for performing blood cell counts on the Coulter Counter are 1:500 for white blood cell and 1:50 000 for erythrocytes. The diluter uses a one dilution sequence for a 1:500 ratio and a second dilution sequence for a 1:50 000 ratio.

The dilution can be divided into two sequences:

- a) aspirate - blood is drawn into the diluter
- b) deliver - blood and Isoton are discharged from the aspirator tip and Isoton from the main flow tip.

The diluter is controlled by a touch bar on the front panel and a white blood cell/erythrocyte selector lever. A dispense indicator lights up to indicate when the instrument is ready to dispense. The diluter is an accurate ratio diluter and therefore the diluent amount dispensed is approximately 20 ml.

3·1·4. Blood Collection and Analyses:

Blood samples were collected from volunteer students by venous puncture, classified according to age and sex, and donors of the Western Province Blood Transfusion Service. The blood was immediately placed in EDTA anti-coagulation test tubes. Analyses of the samples took place within 3 hours after collection via the Coulter Counter model ZF.

3.1.4.1. Dilution of Blood:

The samples were first diluted using the Coulter Dual Diluter III. The diluter was charged with Isoton II. The selection lever was set to the white blood cell position. A whole blood sample test tube was placed under the aspirator tip immersing it into the blood. Blood was drawn up into the aspirator via operation of the touch bar. The sample test tube was removed and the aspirator tip wiped with a tissue to prevent blood contamination with other samples. The dispense lights up to signal readiness for the next stage.

An exact reproducible volume, approximately 20 ml, of diluted sample containing blood and Isoton was then dispensed into a clean accuvette. As the solution is dispensed an identical amount of Isoton is sucked from a reserve bottle to replace the dispensed Isoton. The diluter is therefore immediately ready for another dilution sequence.

This is a 1:500 dilution used for white blood cell counting after the addition of Zap-oglobin. Zap-oglobin hemolyzes the erythrocyte, releases the hemoglobin and converts it to cyanmethemoglobin for direct reading on the Coulter Hemoglobinometer.

For the erythrocyte dilution (1:50 000) the selection lever was moved to the erythrocyte position. The accuvette with the white blood cell dilution was placed under the aspirator tip and (this is done before Zap-oglobin is added to the white blood cell dilution) a certain volume of the white blood cell dilution was drawn up into the aspirator. An exact reproducible volume, approximately 20 ml of diluted sample, was then dispensed into a clean accuvette. This forms the 1:50 000 erythrocyte dilution.

The white blood cell dilution accuvette is a slight murky red colour due to the presence of many erythrocytes and hemoglobin, while the erythrocyte dilution accuvette is almost colourless due to the 1:50 000 dilution causing the erythrocytes to be spread out thinly in the dilution.

When the selection lever is moved to the erythrocyte position, a 1:100 dilution is made of the white blood cell dilution (1:500). Together a 1:50 000 dilution is therefore made. After addition of Zap-oglobin to the white blood cell dilution the fluid turns a clear red after it is shaken. This is due to the hemolysis of the erythrocytes and released hemoglobin.

3-1-4-2. Coulter Counter Analyses Procedure:

The Coulter Counter and Coulter Erythrocyte/MCV/Hct Computer was switched on and allowed to warm up for a few minutes. The threshold dial was set at 10, the amplification at 0,707 and the aperture current at 1.

On the front panel is a debris monitor which provides a view of the aperture. This allows a check on the aperture for blockages. The threshold setting determines the lower size limit of the count. The amplification adjusts the gain of the amplifier and the aperture setting controls the amount of current flow through the aperture. The sizing monitor is an oscilloscope which provides a pulse pattern of cells or particles passing through the aperture.

For operation a sample accuvette was placed on the beaker platform. The platform was raised till the aperture tube and external electrode were immersed in the blood dilution sample. The accuvette was placed on the platform so that an image of the aperture appeared on the debris monitor. (During the counting phase this screen must be monitored for any debris blocking the aperture. If the aperture gets blocked, the beaker platform can be lowered till the aperture tube is clear of the accuvette. The aperture can then be cleaned using a fine hair brush.) The control stopcock was opened and then closed as soon as cell pulses appeared on the oscilloscope.

After approximately 12 seconds the counter stops. The corrected erythrocyte count, MCV and hematocrit were then read from the computer. The erythrocyte count must be multiplied by the factor 10^4 to interpret results in cells per mm^3 .

This process was repeated 5 times for each sample accuvette to obtain the best average for each sample.

The aperture tube and external electrode was rinsed with Isoton between different samples to prevent carryover.

For white blood cell counts the process was the same. The only difference being that the reading was taken from the numeric display on the Coulter Counter itself.

Hemoglobin readings were done on the Coulter Hemoglobinometer. Samples used were the white blood cell dilution accuvettes. The reason for this was due to the usage of Zap-oglobin which hemolyzes the thus releasing the hemoglobin.

Part 2

3.2. ASSAY OF VITAMIN B₁₂

3.2.1. INTRODUCTION

The method followed for the determination of vitamin B₁₂ and folate was via the Quantaphase B₁₂/Folate Radioassay combination kit available from Bio-Rad. This kit is used for the simultaneous determination of vitamin B₁₂ and folate in serum or plasma.

3.2.2. Principle of the Vitamin B₁₂ Assay Procedure:

A serum or plasma sample is combined with vitamin B₁₂ (⁵⁷Co) and folate (¹²⁵I) in a solution containing dithiothreitol (DTT) and cyanide. This mixture is boiled to inactivate endogenous binding proteins and to convert the various forms of vitamin B₁₂ to cyanocobalamin. During the heating the folate and its analogues are stabilized by DTT. The mixture is cooled and then combined with immobilized affinity-purified porcine intrinsic factor and folate binding proteins. These additions adjust and buffer the pH of the reaction mixture to 9,2.

During incubation, the endogenous and labeled vitamins compete for the limited binding sites on the basis of their relative concentrations. The mixtures are then centrifuged and the supernatant is decanted. Labeled and unlabeled vitamins binding to the immobilized binding proteins are concentrated at the bottom of the tube in the form of a pellet. The supernatant containing the unbound vitamins is decanted and discarded. The radioactivity associated with the pellet is then counted. Standard curves are prepared using precalibrated vitamin B₁₂/folate standards in a human serum albumin (HSA) base. The concentrations of the vitamin B₁₂ and folate in the patient serum/plasma is then determined from these curves.

Test Components: (sufficient for 100 tests)

The test kit contains the following components:

- | | |
|------------------------------------|--|
| a) vitamin B ₁₂ /folate | 4 ml of a vitamin B ₁₂ /folate free |
| zero standard | HSA base and sodium azide (<0,5%). |

- b) **vitamin B₁₂/folate standards** 5 vials each containing 2ml of vitamin B₁₂ (cyanocobalamin) and PGA (pteroylglutamic acid) in HSA base with sodium azide (<0,5%) at levels equivalent to:
- vitamin B₁₂:**
- | | | | | | |
|-------|-----|-----|-----|------|-----|
| pg/ml | 100 | 250 | 500 | 1000 | 200 |
|-------|-----|-----|-----|------|-----|
- folate:**
- | | | | | | |
|-------|-----|-----|-----|----|----|
| ng/ml | 1,0 | 2,5 | 5,0 | 10 | 20 |
|-------|-----|-----|-----|----|----|
- c) **microbead reagent** 10,5 ml suspension of affinity purified porcine intrinsic factor and folate binding proteins covalently bound to polymer beads in phosphate buffer with sodium azide (<0,5%).
- d) **blank reagent** 3 ml of a polymer bead suspension in phosphate buffer with sodium azide (0,5%).
- e) **dithiothreitol (DTT)** 1 vial containing lyophilized dithiothreitol.
- f) **vitamin B₁₂/folate tracer** 100 ml of < 2 μ Ci of radioactively labeled vitamin B₁₂ (⁵⁷Co) as cyanocobalamin and < 8 μ Ci of radioactively labeled folate (¹²⁵I) in 0,05 M borate buffer with 10 μ g/ml of potassium cyanide.

Controls were also assayed with the samples and standards. In this case three controls (a, b and c) obtained from Tygerberg Hospital in Parow, were used.

3·2·3. ASSAY PROCEDURE

3·2·3·1. Collection of Blood and Storage:

Blood was collected as described in Section 3·1·4. Whole blood not used in the Coulter analysis was centrifuged at 4000 x g for 15 minutes. The plasma was drawn off and frozen in preparation for the RIA (radio-isotope assay) for vitamin B₁₂/folate.

3·2·3·2. Preparation of working tracer:

The working tracer was prepared as follows:

The DTT was mixed with 10 ml of distilled or deionized water and then gently shaken till dissolved. The activated solution was then left to stand for five minutes.

3·2·3·3. Assay Steps:

All reagents and specimens were allowed to come to room temperature. This is to prevent the inactivation of the binding proteins and to avoid inaccurate results.

Test tubes were labelled for each standard, blank, TC (total count), control and sample. The total amount of test tubes used was 98. There were 3 TC, 5 standard, 3 blank, 3 control and 84 sample test tubes used. To each standard, control and sample, 200 μ l of the appropriate substance was added and 200 μ l of zero standard was added to the blank tubes. To all the tubes, 1,0 ml of working tracer was added. The tubes were vortexed. The TC tubes were prepared by adding 1,0 ml of working tracer only to the test tubes. All the tubes were placed in boiling water (100 °C) in a rack. The tubes were then incubated for a minimum of 20 minutes and then allowed to cool to room temperature by being placed in cold water. Each tube, except

the blank and TC tubes, then received 100 μ l of the microbead agent. The blank tubes each received 100 μ l of the blank reagent. All tubes were then vortexed. The tubes were incubated for one hour after which they were centrifuged for 10 minutes at 1 500 x g. The supernatant was then decanted and discarded. All the tubes were counted for one minute on a gamma counter.

The assay procedure can be displayed as follows:

Number of

Test Tubes: (98)	3	5	3	3	84
	<u>TC</u>	<u>Standard</u>	<u>Blank</u>	<u>Control</u>	<u>Sample</u>
Appropriate	--	200 μ l	200 μ l	200 μ l	200 μ l

Addition

Working	1,0 ml				
----------------	--------	--------	--------	--------	--------

Tracer Reagent

Vortex all Test Tubes.

Place in boiling water (100°C) for 20 min.

Cool to room temperature in cold water.

	<u>TC</u>	<u>Standard</u>	<u>Blank</u>	<u>Control</u>	<u>Sample</u>
Microbead	--	100 μ l	--	100 μ l	100 μ l

Reagent

Blank	--	--	100 μ l	--	--
--------------	----	----	-------------	----	----

Reagent

Vortex all Test Tubes.

**Incubate all test tubes for 1 hour
at room temperature.**

**Centrifuge the test tubes for
10 minutes at 1 500 x g.**

**Decant and discard the supernatant
from the test tubes.**

Count all tubes for 1 minute in a gamma counter.

Part 3

3.3. PREPARATION OF HEALTHY AND PERNICIOUS ANEMIC BLOOD AND BONE MARROW SMEARS

3.3.1. INTRODUCTION:

For the microscopic study of bone and blood, samples were taken from patients with pernicious anemia and normal healthy subjects. The blood and bone samples were mounted on glass slides and coloured with Romanowsky stains. The normal peripheral blood smear was coloured with Leishman's stain and the normal bone marrow and abnormal peripheral blood and bone marrow were coloured with the May-Grünwald-Giemsa stain. Both these two stains fall under the Romanowsky type of stains.

3.3.2. Description and Preparation of Stains Used:

A Romanowsky stain is made up of an acidic (eosin) dye and a basic (methylene blue) dye (Dacie and Lewis, 1975). The mechanism whereby certain components of a cell's structure stain with particular dyes and other components do not stain depends on complex differences in chemical structure and affinities (Dacie and Lewis, 1975). With Romanowsky dyes, the acidic groupings of the nucleic acids and proteins of the cell nuclei and primitive cytoplasm determine their uptake of the basic dye methylene blue. The presence of basic groupings on the hemoglobin molecule results in its affinity for acidic dyes and its staining by eosin (Dacie and Lewis, 1975). In other words, Romanowsky dyes are sensitive to the hydrogen ion concentration in certain structures of a cell. The various types of Romanowsky dyes differ in the ratio mixture of eosin to methylene blue.

3.3.2.1. Preparation of Leishman's Stain:

Leishman's stain is a type of Romanowsky stain widely used for the staining of blood smears. The Leishman stain was prepared as follows:

To a conical flask of 200 - 250 ml capacity, 0,2 g of the powdered dye was weighed out and added. To this, 100 ml of methanol was added and the mixture was heated to 50°C for 15 minutes and shaken occasionally. The solution was filtered and allowed to stand before use (Dacie and Lewis, 1975).

3·3·2.2. Preparation of the May-Grünwald-Giemsa Stain:**May-Grünwald stain:**

In a conical flask of 200 - 250 ml capacity, 0,3 g of the powdered dye was added. To this, 100 ml of methanol was added and the mixture was warmed to 50°C. The flask was cooled to room temperature and shaken several times. After standing for 24 hours, the solution was filtered and ready for use (Dacie and Lewis, 1975).

Giemsa's stain:

A mass of 1,0 g of the powdered stain was weighed out and placed into a conical flask. To this, 66 ml of glycerol was added and the mixture was heated at 56°C for 90 minutes. Then 66 ml of methanol was added and after thoroughly mixing the solution it was allowed to stand for 7 days at room temperature before being filtered and being ready for use (Dacie and Lewis, 1975).

3·3·2.3. Preparation of Buffer:

The buffer used in this case was Sørensen's phosphate buffer and was prepared as follows:

The buffer was made up from two components, KH_2PO_4 and Na_2HPO_4 . These two components were mixed in a ratio of 50,8 : 49,2 respectively to achieve a desired pH of 6,8. Then 50 ml of the buffer was made up to 1,0 l of water (Dacie and Lewis, 1975)

3·3·3. COLLECTION OF SPECIMENS

3·3·3·1. Collection of Peripheral Blood Smears:

Pernicious anemic blood was obtained from patients suffering from a vitamin B₁₂ deficiency at Tygerberg Hospital. Healthy normal blood was obtained from apparently healthy volunteers via the venous puncture technique. The area of puncture was the median-basilic vein of the antecubital fossa in the bend of the elbow. The area was cleaned with an alcohol sponge. A tourniquet was placed about 2 inches above the puncture site on the upper arm. A 10 cc. regular disposable syringe was inserted into the vein and used to draw the blood. The tourniquet was released and the syringe was removed. The blood was used within the hour of removal, thereafter the blood starts to deteriorate showing signs of crenation and spherocytosis. A dry smear of the blood was made on a glass slide and stained with a Romanowsky stain.

3·3·3·2. Collection of Bone Marrow:

Bone marrow was obtained by needle aspiration from the posterior superior iliac spine by a medical practitioner (Dacie and Lewis, 1975; Platt, 1979). Bone marrow obtained via this method has a rusty-red colour and has a thick fluid-like consistency with varying amounts of fatty material and pale grey-white marrow fragments.

Various needle types are available for the puncture of bone for marrow samples. In this case the Jamshidi biopsy aspiration needle was used (Platt, 1979). This instrument is made up of three parts, namely the stylette, obturator and needle. The Jamshidi needle has an uniform external form with a constant internal diameter core, except for the tapered distal portion. The distal portion is beveled with a sharp cutting edge. The interior of the needle is used for the bone marrow collection. A syringe can be attached to the proximal section of the needle. The stylet is designed to fit into the needle and to project 1 to 2 mm beyond the needle tip for entry into the marrow. The needle used was the 4 inch, 8-gauge size.

3-3-4. STAINING OF BLOOD AND BONE MARROW SPECIMENS

3-3-4-1. Staining of Bone Marrow:

Particle smears were made from the bone marrow as follows:

A drop of the aspirate was put, near the end, on a slide. Most of the blood was sucked off with a fine Pasteur pipette. The marrow fragments adhere to the glass and therefore were not sucked off. A smear of the remaining blood and marrow fragments was made with a spreader, similarly as in the preparation of the peripheral blood smears. The marrow fragments are dragged behind the spreader and leave a trail of cells behind them.

3-3-4-1-1. Staining of Bone Marrow with the May-Grünwald-Giemsa Stain:

The bone marrow smears were air dried and then fixed by immersion in a jar containing methanol for 10 minutes. The smear was transferred to a staining jar containing May-Grünwald's stain freshly diluted with an equal volume of buffered water. After 5 minutes of staining, the smear was transferred to a jar containing Giemsa's stain freshly diluted with nine volumes of buffered water. After 10 minutes the smears were transferred to a jar of buffered water. The smears were washed in three to four changes of water and then allowed to stand undisturbed for 3 to 4 minutes in water for differentiation to take place. When differentiation was completed, the smears were stood upright and allowed to dry. The smears were then mounted with a coverslip using Depex as mounting liquid.

3.3.4.2. Staining of Blood Smears with Leishman's Stain:

A drop of blood was placed on the end of a clean slide. Using a spreader, the blood was then spread out towards the opposite end. The smear was allowed to air dry and then flooded with Leishman's stain. Buffered water was added after 30 to 60 seconds. The amount of buffered water added was double the amount of stain used. The water was allowed to mix with stain and staining took place for 10 minutes. The smear was then washed with distilled water until the smear achieved a pink colour. The stained smear was then set upright to dry. The dry stained blood smear was mounted with Depex and a coverslip.

A microscope equipped with an oil immersion lens at a magnification of 100x was used to investigate the smears.

3.4. PHOTOGRAPHIC PREPARATION

The camera-microscope used to take the photographs was the NIKON Microflex HFM with photomicrographic attachments. Basically it is a microscope with a camera attached to the top of the microscope.

3.4.1. Operation of Camera:

An Agfa ASA 100 colour film with 24 exposures was used and loaded into the camera in the usual way. The automatic dial on the shutter control box was set to 100 ASA. The eyepiece selection lever on the shutter box was pulled out. The diopter adjustment was set in till the double crosshairs of the ocular finder was clear. The lever was pushed back in. With the microscope eyepiece the desired object was looked for using the 10x lense on a slide placed on the slide platform. A drop of oil was then placed onto the slide and the 100x oil immersion lense was then used for higher magnification and finer detail. The eyepiece selection lever was pulled out and the object was then centered on the crosshairs and focussed. The rheostat for the

light source was set to ± 10 V and the blue filter with a medium NDF filter was selected. With the exposure time set between 1/8 and 1/60, the shutter was released and a photograph was taken. The camera automatically loads for another exposure. The photomicrographs were all taken at 1000x magnification except for photomicrograph 6 which was taken with a 100x magnification.

3.5. CONCLUSION OF MATERIALS AND METHODS

There are many different methods of obtaining blood and bone marrow. There are also different stains which can be used to stain blood and marrow specimens. The methods mentioned in this chapter were found to be satisfactory. The next chapter will show the results obtained with the help of the above mentioned processes.

Chapter 4

RESULTS

4.1. INTRODUCTION

In this chapter the results obtained using the techniques and methods described in Chapter 3 are presented and laid out. The results from the Coulter Counter and RIA are shown in table form as well as in graphic form. Statistics were derived from the tables to facilitate the comparison of results between healthy and pernicious anemic blood. The mean was calculated for each group of results per table, as well as the one standard deviation (1SD) and two standard deviations (2SD) and the minimum and maximum ranges. Photomicrographs were taken of healthy and pernicious anemic blood and bone marrow smears to visually show the effects caused by a vitamin B₁₂ deficiency in the blood.

Part 1

4.2. COULTER COUNTER AND VITAMIN B₁₂ ASSAY RESULTS OF HEALTHY AND PERNICIOUS ANEMIC BLOOD FROM MALES AND FEMALES.

Abnormal vitamin B₁₂ RIA readings were obtained from Tygerberg Hospital. Readings for a specific case of pernicious anemia was also received from the hospital. The incidence of pernicious anemia is very rare these days. Obtaining abnormal statistical values is therefore an ongoing task. In one year alone Tygerberg Hospital receives, on the average, approximately thirty to forty cases of pernicious anemia. This is a clear indication of the rarity of this disease, thanks to better dietary control, food education and awareness from society.

The average age of the healthy females from whom blood was collected was 21, the youngest being 18 and the oldest being 33. The average age of the healthy males was 22, of whom the youngest was 16 and the oldest 35.

It can be seen from the Tables that there are certain readings that are above or below normal. This is to be expected since reference values are relative to the group from which the readings were made. If the overall readings are taken into account the hematological pictures are normal.

Blood samples were analyzed from forty healthy male volunteers and thirty healthy female volunteers. Results were received for 13 pernicious anemic males and 21 pernicious anemic females from Tygerberg Hospital. The results obtained from the Coulter analyses only are displayed in Tables 4.1, 4.7, 4.13 and 4.14. Tables 4.1 and 4.7 are represented to show the values obtained for healthy males and healthy females and Tables 4.13 and 4.14 represent pernicious anemic males and females. Table 4.13 represent the values obtained from pernicious anemic females determined via the Coulter Counter and vitamin B₁₂ assay. The data for pernicious anemic males is shown in Table 4.14 and was determined by Coulter Counter and vitamin B₁₂ assay.

4.2.1. Coulter Counter Readings of Blood from Healthy Females:

The data obtained from 30 healthy females via the Coulter Counter is shown in Table 4.1.

TABLE 4.1. The erythrocyte, MCV, Hct, Hb, MCHC and MCH values of apparently healthy females between the ages of 18 and 33 years.

No.	Age	Erythrocyte ($\times 10^{10}$ /l)	MCV (fl)	Hct (%)	Hb (g/dl)	MCHC (g/dl)	MCH (pg)
1	25	401	99,5	41,2	12,6	30,6	31,4
2	21	685	99	68,8	15,3	22,2	22,3
3	19	525	60	32,5	14,6	44,9	27,8
4	22	529	77,4	47	14,8	31,5	28
5	21	454	91	42,5	13	30,6	28,6
6	21	500	97	49,1	14,6	29,7	19,2
7	21	549	89	50	12,8	25,6	23,3
8	33	520	78	42	14,9	35,5	29,5
9	21	505	96	49	14,9	30,4	29,5
10	19	506	93	47,9	15,2	31,7	30
11	18	512	87	45,9	12,5	27,2	29,7
12	19	450	89	41,4	12,4	30	27,6
13	18	360	99	37	12,2	33	33,9
14	18	426	87	36,4	12,8	35,2	30
15	18	429	95	42,5	13	30,6	30,3
16	19	381	102	40	11,9	29,8	31,2
17	23	405	97	40,5	13,2	32,6	32,6
18	19	391	96	38,8	12,8	33	32,7
19	20	378	80	30,3	9,2	30,4	24,3
20	18	381	95	37,4	12	32,1	31,5
21	20	403	96	39,7	10,7	27	26,6
22	21	361	80	32,3	12,1	37,5	33,5
23	21	360	92	34,2	11,4	33,3	31,7
24	21	329	96	32,5	11,7	36	35,6
25	21	372	99	38,3	12,8	33,4	34,4
26	31	453	91	42,6	14,7	34,5	32,5
27	18	497	86	44,3	12,9	29,1	26
28	31	391	97	39,1	12,1	30,9	30,9
29	20	427	99	43,8	12,6	28,8	29,5
30	23	458	129	61,3	10,1	16,5	22,1

From Table 4.1, it can be seen that the normal erythrocyte counts ranged from 329×10^{10} to 685×10^{10} /l for women, all within the reference limits. The MCV for the females varied between 60 and 129 fl and the average was 92,4 fl. The minimum Hct for the females was 30,3 % and the maximum was 68,8% with an average of 42,3%. The average Hb for the females was 12,9 g/dl with a minimum of 9,2 g/dl

and a maximum of 15,3 g/dl. The average MCHC for the females was 31,1 g/dl with a minimum of 16,5 g/dl and a maximum of 44,9 g/dl. The average MCH for the females was 29,5 pg with a minimum of 22,3 pg and a maximum of 35,6 pg.

4.2.1.1. Statistical Analyses of Healthy Female Values from Table 4.1:

The statistical analyses of the data from Table 4.1 is shown in Tables 4.2 and 4.3.

TABLE 4.2. Statistical explanation of the results from Table 4.1.

	Erythrocyte ($\times 10^{10}$ /l)	MCV (fl)	Hct (%)	Hb (g/dl)	MCHC (g/dl)	MCH (pg)
Mean	444,6	92,4	42,3	12,9	31,1	29,5
1SD	75,4	11,1	8	1,5	4,8	3,4
2SD	150,8	22,2	16	3	9,6	6,8
minimum	329	60	30,3	9,2	16,5	22,3
maximum	685	129	68,8	15,3	44,9	35,6

From the above Table the 1SD and 2SD ranges were set up and are displayed in Table 4.3.

TABLE 4-3. One and two standard deviations from Table 4-2.

	1SD range	2SD range
Erythrocyte (x 10 ¹⁰ /l)	444,6 ± 75,4 x 10 ¹⁰ /l	444,6 ± 150,8 x 10 ¹⁰ /l
MCV (fl)	92,4 ± 11,1 fl	92,4 ± 22,2 fl
Hct (%)	42,3 ± 8 %	42,3 ± 16 %
Hb (g/dl)	12,9 ± 1,5 g/dl	12,9 ± 3 g/dl
MCHC (g/dl)	31,1 ± 4,8 g/dl	31,1 ± 9,6 g/dl
MCH (pg)	29,5 ± 3,4 pg	29,5 ± 6,8 pg

From the Table above the following normal reference values using the 2SD and the mean values, were deduced:

For the erythrocytes the reference range was 293,8 x 10¹⁰ /l to 595,4 x 10¹⁰ /l. From Table 4-1 it can be seen that 29 (96,7%) of the 30 values fell within this range. The reference range for the MCV was 70,2 fl to 114,6 fl. From Table 4-1, of the 30 values 28 (93,3%) were inside this range. The Hct reference range was 26,3% to 58,3%. Of the 30 values in Table 4-1, 28 (93,3%) fell within the range. The reference range for Hb was 9,3 g/dl to 15,9 g/dl. From Table 4-1 it can be seen that 29 (96,7%) of the 30 values were within the range. The MCHC reference range was 21,5 g/dl to 40,7 g/dl. Of the 30 values in Table 4-1, 28 (93,3%) fell within the range. The reference range for the MCH was 22,7 pg to 36,3 pg. From the 30 values in Table 4-1, 28 (93,3%) were within the range.

4.2.1.2. RIA Readings of Serum Vitamin B₁₂ from Healthy Females:

Blood samples were collected from 30 apparently healthy females and analyzed. The results of the vitamin B₁₂ assay are displayed in Table 4.4 and statistical analyses thereof is shown in Tables 4.5 and 4.6.

TABLE 4.4. The RIA vitamin B₁₂ and folate values for apparently healthy females between the ages of 18 and 33 years.

No.	Age	Vitamin B ₁₂ (pg/ml)	Folate (ng/ml)
1	25	532,8	4,3
2	21	649,2	5,2
3	19	468,7	2,3
4	22	397,2	3,6
5	21	612,2	3,7
6	21	653,7	5,1
7	21	598,2	4,7
8	33	463,5	5,5
9	21	592,6	6,1
10	19	588,5	2,7
11	18	362,4	3,9
12	19	794,3	5,6
13	18	644,6	4,7
14	18	682,6	4,3
15	18	590,2	5,6
16	19	430,7	2,4
17	23	644,9	3,3
18	19	582,5	3,7
19	20	561,4	5,2
20	18	461,9	5,7
21	20	590,2	4,3
22	21	582,3	5,3
23	21	648,9	4,6
24	21	603,1	2,2
25	21	612,2	3,2
26	31	413,7	2,8
27	18	537,2	2,5
28	31	332,4	2,5
29	20	364,2	3,5
30	23	949,2	4,4

From Table 4.4 the vitamin B₁₂ results for apparently healthy females can be observed. All the readings fell within the reference range. The maximum vitamin B₁₂ reading was 949,2 pg/ml with a minimum of 332,4

pg/ml and the average was 564,9 pg/ml. The folic acid for the females was well within the reference range of 1,7 ng/ml to 6,5 ng/ml.

4.2.1.3. Statistical Analyses of Healthy Female Values from Table 4.4:

The statistical analyses of the data from Table 4.4 is shown in Tables 4.5 and 4.6.

TABLE 4.5. Statistical explanation of the results from Table 4.4.

	Vitamin B12 (pg/ml)	Folate (ng/ml)
Mean	564,9	4,1
1SD	128,5	1,2
2SD	257	2,4
minimum	332,4	2,2
maximum	949,2	6,1

From the above Table the 1SD and 2SD ranges were set up.

TABLE 4.6. One and two standard deviations from Table 4.5.

	1SD	2SD
Vitamin B₁₂ (pg/ml)	564,9 ± 128,5	564,9 ± 257
Folate (ng/ml)	4,1 ± 1,2	4,1 ± 2,4

From the Table above the following normal reference values using the 2SD and the mean values, were deduced:

The reference range for the vitamin B₁₂ level was 307,9 pg/ml to 821,9 pg/ml. Of the 30 values from Table 4-4 it can be seen that 29 (96,7%) were within the range. The folate reference range was 1,7 ng/ml to 6,5 ng/ml. None of the values in Table 4-4 were outside this range.

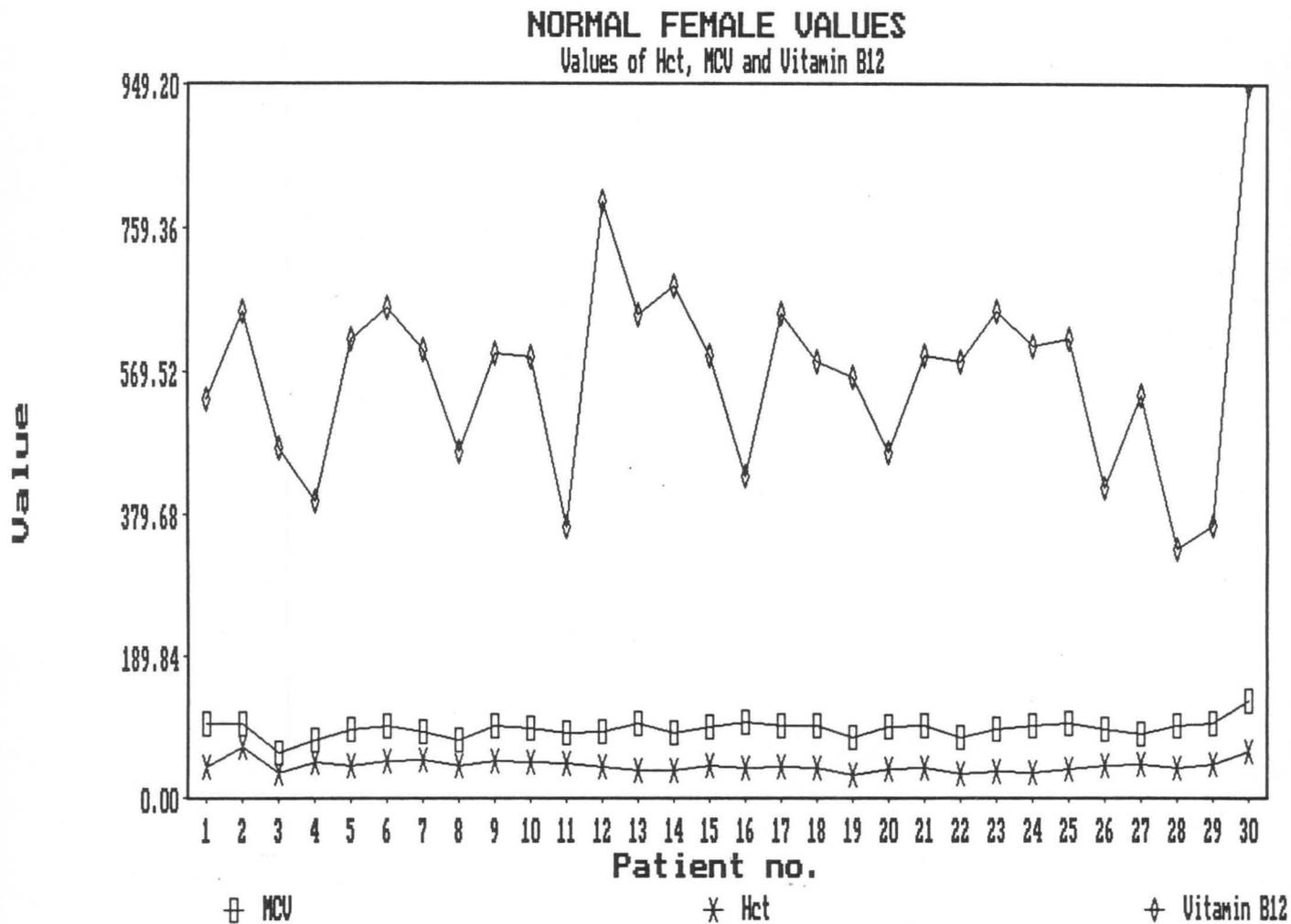


FIGURE 4-2-1. Graphic presentation of the Hct, MCV and vitamin B₁₂ values of 30 healthy females.

Figure 4.2.1 displays the Hct, MCV and vitamin B₁₂ values of healthy females. The values were obtained from Tables 4.1 and 4.4. The Hct values ranged between 26,3% and 58,3%. The values for the MCV all fell within the range of 70,2 fl to 114,6 fl. The vitamin B₁₂ values were within the range of 307,9 pg/ml to 821,9 pg/ml.

4.2.2. Coulter Counter Readings of Blood from Healthy Males:

The data obtained from 40 healthy males via the Coulter Counter is shown in Table 4.7.

TABLE 4-7. The erythrocyte, MCV, Hct, Hb, MCHC and MCH values of apparently healthy males between the ages of 16 and 35 years.

No.	Age	Erythrocyte ($\times 10^{10}$ /l)	MCV (fl)	Hct (%)	Hb (g/dl)	MCHC (g/dl)	MCH (pg)
1	22	521	94	50,5	15,1	29,9	29
2	19	517,5	91	48,4	16,1	33,3	31,1
3	23	523	94	52,4	16,1	30,7	30,8
4	23	595	89,5	49,5	16,8	33,9	28,2
5	22	476,5	85	42,5	14,5	34,1	30,4
6	22	543	84	47,3	15,7	33,2	28,9
7	25	687,5	100	71,1	18,2	25,6	26,5
8	24	661,5	90,5	62	15,7	25,3	23,7
9	21	744	88,5	68	16,5	24,3	22,2
10	18	505	98	49,5	16	32,3	31,7
11	19	575	89	53,4	15,7	29,4	27,3
12	27	538	95	53,1	17,5	33	32,5
13	23	549	93	52,7	14,9	28,3	27,1
14	26	505	100	102	16,1	15,8	31,9
15	16	483	64	37	13,3	35,9	27,5
16	35	513	84	44,4	15,3	34,5	29,8
17	24	517	87	47,5	16,6	34,9	32,1
18	21	522	107	57,3	16,6	29	31,8
19	21	427	73	31,9	14,2	44,5	33,3
20	21	462	74	36,1	15,1	41,8	32,7
21	20	404	83	33,6	14,2	42,3	35,1
22	22	428	93	40,7	15,5	38,1	36,2
23	18	410	96	40,6	13,5	33,3	32,9
24	22	414	101	43,5	14,6	33,6	35,3
25	22	551	97	54,9	15,2	27,7	27,6
26	19	440	96	44	14,8	33,6	33,6
27	23	392	99	40,8	14	34,3	35,7
28	21	433	100	44,5	15,3	34,4	35,3
29	21	448	95	43,8	14,9	34	33,3
30	25	498	99	50,9	15,8	31	31,7
31	21	444	97	44,3	13,3	30	30
32	19	456	97	45,3	13,6	30	29,8
33	21	428	102	44,8	13,9	31	32,5
34	20	436	94	42	15	35,7	34,4
35	27	411	97	41,4	13,6	32,9	33,1
36	20	446	94	43,7	15,3	35	34,3
37	26	400	78	32,2	14,7	45,7	36,8
38	22	487	47,4	47,4	15,1	31,9	31
39	28	436	46,5	46,5	13,9	29,9	31,9
40	23	445	35,7	35,7	14	39,2	31,5

From Table 4.7 it can be seen that the normal erythrocyte counts for healthy males ranged from 392×10^{10} to $744 \times 10^{10} /l$ which fell within the reference limits. The MCV varied between 64 and 107 fl with an average of 91,7 fl. The average Hct for the healthy males was 47,9% with a minimum of 31,9% and a maximum of 102%. The minimum Hb was 13,3 g/dl and the maximum was 18,2 g/dl with an average of 15,2 g/dl. The maximum MCHC for the healthy males was 45,7 g/dl with a minimum of 15,8 g/dl and an average of 32,8 g/dl. The minimum MCH was 22,2 pg with a maximum of 36,8 pg and an average of 31,3 pg.

4.2.2.1. Statistical Analyses of Healthy Male Values from Table 4.7:

The statistical analyses of the data from Table 4.7 is shown in Tables 4.8 and 4.9.

TABLE 4.8. Statistical explanation of the results from Table 4.7.

	Erythrocyte ($\times 10^{10} /l$)	MCV (fl)	Hct (%)	Hb (g/dl)	MCHC (g/dl)	MCH (pg)
Mean	491,8	91,7	47,9	15,2	32,8	31,3
1SD	78,4	9,2	12,1	1,1	5,4	3,2
2SD	156,8	18,4	24,2	2,2	10,8	6,4
minimum	392	64	31,9	13,3	15,8	22,2
maximum	744	107	102	18,2	45,7	36,8

From the above Table the 1SD and 2SD ranges were set up.

TABLE 4-9. One and two standard deviations from Table 4-8.

	1SD	2SD
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Erythrocyte ($\times 10^{10}$ /l)	491,8 \pm 78,4 $\times 10^{10}$ /l	491,8 \pm 156,8 $\times 10^{10}$ /l
MCV (fl)	91,7 \pm 9,2 fl	91,7 \pm 18,4 fl
Hct (%)	47,9 \pm 12,1 %	47,9 \pm 24,2 %
Hb (g/dl)	15,2 \pm 1,1 g/dl	15,2 \pm 2,2 g/dl
MCHC (g/dl)	32,8 \pm 5,4 g/dl	32,8 \pm 10,8 g/dl
MCH (pg)	31,3 \pm 3,2 pg	

From the Table above the following normal reference values using the 2SD and the mean values, were deduced:

For the erythrocytes the reference range was 335×10^{10} /l to $648,6 \times 10^{10}$ /l. From Table 4-7 it can be seen that 37 (92,5%) of the 40 values fell within this range. The reference range for the MCV was 73,3 fl to 110,1 fl. From Table 4-7, of the 40 values 37 (92,5%) were inside this range. The Hct reference range was 23,7% to 72,1%. Of the 40 values in Table 4-7, 39 (97,5%) fell within the range. The reference range for Hb was 13 g/dl to 17,4 g/dl. From Table 4-7 it can be seen that 39 (97,5%) of the 40 values were within the range. The MCHC reference range was 22 g/dl to 43,6 g/dl. Of the 40 values in Table 4-7, 38 (95%) fell within the range. The reference range for the MCH was 24,9 pg to 37,7 pg. From the 40 values in Table 4-7, 38 (95%) were within the range.

4.2.2.2. RIA Readings of Serum Vitamin B₁₂ from Healthy Males:

Blood samples were collected from 40 apparently healthy males and analyzed. The results of the vitamin B₁₂ assay are displayed in Table 4.10 and the statistical analyses thereof is shown in Tables 4.11 and 4.12.

TABLE 4.10. The RIA vitamin B₁₂ and folate values for apparently healthy males between the ages of 16 and 35 years.

No.	Age	Vitamin B ₁₂ (pg/ml)	Folate (ng/ml)
1	22	767,9	7,3
2	19	300,5	6,4
3	23	593,3	5,6
4	23	853,5	5
5	22	451,2	7,7
6	22	427,8	4,8
7	25	865,1	1,7
8	24	986,5	5,5
9	21	616,7	2,7
10	18	566,7	3,2
11	19	801,4	6,3
12	27	551,5	4,9
13	23	528,8	5,8
14	26	801,9	2,8
15	16	978,5	7,1
16	35	946,6	3,7
17	24	550,8	4,2
18	21	611,3	7,7
19	21	397,6	4,6
20	21	569	1,7
21	20	532,8	6,3
22	22	657,2	3,2
23	18	802,5	7,5
24	22	498,4	7,3
25	22	523,1	6,5
26	19	503	6,6
27	23	545,4	3,4
28	21	651,2	2,6
29	21	949,8	7,5
30	25	414,5	3,7
31	21	680	3,4
32	19	738,5	5,2
33	21	594,6	6,6
34	20	951,1	1,8
35	27	908,9	3,5
36	20	628,1	7,9
37	26	844,3	4,5
38	22	918,8	3
39	28	930,1	2,4
40	23	531	8,1

From Table 4.10 the vitamin B₁₂ results for apparently healthy males can be observed. All the readings fell within the reference range. The maximum vitamin B₁₂ reading was 986,5 pg/ml with a minimum of 300,5 pg/ml and the average was 764,2 pg/ml. The folic acid for the males was well within the reference range of 1,2 ng/ml to 8,8 ng/ml.

4.2.2.3. Statistical Analyses of Healthy Male Values from Table 4.10:

The statistical analyses of the data from Table 4-10 is shown Tables 4-11 and 4-12.

TABLE 4-11. Statistical explanation of the results from Table 4-10.

	Vitamin B₁₂ (pg/ml)	Folate (ng/ml)
Mean	764,2	5
1SD	186,3	1,9
2SD	372,6	3,8
minimum	300,5	1,7
maximum	986,5	8,1

From the above Table the 1SD and 2SD ranges were set up.

TABLE 4-12. One and two standard deviations from Table 4-11.

	1SD	2SD
Vitamin B₁₂ (pg/ml)	764,2 ± 186,3	764,2 ± 372,6
Folate (ng/ml)	5 ± 1,9	5 ± 3,8

From the Table above the following normal reference values using the 2SD and the mean values, were deduced:

The reference range for the vitamin B₁₂ level was 391,6 pg/ml to 1136,8 pg/ml. Of the 40 values from Table 4-10 it can be seen that 39 (97,5%) were within the range. The folate reference range was 1,2 ng/ml to 8,8 ng/ml. None of the values in Table 4-10 were outside this range.

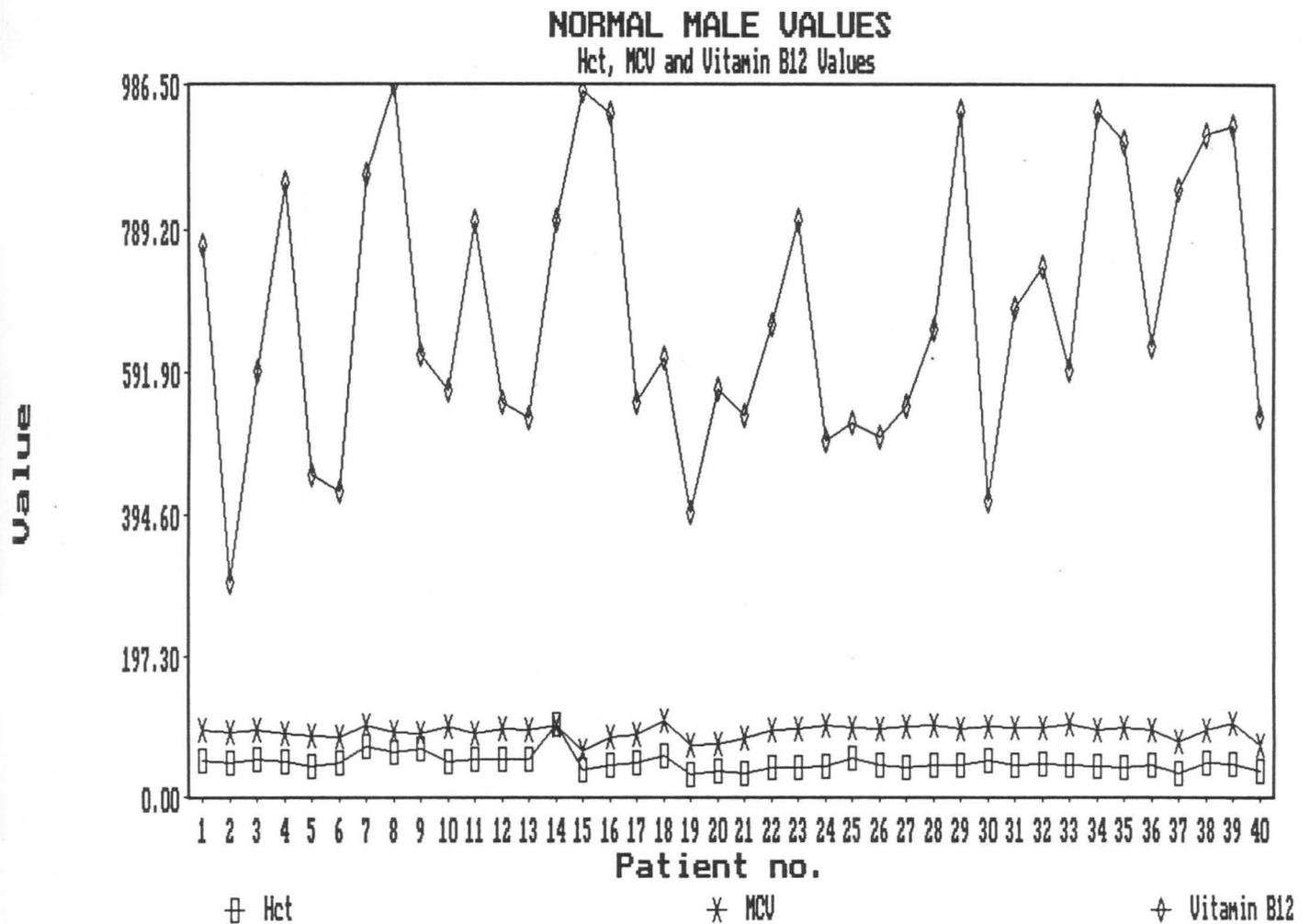


FIGURE 4-2-2. Graphic presentation of the Hct, MCV and vitamin B₁₂ values of 40 healthy males.

Figure 4.2.2 displays the Hct, MCV and vitamin B₁₂ values of healthy males. The values were obtained from Tables 4.7 and 4.10. The Hct values ranged between 23,7% and 72,1%. The values for the MCV all fell within the range of 73,3 fl to 110,1 fl. The vitamin B₁₂ values were within the range of 307,9 pg/ml to 821,9 pg/ml.

4.2.3. Coulter Counter and RIA Readings of Blood from Pernicious Anemic Females:

The results obtained from pernicious anemic females via the Coulter Counter and vitamin B₁₂ assay are displayed in Table 4.13.

TABLE 4-13. The MCV, Hct, folate and vitamin B₁₂ values of females suffering from pernicious anemia.

No.	Hct (%)	MCV (fl)	Vitamin B ₁₂ (pg/ml)	Folate (ng/ml)
1	27	103,9	116,3	5,4
2	18,8	107,8	147,6	5,3
3	23,7	112,7	109,5	5,4
4	21,4	111	104,6	15,3
5	19,1	105,4	140	3,3
6	24	105,7	82,3	6,8
7	29,2	109,8	120,1	5,3
8	21,2	110,7	153,3	2
9	23,3	149,9	54,4	4
10	26,6	116,4	65,2	7,2
11	22,5	106,5	139,6	6,3
12	17,6	113,3	37,6	7
13	19,1	107,2	51,2	22,1
14	20	123	83	4,7
15	18,6	130	71,9	4,9
16	16,2	127	150,8	34,3
17	23,7	130,2	97,8	2,2
18	14,1	125,3	159,1	13,9
19	15,3	121,1	146,2	17
20	12,7	123,7	141	4,8
21	20,1	116,6	156	10,8

4.2.4. Coulter Counter and RIA Readings of Blood from Pernicious Anemic Males:

The results obtained from pernicious anemic males via the Coulter Counter and vitamin B₁₂ assay are shown in Table 4.14.

TABLE 4-14. The MCV, Hct, folate and vitamin B₁₂ values of males suffering from pernicious anemia.

No.	Hct (%)	MCV (fl)	Vitamin B ₁₂ (pg/ml)	Folate (ng/ml)
1	19,3	138,5	138,8	54
2	15,9	129,3	126,2	7,2
3	17,7	115,9	133,9	4
4	21,1	118,7	131,4	2,9
5	23,2	132,6	139,3	17,9
6	15,6	129,2	160,5	1,7
7	14,5	134,8	125,2	6,8
8	19,2	126,1	58,6	8,8
9	21,3	109,9	141,8	14
10	14,7	115,3	160,8	7,4
11	13,1	123	71,6	8,7
12	11,9	107,2	52,9	5,3
13	14	119,6	102,7	1,4

From Table 4.14 the MCV, Hct and vitamin B₁₂ of males suffering from pernicious anemia are shown. The minimum Hct was 11,9% with a maximum of 23,2% and an average of 17%. The average MCV for the males was 123,1 fl with a minimum of 107,2 fl and a maximum of 138,5 fl. The average vitamin B₁₂ value was 118,8 pg/ml with a minimum of 52,9 pg/ml and a maximum of 160,8 pg/ml. All these readings were well outside the healthy parameters.

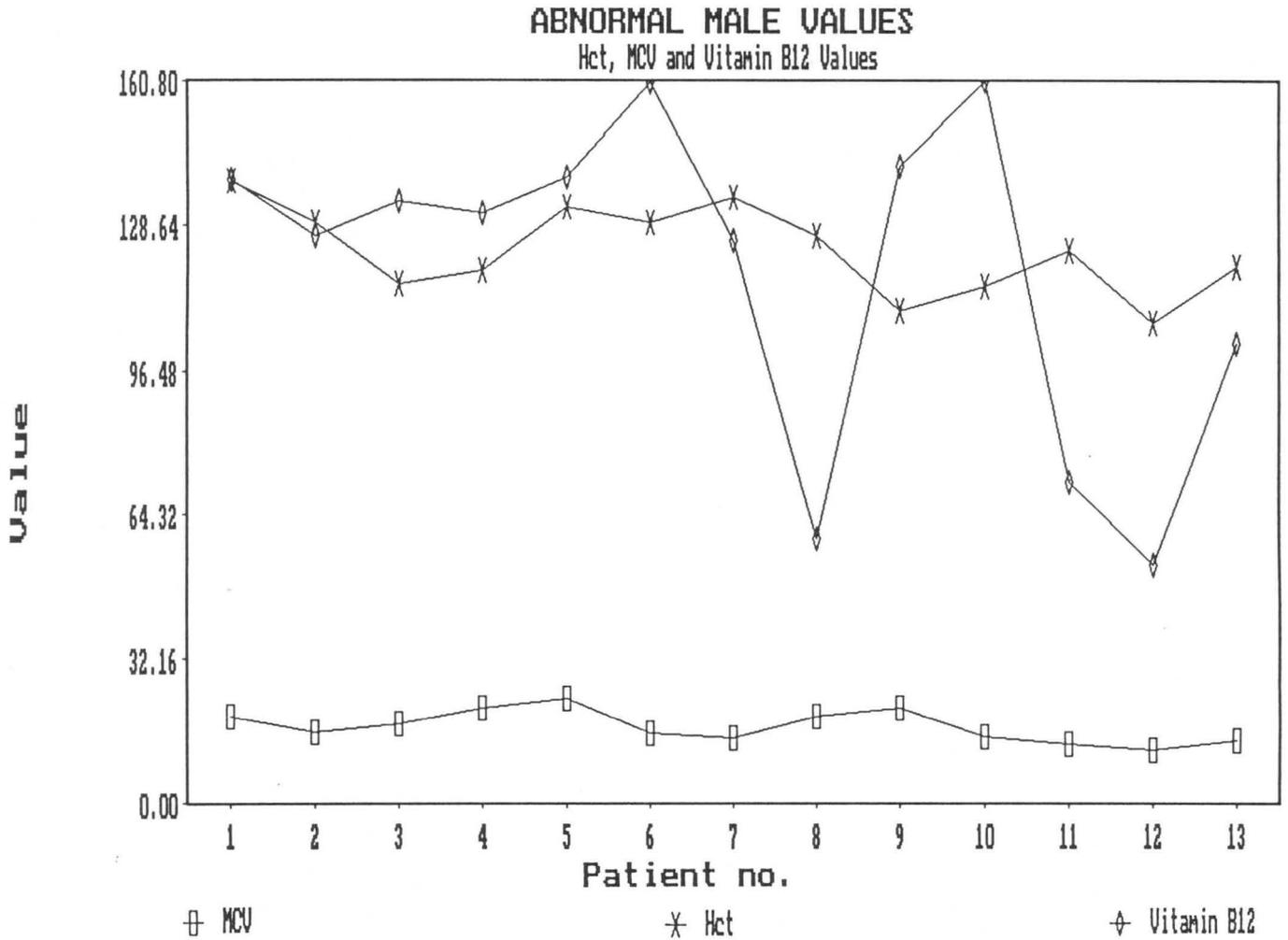


FIGURE 4.2.4. Graphic presentation of the Hct, MCV and vitamin B₁₂ values of 13 males suffering from pernicious anemia.

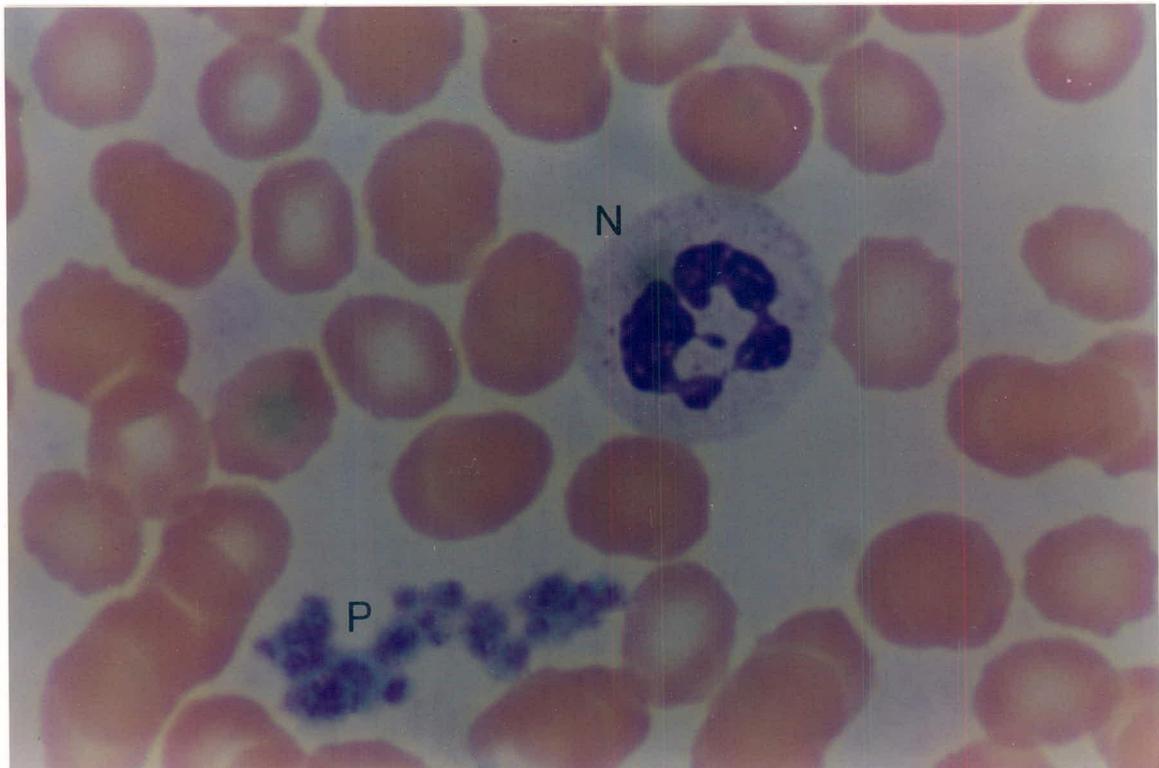
Figure 4.2.4 displays the Hct, MCV and vitamin B₁₂ values of pernicious anemic males. The values were obtained from Table 4.14. The Hct values ranged between 11,9% and 23,2%. The MCV values were in the range of 107,2 fl and 138,5 fl. The vitamin B₁₂ values were within the range of 52,9 pg/ml and 160,8 pg/ml.

Part 2

4.3. MORPHOLOGY OF THE BLOOD CELLS PRESENT IN HEALTHY AND PERNICIOUS ANEMIC BLOOD AND BONE MARROW SMEARS

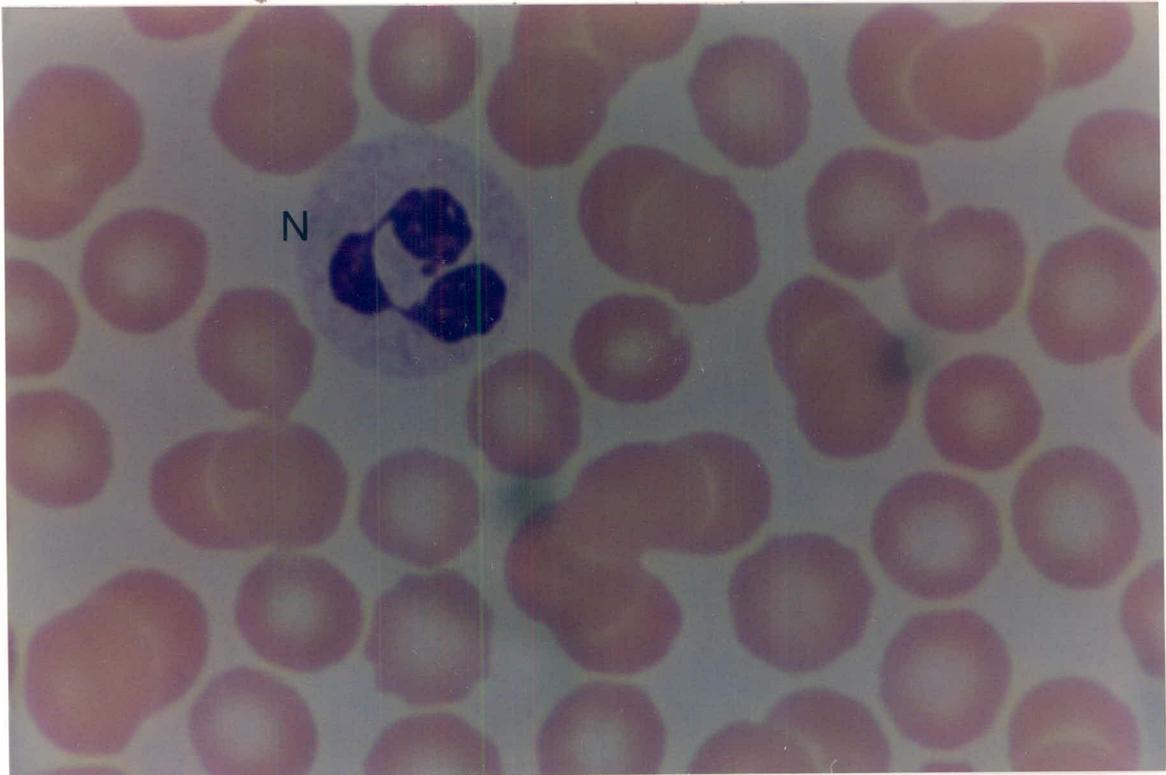
Photomicrographs were taken with a NIKON camera mounted on a NIKON microscope as explained in Chapter 3 to demonstrate the effect that pernicious anemia has on the blood cells as seen in peripheral blood and bone marrow smears. Photomicrographs were taken of healthy and pernicious anemic peripheral blood and bone marrow smears. The pernicious anemic blood and bone marrow was obtained from Tygerberg Hospital.

4.3.1. NORMAL PERIPHERAL BLOOD SMEARS FROM VOLUNTEERS.



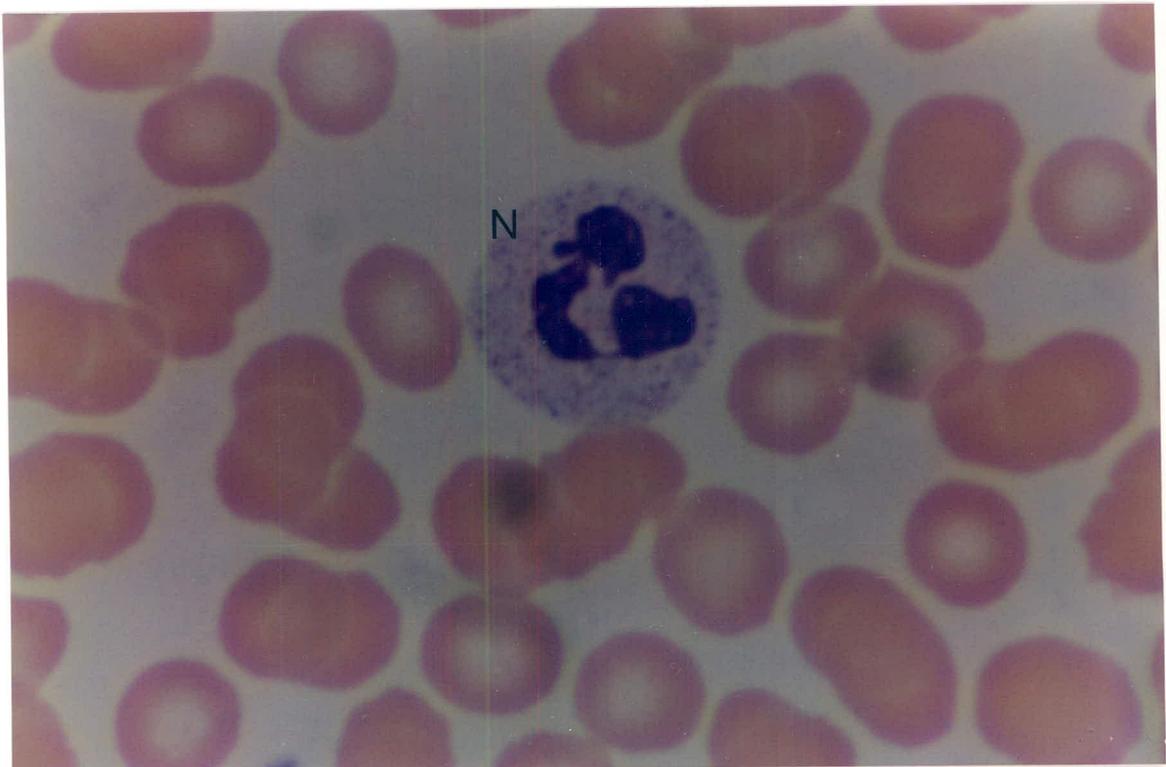
Photomicrograph 1:

A normal healthy neutrophil (N) with a group of platelets (P). (x1000)



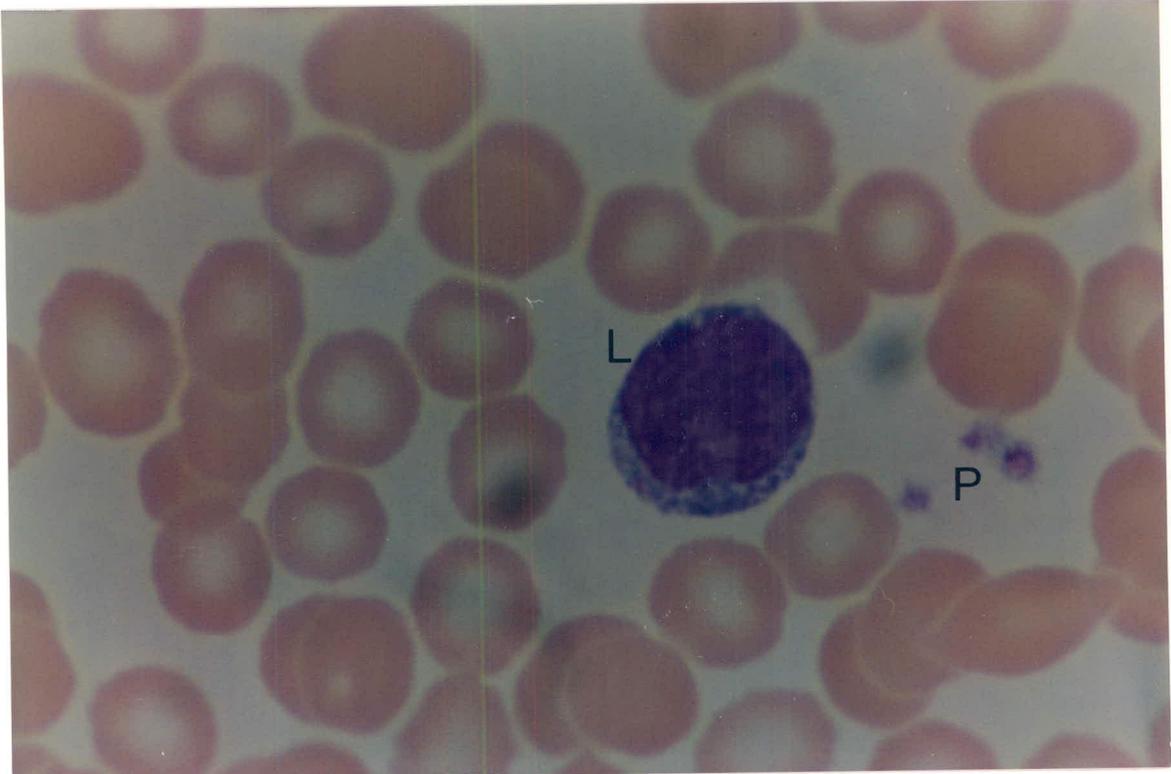
Photomicrograph 2:

A normal healthy neutrophil (N) with 3 nuclear lobes surrounded by erythrocytes. (x1000)



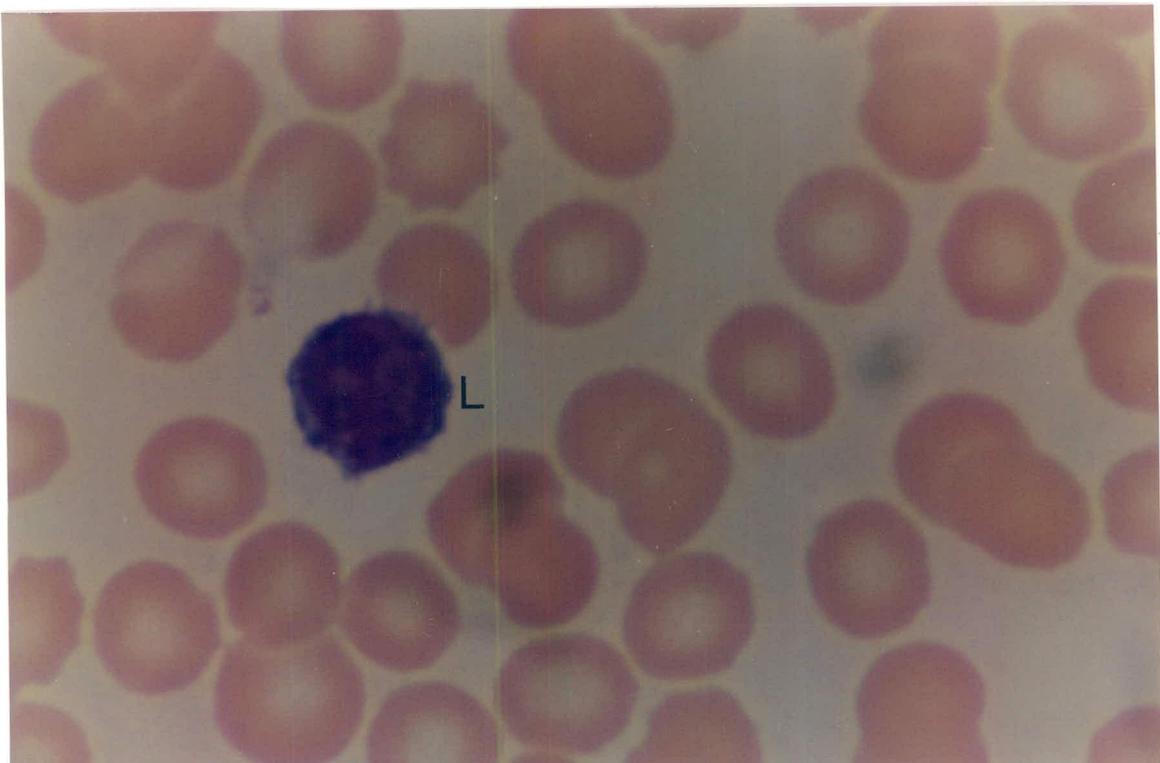
Photomicrograph 3:

A normal healthy neutrophil (N) surrounded by erythrocytes. (x1000)



Photomicrograph 4:

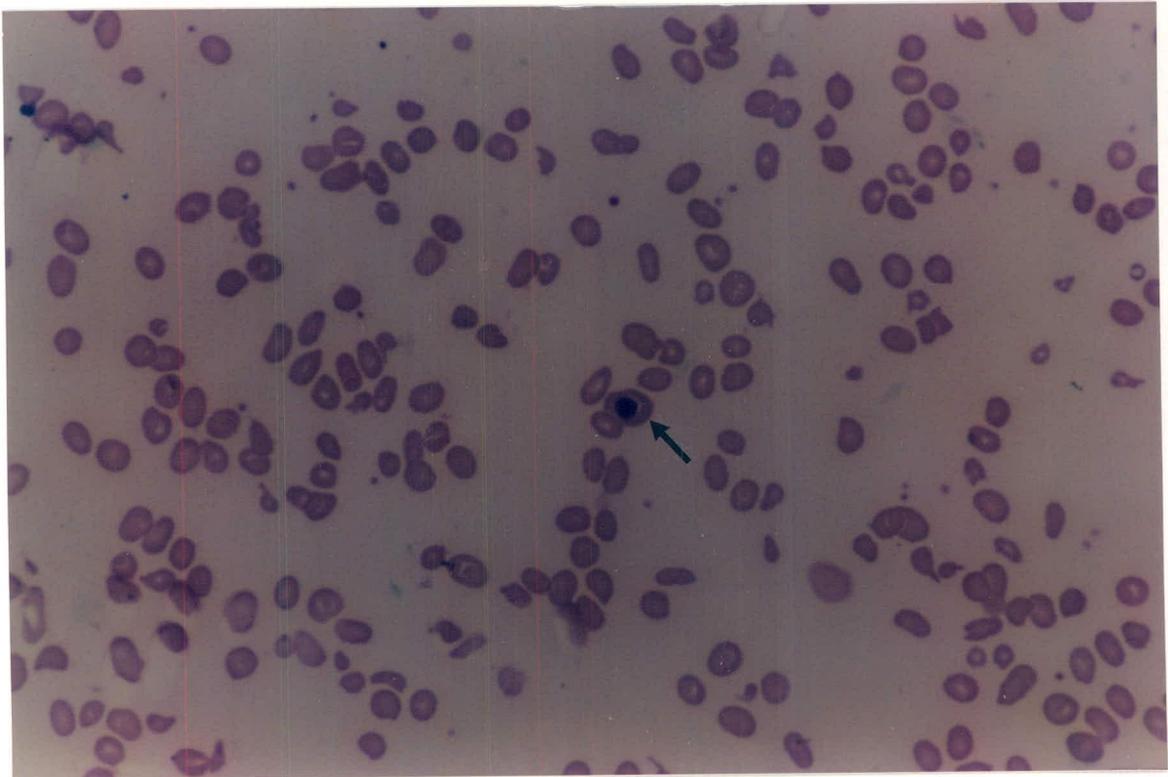
A large normal healthy lymphocyte (L) amongst normal erythrocytes. There is no sign of poikilocytosis or anisocytosis. A group of normal platelets (P) can also be seen. (x1000)



Photomicrograph 5:

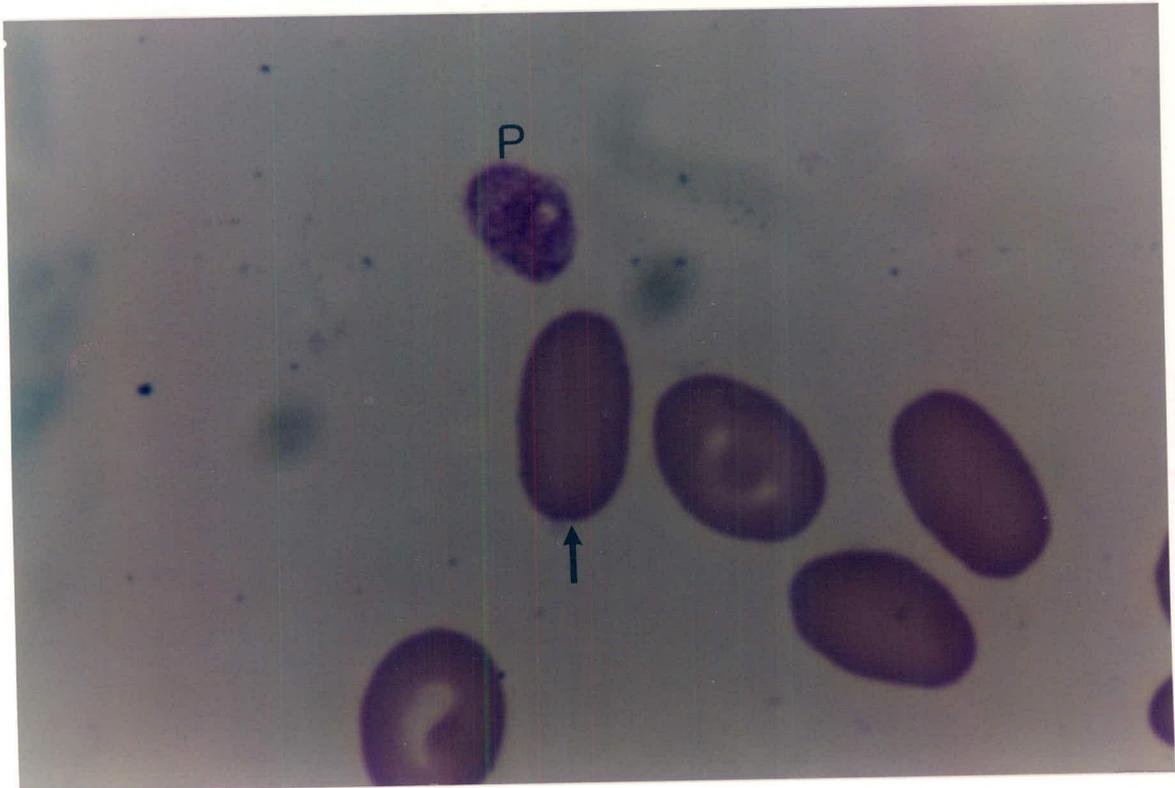
A small normal healthy lymphocyte (L). If the size of this cell is compared to the size of the normal erythrocytes, it can be seen that the erythrocytes are smaller than the lymphocyte. The erythrocytes are also uniform in shape and size (see photomicrograph 9). (x1000)

4-3-2. PERNICIOUS ANEMIC PERIPHERAL BLOOD SMEARS FROM PATIENTS



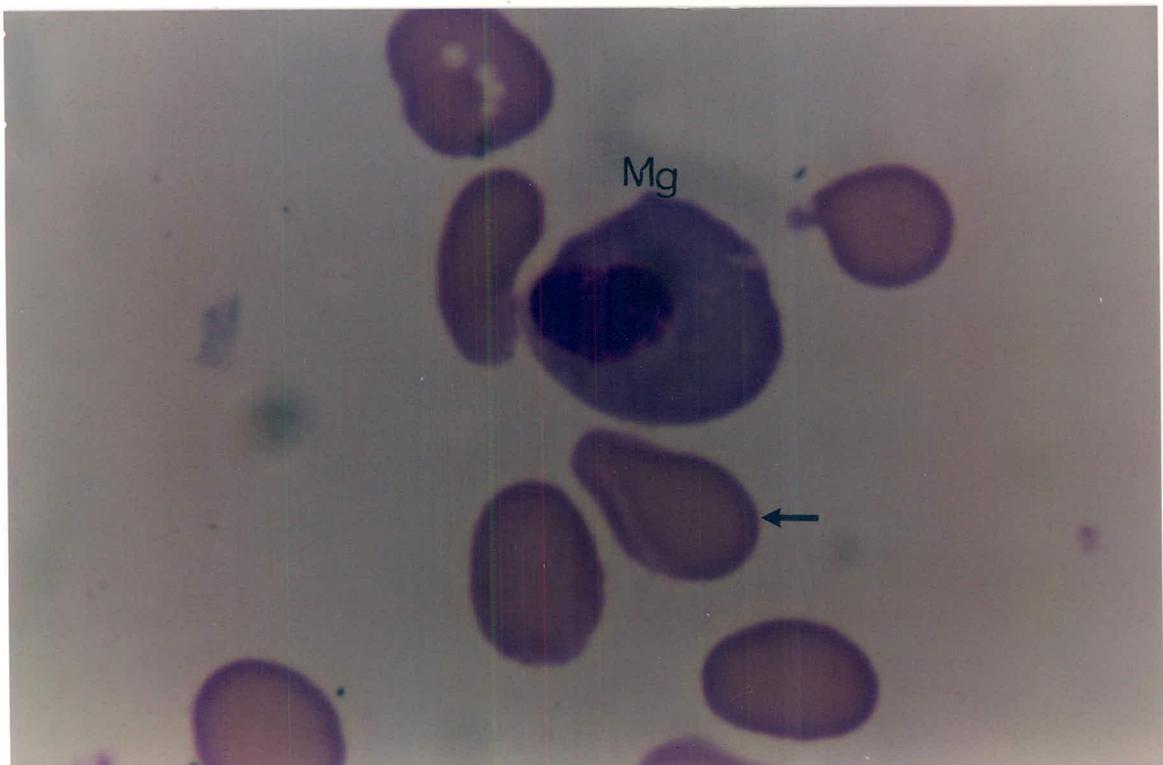
Photomicrograph 6:

An overall view of macrocytic blood showing irregularly shaped erythrocytes. A late megaloblast (↑) is also present still containing its nucleus. (x100)



Photomicrograph 7:

An abnormally large platelet (P) surrounded by erythrocytes showing signs of poikilocytosis (↑) present in pernicious anemic blood. (x1000)



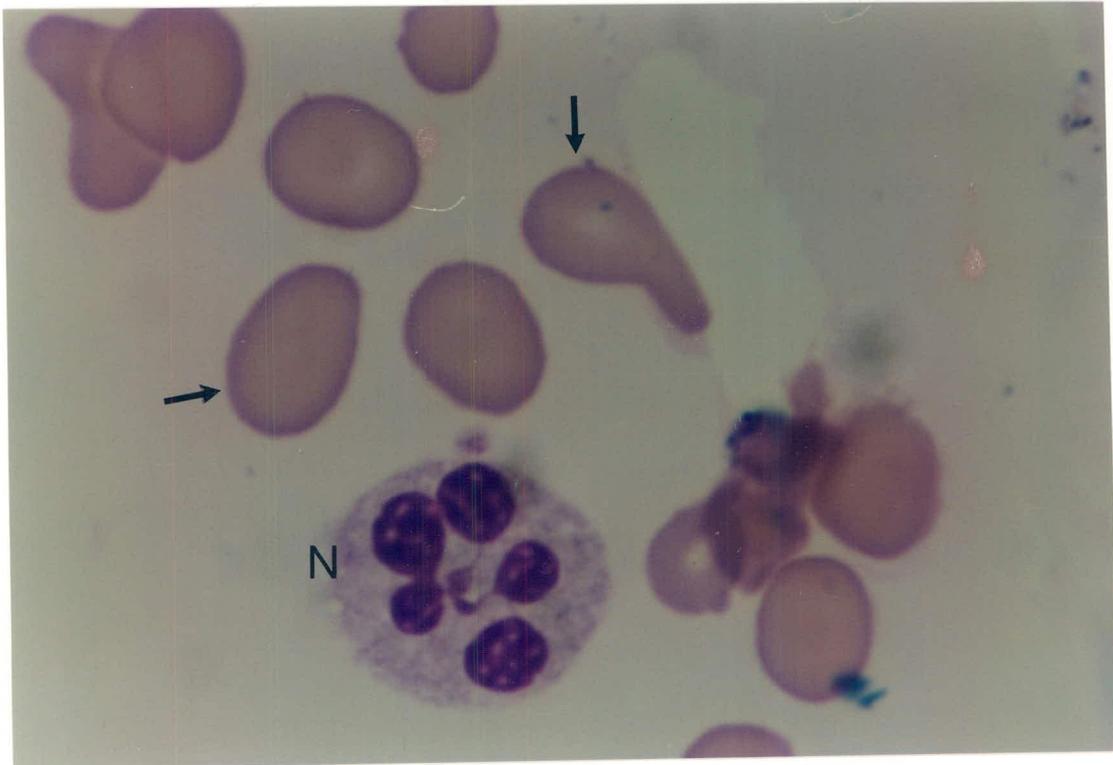
Photomicrograph 8:

An abnormally large megaloblast (Mg) with its nucleus. Also present are poikilocytic erythrocytes (↑) found in pernicious anemic blood. (x1000)



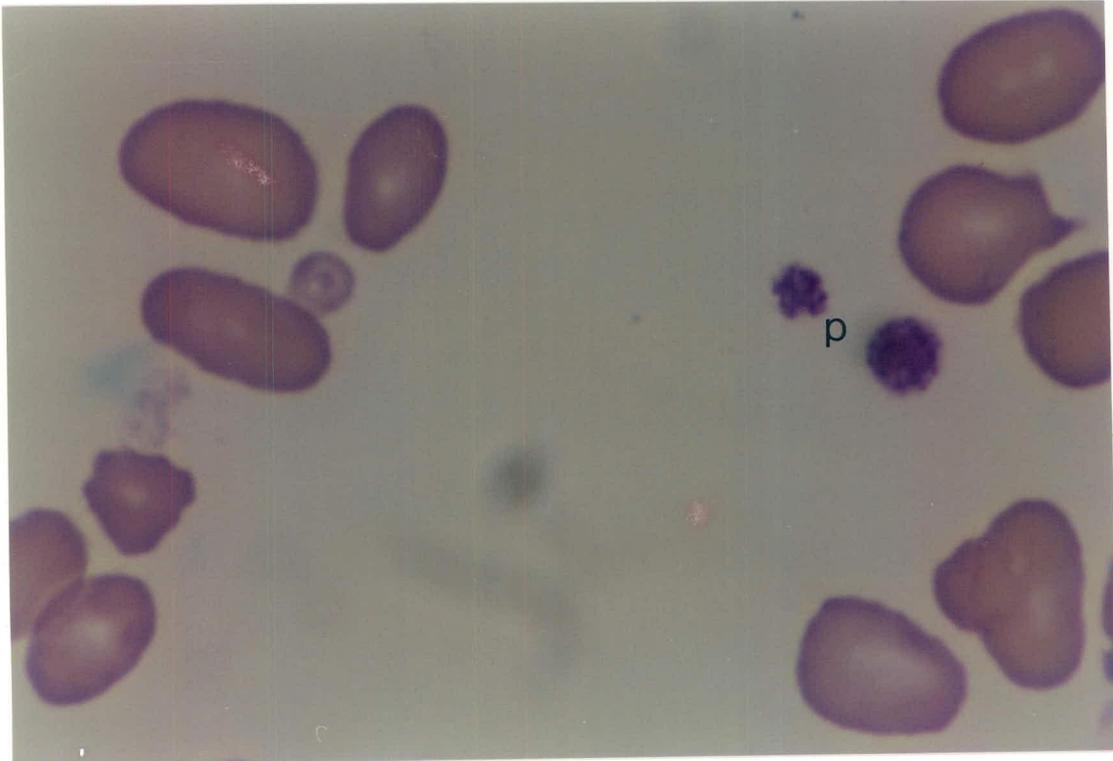
Photomicrograph 9:

A small lymphocyte (L) surrounded by abnormally large erythrocytes (↑) as seen in pernicious anemic blood. It can be seen that the erythrocytes show variation in size as well as in shape (↑). The erythrocytes shown here are clearly larger or equal to the lymphocyte's size. (x1000)



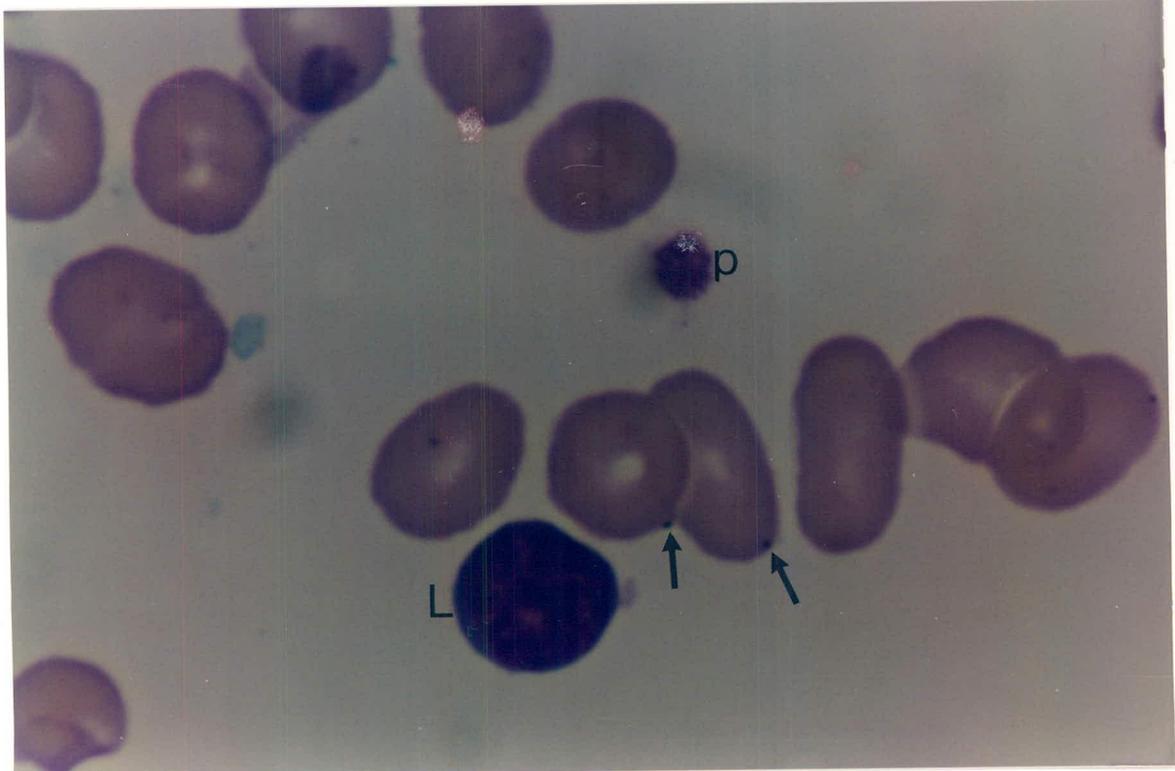
Photomicrograph 10:

An abnormal hypersegmented polymorphonuclear neutrophil (N) typically found in pernicious anemia. The neutrophil shown here has five segments of nuclear material. Also present are poikilocytes (↑) showing oval and pear-shaped erythrocytes. (x1000)



Photomicrograph 11:

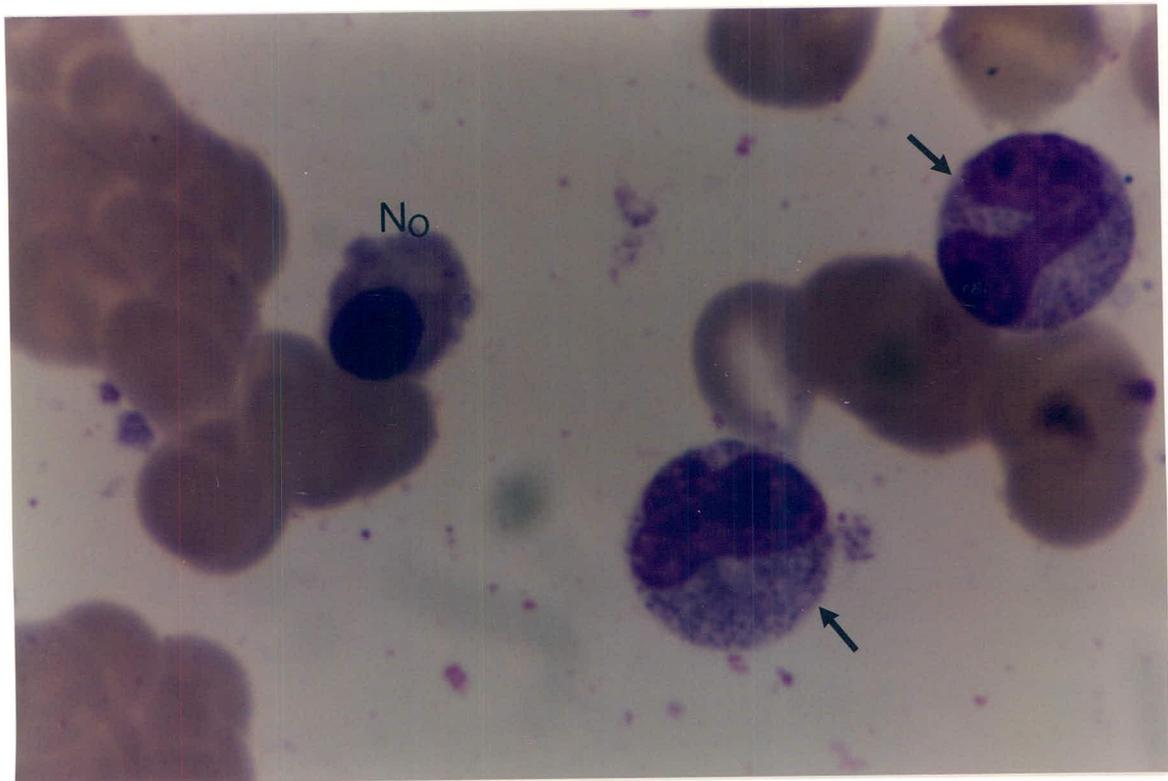
Abnormally large platelets (P) found in pernicious anemia. Compared to the platelets shown in photomicrographs 16 and 4, it can be seen that these platelets are abnormally large. Poikilocytosis and anisocytosis of the erythrocytes is also present. (x1000)



Photomicrograph 12:

A small lymphocyte (L), an abnormally large platelet (P) and abnormal large erythrocytes with poikilocytosis and anisocytosis found in pernicious anemic blood. The three erythrocytes (↑) above the lymphocyte contain Howell-Jolly bodies. (x1000)

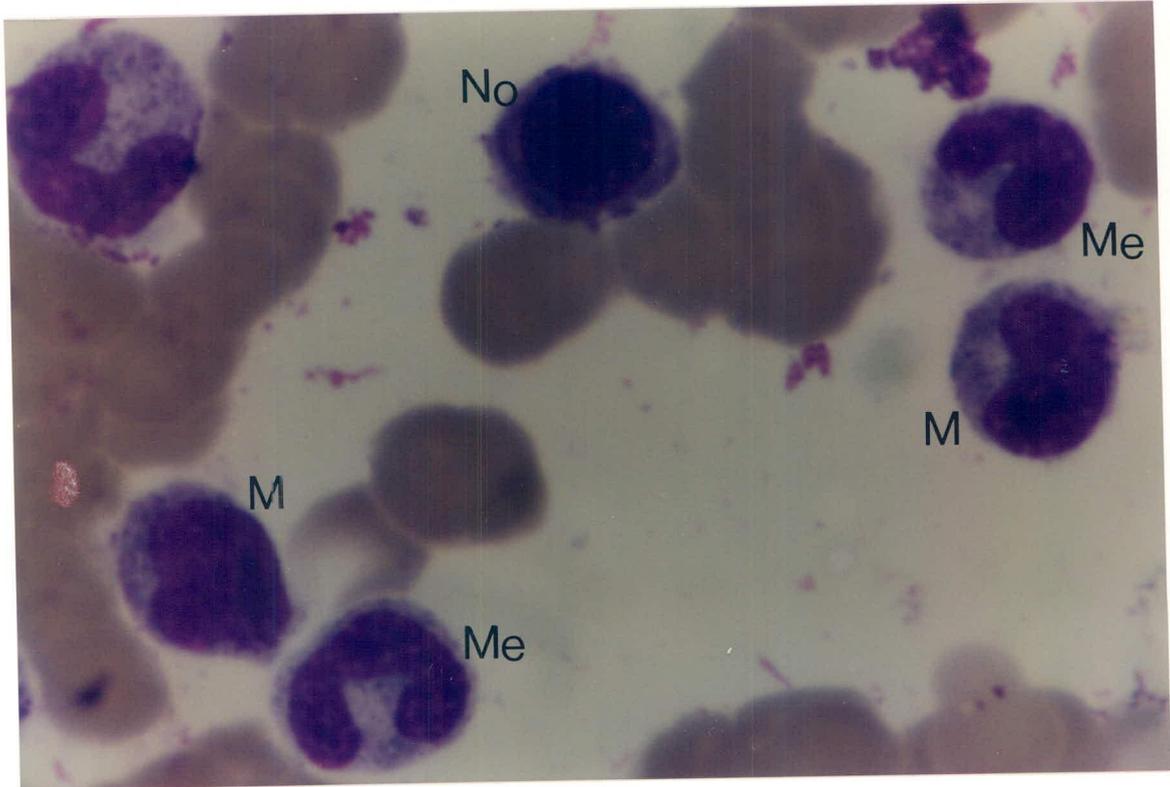
4-3-3. NORMAL BONE MARROW SMEARS FROM VOLUNTEERS



Photomicrograph 13:

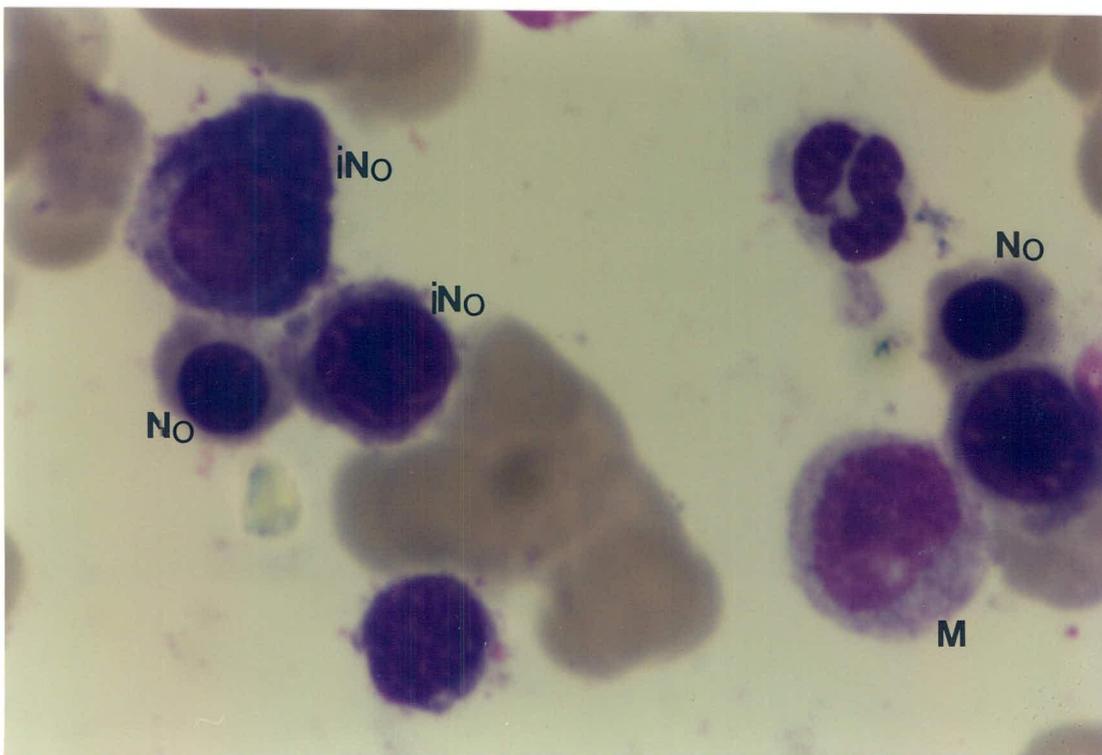
A normal late normoblast (No) with its eccentrically placed nucleus present in normal bone marrow.

Also present are two granulocytes (↑). (x1000)



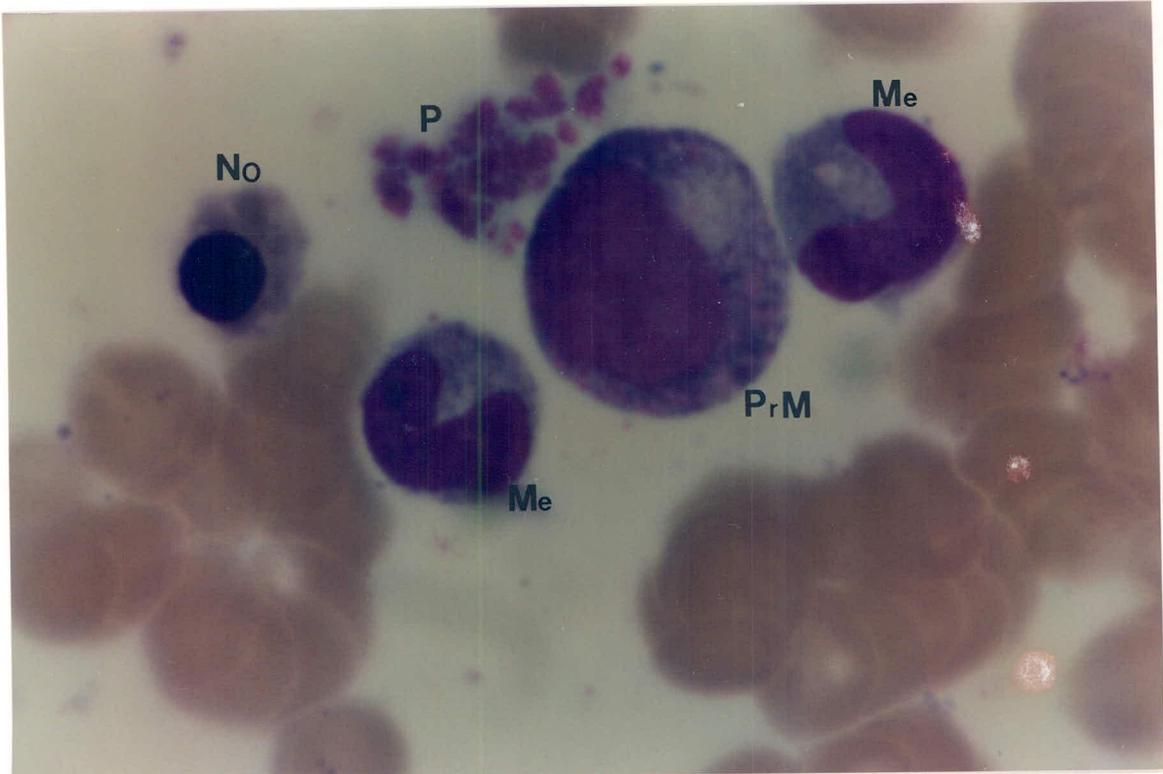
Photomicrograph 14:

One normal normoblast (No) is present. The rest are stages in granulocytic development. This includes metamyelocytes (Me) and myelocytes (M) as seen in normal bone marrow. (x1000)



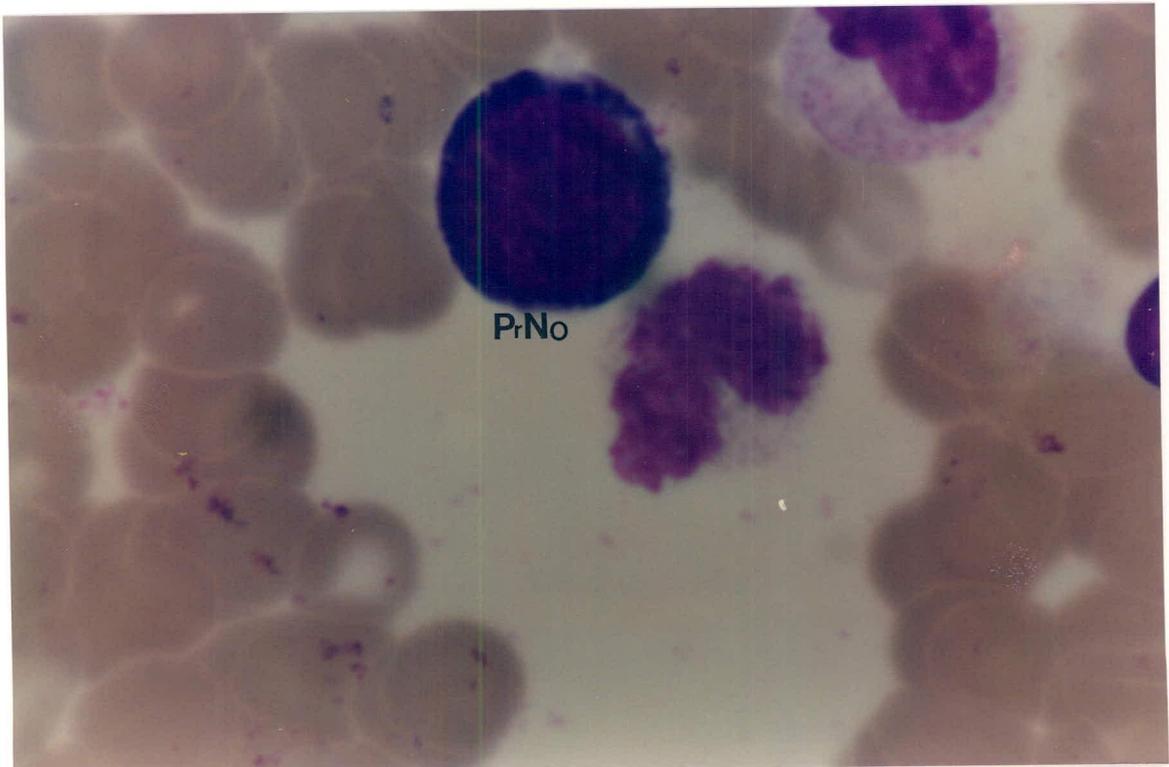
Photomicrograph 15:

Different stages of erythrocytic development in normal bone marrow. There are two normal late normoblasts (No) as well as intermediate normoblasts (iNo). A normal myelocyte is also present (M). (x1000)



Photomicrograph 16:

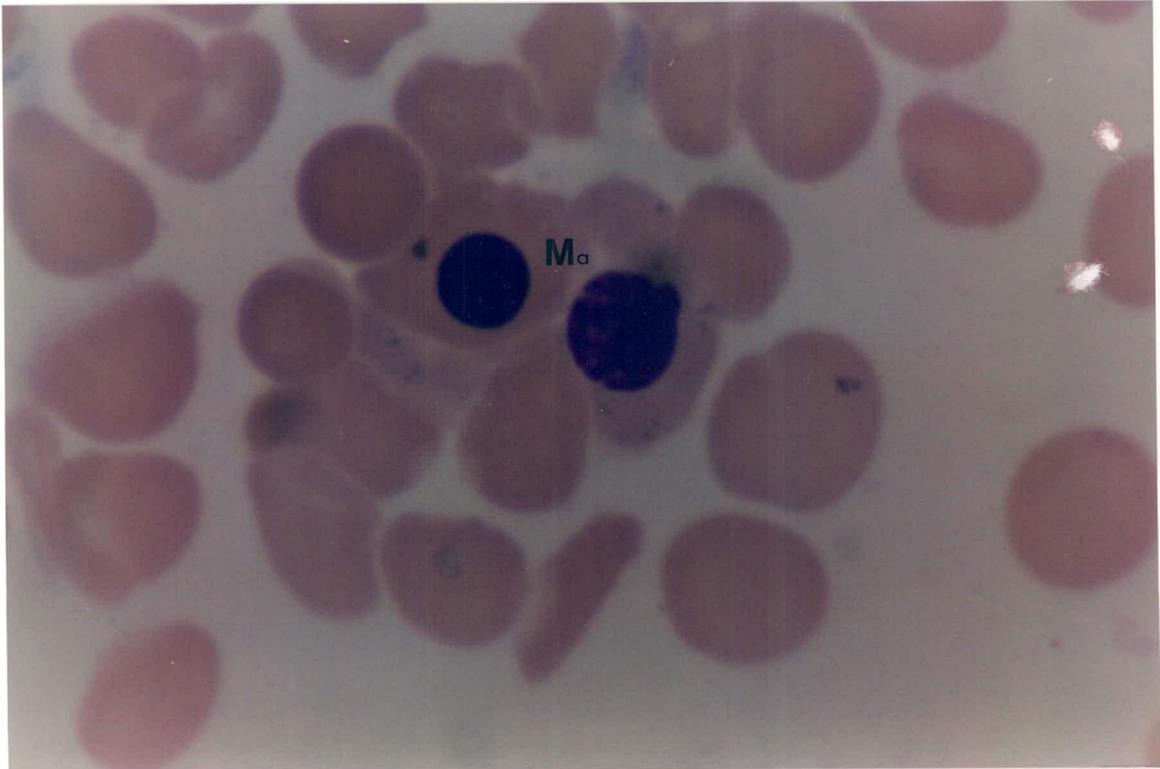
A group of platelets (P) is shown present in normal bone marrow. Also present is a normal promyelocyte (PrM) and two neutrophilic metamyelocytes (Me). In addition a normal late normoblast (No) with a dark picnotic nucleus can be seen. (x1000)



Photomicrograph 17:

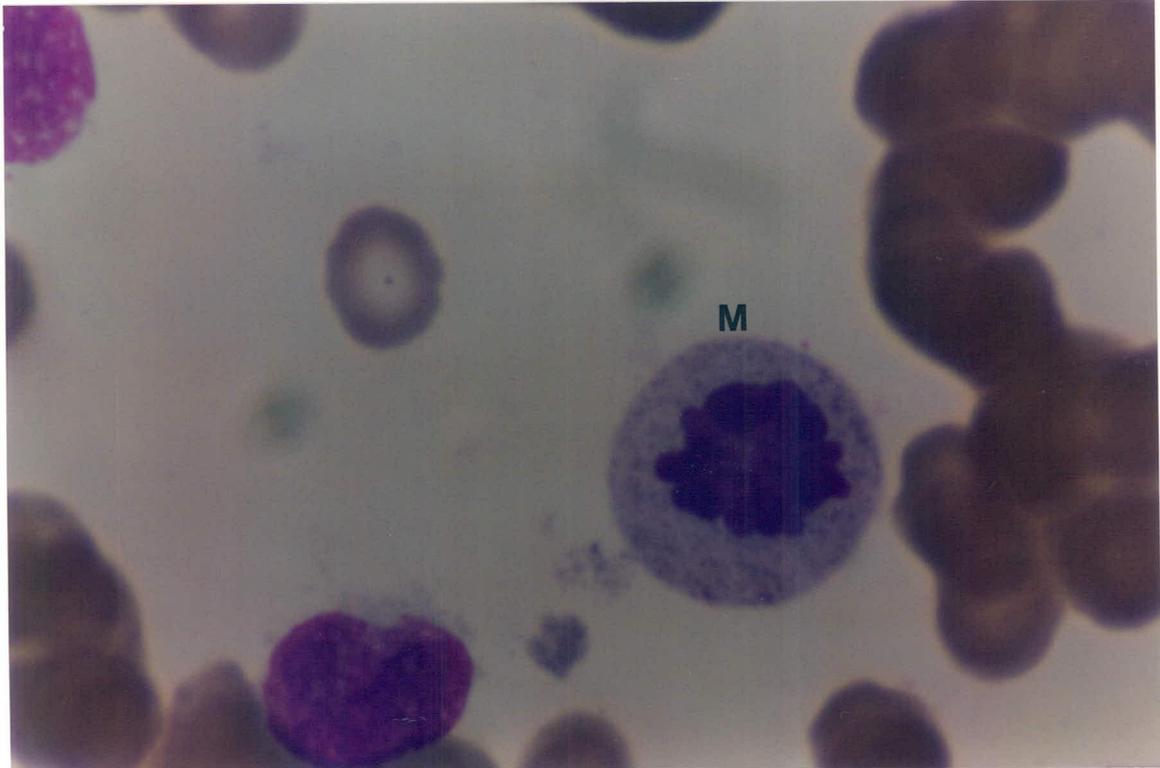
A normal basophilic pronormoblast (PrNo) as seen in normal bone marrow. (x1000)

4-3-4. PERNICIOUS ANEMIC BONE MARROW SMEARS FROM PATIENTS



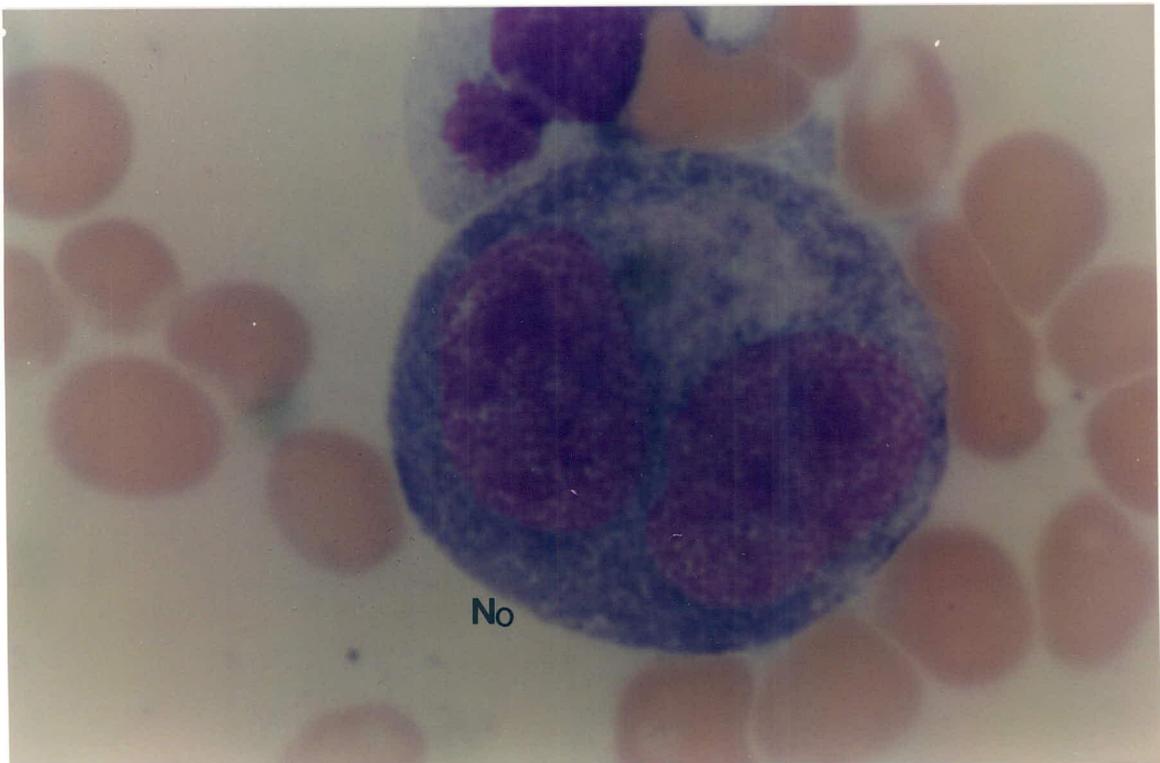
Photomicrograph 18:

Two fully hemoglobinized abnormal macrocytes (Ma) still with their nuclei present in pernicious anemic bone marrow. (x1000)



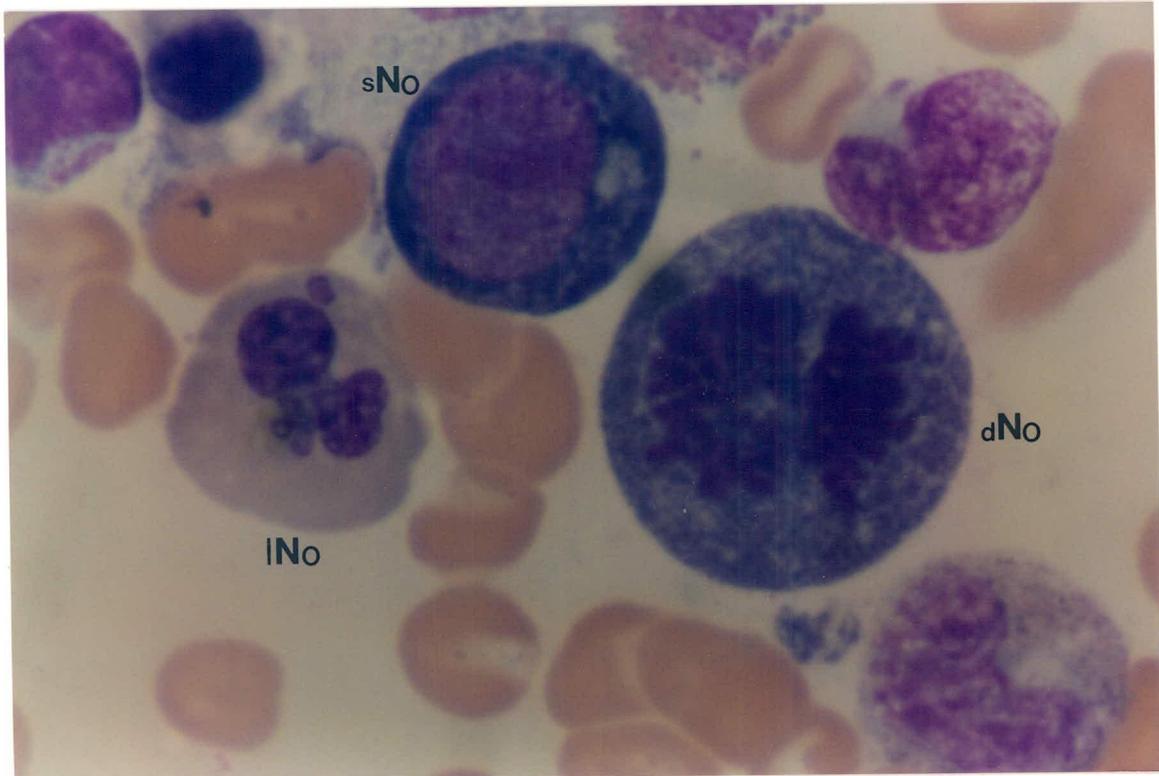
Photomicrograph 19:

An abnormal neutrophilic myelocyte seen in pernicious anemic bone marrow (M) with a nucleus showing signs of mitosis. (x1000)



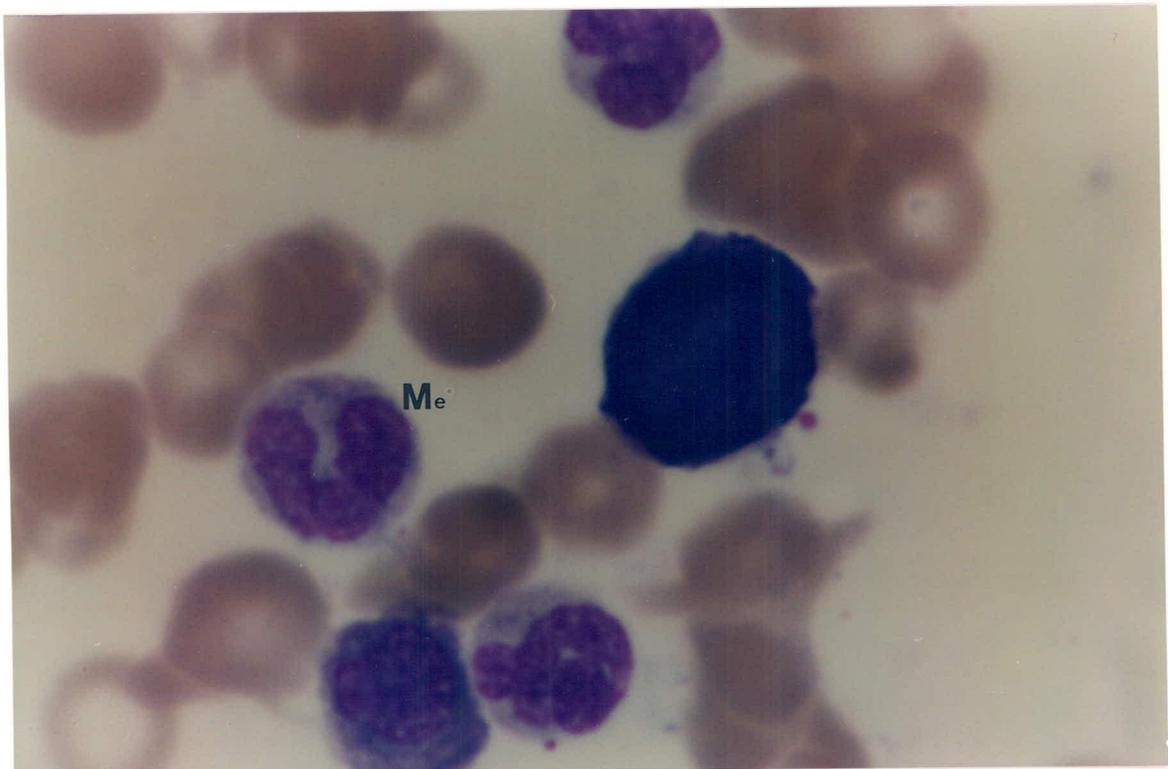
Photomicrograph 20:

An abnormal megaloblastic normoblast (No) containing two nuclei in pernicious anemic bone marrow. (x1000)



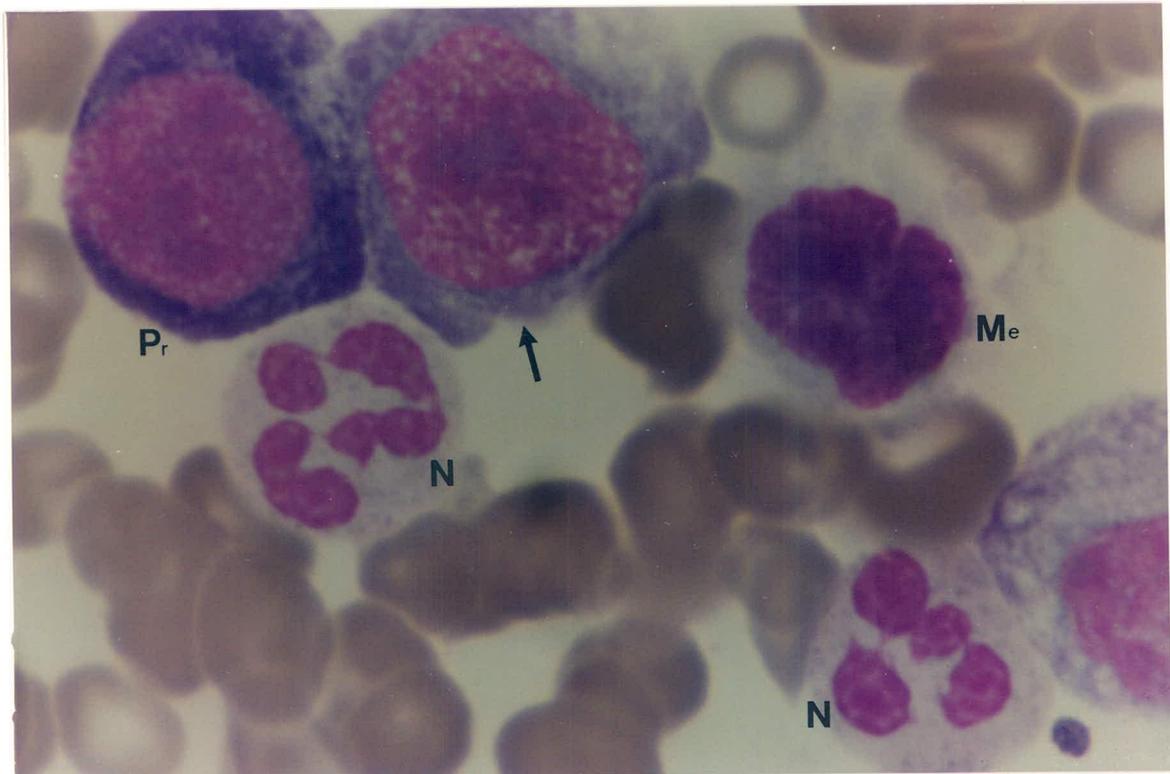
Photomicrograph 21:

An abnormal binuclear late normoblast (INo) present in pernicious anemic bone marrow. An abnormal dividing megaloblastic normoblast (dNo) is also shown as well as a smaller abnormal megaloblastic normoblast (sNo). (x1000)



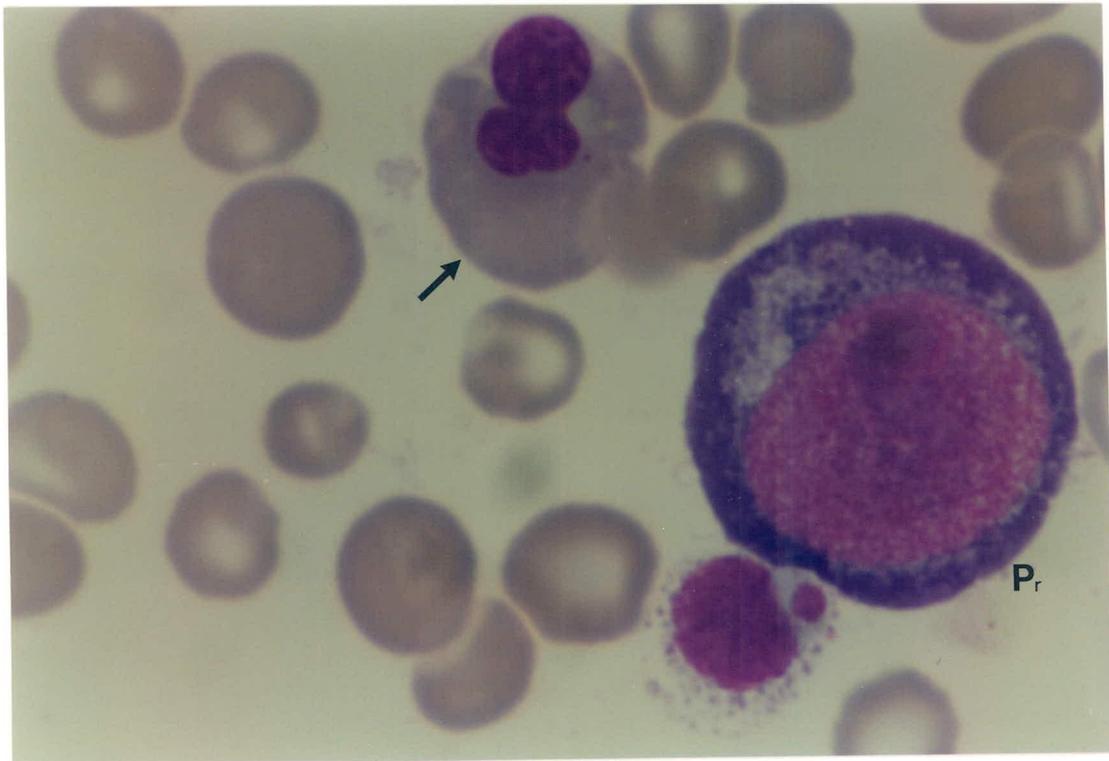
Photomicrograph 22:

An abnormal doughnut-shaped metamyelocyte (Me) in pernicious anemic bone marrow. The cytoplasm is poorly stained light blue and relatively agranular. (x1000)



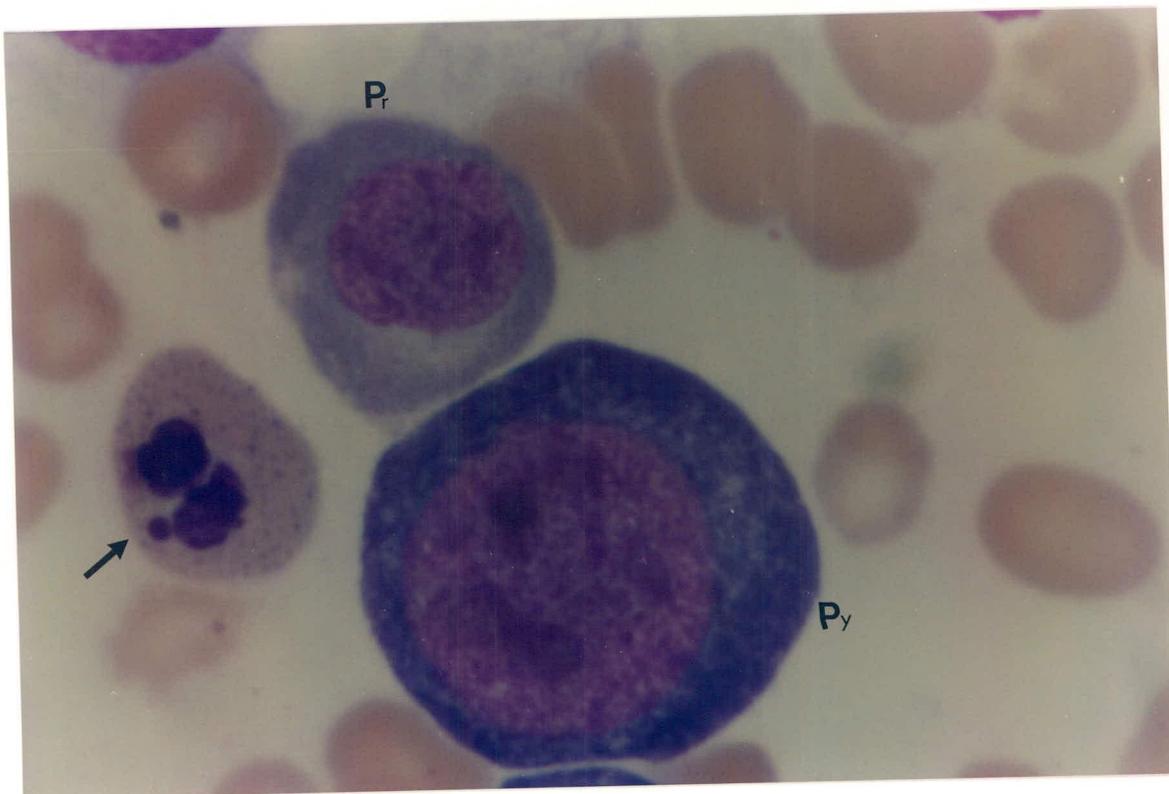
Photomicrograph 23:

An abnormal promegaloblast (Pr) containing basophilic cytoplasm is present with two polymorphonuclear neutrophils (N) and a giant promyelocyte (↑) as well as a large metamyelocyte (Me) with a purple nucleus as seen in pernicious anemic bone marrow. (x1000)



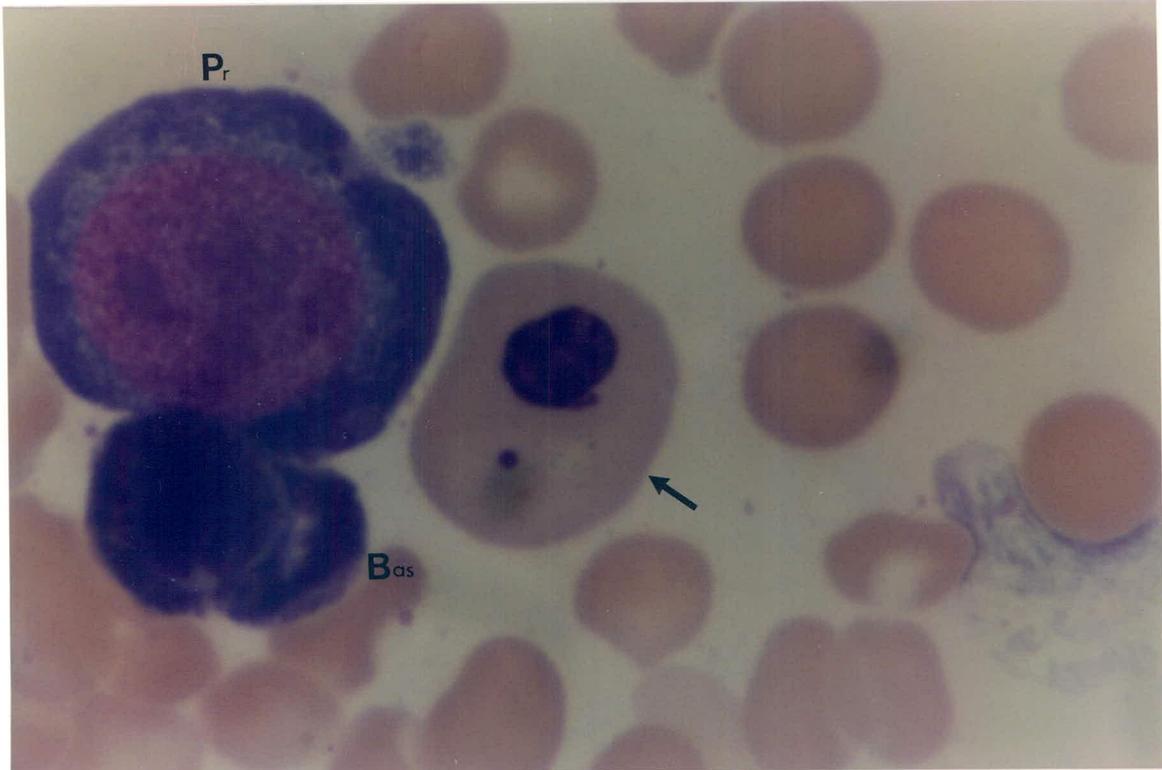
Photomicrograph 24:

An abnormal large promegaloblast (Pr) and abnormal late megaloblast (↑) found in pernicious anemic bone marrow. The promegaloblast has the characteristic big nucleus with the spread out chromatin. The late megaloblast is binucleated. (x1000)



Photomicrograph 25:

An abnormal large promegaloblast (Pr) can be seen with deeply basophilic cytoplasm present in pernicious anemic bone marrow. Also present is an abnormal polychromatophilic megaloblast (Py) and a late binuclear megaloblast (↑) containing a Howell-Jolly body. (x1000)



Photomicrograph 26:

An abnormal large promegaloblast (Pr), basophilic megaloblast (Bas) and a late megaloblast (↑) with a Howell-Jolly body present in pernicious anemic bone marrow. From the promegaloblast it can be seen that the nuclear material is fine and spread out. The cytoplasm is also more and lighter in stain than its normal counterpart. (x1000)

4.4. CONCLUSION OF RESULTS

From the results found during this study the following reference values for healthy males and females were calculated:

Vitamin B ₁₂	-	males: 391,6 to 1136,8 pg/ml
		females: 307,9 to 821,9 pg/ml
Folate	-	males: 1,2 to 8,8 ng/ml
		females: 1,7 to 6,5 ng/ml
Erythrocyte count	-	males: 335 to 648,6 x 10 ¹⁰ /l
		females: 293,8 to 595,4 x 10 ¹⁰ /l
Hemoglobin	-	males: 13 to 17,4 g/dl
		females: 9,3 to 15,9 g/dl
Hematocrit	-	males: 23,7 to 72,1 %
		females: 26,3 to 58,3 %
MCV	-	males: 73,3 to 110,1 fl
		females: 70,2 to 114,6 fl
MCH	-	males: 24,9 to 37,7 pg
		females: 22,7 to 36,3 pg
MCHC	-	males: 22 to 43,6 g/dl
		females: 21,5 to 40,7 g/dl

The results obtained above will be discussed in the next chapter. Interpretations of the graphs and photomicrographs will also be done in addition to comparisons between pernicious anemic values and the normal healthy reference values.

Chapter 5

DISCUSSION AND CONCLUSION

5.1. INTRODUCTION

In this chapter the results obtained in the preceding chapter are discussed. The results from the experimental work is compared to healthy and pernicious anemic readings from males and females and a general conclusion is given. Pernicious anemia occurs when there is a lack of vitamin B₁₂ in the diet or when absorption of the vitamin cannot take place. Megaloblastic anemia can be suspected if macrocytes, oval erythrocytes, pear-shaped poikilocytes and polymorphonuclear neutrophils with hypersegmented nuclei are present in a blood smear (Platt, 1979). This diagnosis can be confirmed by finding megaloblasts and giant metamyelocytes in the bone marrow and the assay of serum vitamin B₁₂ and folate can provide additional evidence for a firm diagnosis. The incidence of pernicious anemia is very rare these days, obtaining abnormal statistical values is therefore an ongoing task. In one year alone Tygerberg Hospital receives, on the average, approximately thirty to forty cases of pernicious anemia. This is a clear indication of the rarity of this disease, thanks to better dietary control and food education.

Part 1

5.2. HEMATOLOGICAL REFERENCE VALUES OF HEALTHY MALES AND FEMALES AND PERNICIOUS ANEMIC MALES AND FEMALES

It is extremely difficult to state the hematological values in health. Observed ranges are large and age, sex and altitude determine important differences. Variation in technique may also explain differences between various observers data (Dacie and Lewis, 1975; Cronje, 1987).

The borderline between health and ill-health is not very clear and so it is with hematological values, due to the overlap of the normal with the abnormal. For example, a value within the recognized reference range could be definitely pathological in a particular subject. There is also a variation between individuals. It is possible to establish a reference range which provides a better guide to the significance of an individual measurement by applying statistical methods to data obtained from a relatively small random sample which is assumed to represent the parent population (Lewis, 1970, Viteri; De Tuna and Guzmán, 1972; Dacie and Lewis, 1975).

It is well known that there is a considerable variation in the erythrocyte count and Hb content at different periods of life (Dacie and Lewis, 1975). At birth the Hb is higher than at any period thereafter. After the immediate postnatal period, the Hb falls fairly steeply to a minimum of about 11,0 g/dl or even less at about the second or third month. The erythrocyte count and Hct also fall, although less steeply and the cells become hypochromic (Dacie and Lewis, 1975). The Hb content and erythrocyte count usually rise gradually to almost adult levels by the time of puberty, thereafter the levels in females tend to be lower than those of males (Dacie and Lewis, 1975). Factors influencing the difference between males and females include the hormonal influence on hemopoiesis and menstrual blood loss (Dacie and Lewis, 1975).

In old age the Hb is reported to fall. In males it falls to a level of 3,4 g/dl at 65, 12 g/dl at 75 and 12,2 g/dl over the age of 85 (Smith and Whitelaw, 1971). With older females the level tends to rise, so that a sex difference of 2 g/dl in younger age groups is reduced to 1,0 g/dl or less in old age (Myers, Saunders and Chalmers, 1968). In addition to the permanent effects of age and sex, other factors seem to have an influence as well. Muscular activity, if at all strenuous, unquestionably raises the erythrocyte count and Hb. This is presumably largely due to the re-entry into the circulation of cells previously secluded in shut-down capillaries or to the loss of circulating plasma. Increases in erythrocytes amounting to $0,5 \times 10^{12}$ /l and in Hb of 1,5 g/dl have been observed (Dacie and Lewis, 1975). Posture also appears to cause transient alterations in the Hb and Hct. A change from an upright to a reclining position results in up to a 5% fall in Hb content. This occurs within about 20 minutes, after which time the Hb is stabilized at the lower level (Ekelund, Eklund and Kaijser, 1971). Diurnal variation also appears to have an effect (McCarthy and Van

Slyke, 1939; Biggs and Allington, 1951). It appears that the Hb is highest in the morning and lowest in the evening with the maximum recorded change being 15% (Dacie and Lewis, 1975).

A rise in altitude also raises the Hb and increases the number of circulating erythrocytes. This appears to be due to both increased erythropoiesis as a result of the anoxic stimulus and to the decrease in plasma volume which occurs at high altitudes (Myhre, Dill, Hall and Brown, 1970; Dacie and Lewis, 1975).

From the above it is clear that it is difficult to obtain normal comparative hematological values. It is therefore necessary to work according to universal standards to avoid confusion. Reference values in hematology are important. From these values it can be determined whether a person is suffering from some or other disease or disorder. It is therefore necessary to have a background to which values can be compared against.

5.2.1. Comparison of Hematology Results between Healthy Males and Females and Pernicious Anemic Males and Females:

Blood was collected from healthy males and females for hematological analyses. The average age of the healthy females from which blood was collected was 21, the youngest being 18 and the oldest being 33. The average age of the healthy males was 22, of which the youngest was 16 and the oldest was 35. Pernicious anemic vitamin B₁₂, MCV and Hct readings were obtained from Tygerberg Hospital of patients suffering from pernicious anemia.

5.2.1.1. Comparative Hematology Tables for Healthy Males and Females

Hematology results derived from the literature is compared to results obtained in this study. The literature results was obtained from Cronje (1987) and the results for this study were obtained from Chapter 4. From the results displayed in Table 5.1 a vitamin B₁₂ and/or folate deficiency can be diagnosed.

TABLE 5.1. Comparison between Results from the Literature and the Present Study.

	column one Literature Results	column two Study Results
Vitamin B ₁₂ :	165 to 1603pg/ml	307,9 to 1136,8pg/ml
Folate (serum):	1,5 to 27,3ng/ml	1,2 to 8,8ng/ml
Erythrocyte- male:	450 to 650 x 10 ¹⁰ /l	335 to 648 x 10 ¹⁰ /l
female:	380 to 580 x 10 ¹⁰ /l	293,8 to 595,5/l
Hemoglobin- male:	13 to 18g/dl	9,3 to 17,4g/dl
female:	11,5 to 16,5g/dl	9,3 to 15,9g/dl
Hematocrit- male:	40 to 54%	23,7 to 72,1%
female:	37 to 47%	26,3 to 58,3%
MCV:	75 to 95fl	70,2 to 114,6fl
MCH:	27,5 to 32,5pg	22,7 to 37,7pg
MCHC:	30 to 36g/dl	21,5 to 43,6g/dl

From Table 5.1 it can be seen that the ranges from the literature and thesis itself do not quite fit together. This is because the ranges found in the literature include the highest and lowest values that are regarded as normal. The ranges for this thesis were also collected from a smaller group of volunteers and therefore the ranges are wider or narrower as compared to the literature ranges. The best correlation between the two types of ranges studies can be seen with the MCH and hemoglobin ranges. The vitamin B₁₂ and folate ranges from the thesis are narrower than the ranges found in the literature. The ranges for erythrocyte count, hemoglobin, hematocrit, MCV, MCH and MCHC are all wider in the thesis study than found in the literature.

The MCV is calculated from the Hct divided by the erythrocyte count. The MCH is calculated from the Hb divided by the erythrocyte count and the MCHC is calculated from the Hb divided by the Hct.

In a typical case of pernicious anemia, the hematological ranges would be as follows:

Vitamin B ₁₂	-	< 160 pg/ml
Folate (serum)	-	< 1,5 ng/ml
Erythrocyte	-	< 380 x 10 ¹⁰ /l
Hemoglobin	-	< 9 g/dl
Hematocrit	-	< 37 %
MCV	-	> 96 fl
MCH	-	> 33 pg
MCHC	-	not above 36 g/dl

All the hematological values for this patient suffering from a vitamin B₁₂ deficiency are below the normal ranges as indicated in Table 5.1. The only hematological parameter that stayed the same, is the MCHC value. The reason for this is because, although the MCV (erythrocyte volume) and the MCH increase, the overall hemoglobin concentration in the erythrocyte stays constant.

5.3. DISCUSSION OF RESULTS OBTAINED FROM PERNICIOUS ANEMIC MALES AND FEMALES AND THE COMPARISON THERE OF WITH RESULTS FROM HEALTHY MALES AND FEMALES

Blood samples were collected and analyzed as described in Chapter 4 with the aid of a Coulter Counter. From the results obtained in the previous chapter, it can be seen that there are values that fall out of the reference range. But as explained previously it is difficult to set normal constant ranges for hematology. Different results will be obtained for different persons. This is demonstrated by the minimum and maximum values obtained for the MCV of females in Table 4.1. But if the averages of the total group are taken the values fall into the acceptable normal ranges. If one of the hematological parameters is too high or too low it does not necessarily mean that the person is suffering from some or other disorder. The other parameters must also be taken into account. This is illustrated in the case of female no. 30 who has a MCV of 129 fl, but has a normal vitamin B₁₂ level of 949,2 pg/ml.

5.3.1. Comparison of Coulter Counter Results from a Specific Case of Pernicious Anemia to Normal Reference Values.

Table 5.2 displays the data from a male patient suffering from a vitamin B₁₂ deficiency. The data from Table 5.2 is compared to data obtained from healthy male volunteers as displayed in Tables 4.7 and 4.10 in Chapter 4.

TABLE 5.2. The hematological results from a patient suffering from pernicious anemia.

	VALUE
Erythrocyte	85 x 10 ¹⁰ /l
Hemoglobin	3,7 g/dl
Hematocrit	10,9 %
MCV	128,1 fl
MCH	43,9 pg
MCHC	34,2 g/dl

To demonstrate the condition of pernicious anemia, a specific case of pernicious anemia is compared to healthy reference values, obtained from Table 5.1, in the following paragraphs. The reference values used in hematology are shown in Table 5.1. The reference values used for comparative study in this thesis are shown in column two in Table 5.1. From the experimental work done it was found that the normal erythrocyte counts varied from 329 x 10¹⁰ to 685 x 10¹⁰ /l for healthy females as seen in Table 4.2 and 392 x 10¹⁰ to 744 x 10¹⁰ /l for healthy males as seen in Table 4.8. The average values for males and females were well within the reference ranges as specified in Table 4.2 for healthy females and Table 4.8 for healthy males. Compared to the erythrocyte count from the patient suffering from pernicious anemia shown in

Table 5.2, it is clear that the erythrocyte value of $85 \times 10^{10} /l$ is well below the normal limit of $335 \times 10^{10} /l$ for males as shown in Table 5.1. The value of $85 \times 10^{10} /l$ is even far below the minimum value of $392 \times 10^{10} /l$ obtained in the reference range for males as shown in Table 4.8. This is already an indication that all is not well with the patient's blood. To find out if this person is only suffering from a loss of blood, other hematological parameters will have to be determined. The Hct of the patient, 10,9%, is well below the normal healthy male limit of 23,7%, but this is to be expected if the patient has lost blood recently. However, when compared to the minimum value shown in Table 4.7 of 31,9%, it can be seen that the patient's Hct is lower by 20%. The patient's Hb level of 3,7 g/dl is also lower than normal i.e. 13 g/dl. If this value is compared to the normal minimum value of 13 g/dl, it is found that the patient's Hb is 9,3 g/dl below normal. This is definitely too low to be normal and is an indicator of a pernicious anemic blood picture. When the patient's MCV of 128,1 pg is compared to reference values of 70,2 to 114,6 fl it can be seen that there is clearly something wrong with the patient's blood. Even the maximum value obtained from normal males of 107 fl in Table 4.7 is lower than the patient's value. The increased MCV indicates that the erythrocytes of the patient are bigger than average. This information taken together with the low erythrocyte count, Hb and Hct shows that megaloblasts are present in the patient's peripheral blood. The evidence mentioned thus far is not sufficient to make a diagnosis for pernicious anemia, however the above mentioned evidence does warrant further investigation. To establish if the patient is suffering from pernicious anemia a RIA has to be performed to determine the level of vitamin B₁₂ in the blood in addition to the above mentioned facts. Once this has been done and the serum levels of vitamin B₁₂ have been found to be decreased, then only can a diagnosis of pernicious anemia be made.

The reference range for the normal MCH is 22,7 to 37,7 pg. Although the maximum and minimum values for the males and females are out of this range as seen in Tables 4.1 and 4.7, the average for both groups are well within the normal range. In pernicious anemia, the MCH is expected to rise due to the increase in erythrocyte size and Hb production. This was found to be the case in the patient's MCH value. This value of 43,9 pg is 6,2 pg above the normal range of 37,7 pg. The MCHC value of the patient is 34,2 g/dl which falls into the normal reference range of 21,5 to 43,6 g/dl. This is as expected because although the MCV and MCH rise, the Hb of the erythrocyte stays the same relative to the size of the erythrocyte. The MCHC does not rise or decrease in pernicious anemia because although the erythrocyte count is decreased, the

hemoglobin concentration in the erythrocyte remains the same. This is because the megaloblastic erythrocytes have a bigger volume for hemoglobin and a megaloblast contains more hemoglobin than a normal erythrocyte. This also explains why the MCH rises (increased Hb production by megaloblast). The MCV rises due to the increased size of the erythrocytes which increases the volume of the erythrocyte. If the MCHC of the females and males in Tables 4-1 and 4-7 are examined, it is clear that some individual values are either above or below the reference range. However, if the averages of both groups are taken into account, it can be seen that the averages do fall into the normal range.

5.3.2. Comparison of Coulter Counter and RIA Readings from Pernicious Anemic Males and Females with Results from Healthy Males and Females:

Blood was collected and analyzed as described in Chapter 3 with the aid of a RIA kit. The normal values for vitamin B₁₂ and folic acid cover a wide range. Reference values for vitamin B₁₂ range from 307,9 to 1136,8 pg/ml and for folic acid the values range from 1,2 to 8,8 ng/ml as seen in Table 5.1. Any value below these ranges indicate the condition of pernicious anemia, i.e. values less than 160 pg/ml (Cronje, 1987) for vitamin B₁₂ and less than 1,2 ng/ml (Table 5.1, column two) for folic acid. The levels of vitamin B₁₂ and folic acid are usually higher in males than in females. This came to light in the values obtained and which compare well with other reference values as shown in Table 5.1. As can be seen from Table 4-10 the average male level was 764,2 pg/ml which is well within the normal reference range of 307,9 to 1136,8 pg/ml. The average level of the females was 564,9 pg/ml, as seen in Table 4.11, which also falls well within the normal range. The minimum of 332,4 pg/ml and maximum of 949,2 pg/ml were also within the normal range.

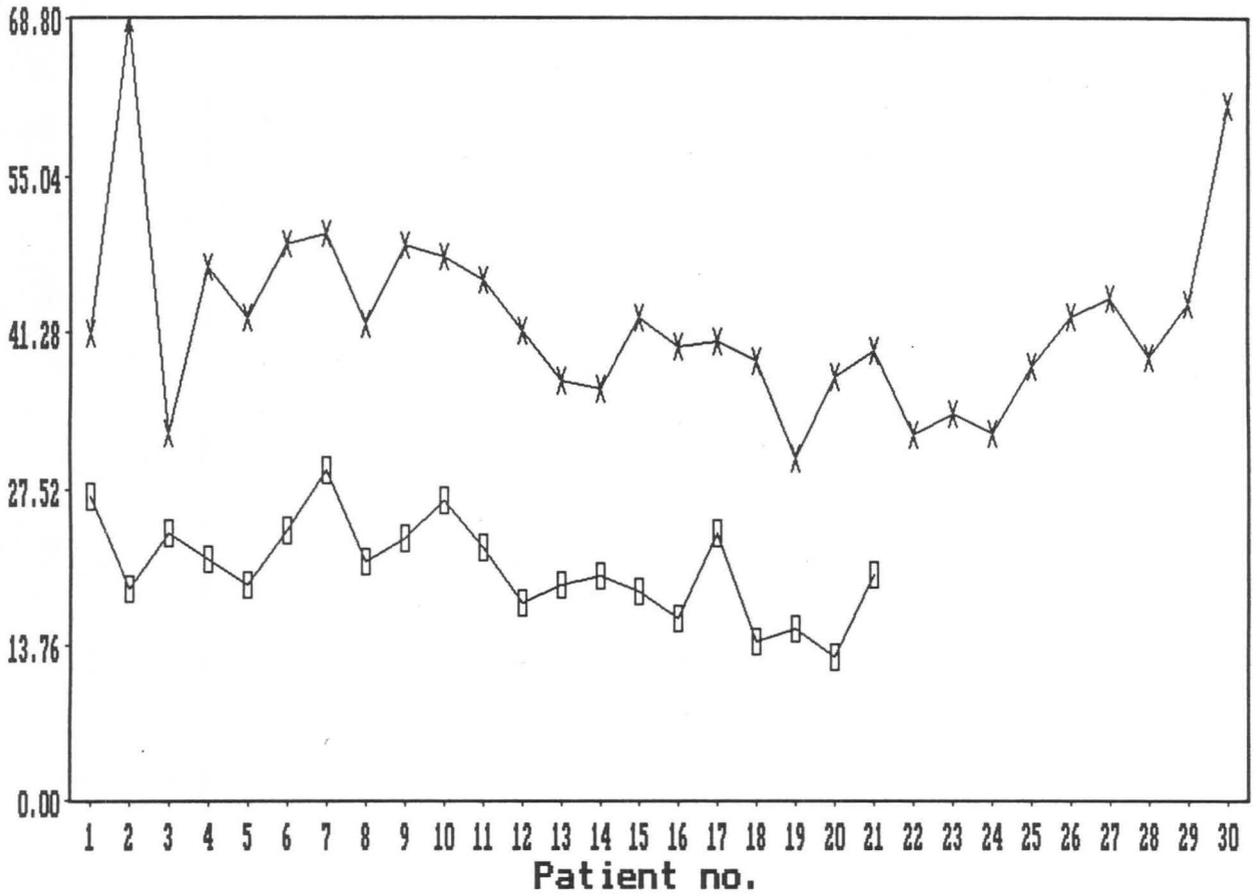
Tables 4-13 and 4-14 show the Hct, MCV and vitamin B₁₂ values of males and females suffering from pernicious anemia. These results were obtained from patient's at Tygerberg Hospital and clearly show the high MCV, low Hct and vitamin B₁₂ levels associated with pernicious anemia. A diagnoses of pernicious anemia can be made from these results. The average and maximum values for Hct and vitamin B₁₂ from Tables 4.13 and 4.14 are below the reference values, both for males and females. The MCV values for

males and females are above normal indicating the presence of macrocytes in the blood. The folic acid levels are all exactly within the reference range, indicating that these patients were suffering purely from a vitamin B₁₂ deficiency. Therefore the values represented in Tables 4.13 and 4.14 all indicate patients suffering from pernicious anemia or in other words a vitamin B₁₂ deficiency. There are some very severe cases of pernicious anemia judging from some of the values in Tables 4.13 and 4.14. For instance patient number (no.) 9 from Table 4.13 is a female with a vitamin B₁₂ value of 54,4 pg/ml. This is a very low concentration when compared to the minimum value of 332,4 pg/ml obtained from the normal females in Table 4.1. If the rest of the patient's hematological values are looked at it can be seen that these values are typical for a patient suffering from pernicious anemia. The Hct, of female patient no. 9, of 23,3% is below the normal value of 23,7% and the MCV value of 149,9 fl is above the normal value of 110,1 fl. Together with the low vitamin B₁₂ value, the values of female patient no. 9 clearly indicate a case of pernicious anemia. Even the border line case of male patient no. 10 in Table 4.14 is indicative of pernicious anemia, although the vitamin B₁₂ value of 160,8 pg/ml is only slightly below the minimum normal value of 160 pg/ml indicating a vitamin B₁₂ deficiency. But once again if the rest of the hematological values are taken into account it is clear that the Hct of male patient no. 10 is decreased and the MCV is above the reference range.

5.4. COMPARATIVE STUDY OF GRAPHS BETWEEN PERNICIOUS ANEMIC MALES AND FEMALES AND HEALTHY MALES AND FEMALES

The four graphs in Chapter 4 show the results obtained from the RIA and Coulter analyses for healthy males and females in graphic form. The data for the the healthy females was obtained from Tables 4. and 4.4. The data for healthy males was obtained from Tables 4.7 and 4.10. The six graphs displayed below show the comparison between healthy and pernicious anemic male and female Hct, MCV and vitamin B₁₂ values. The data for pernicious anemic females was obtained from Table 4.13 and the data for pernicious anemic males was obtained from Table 4.14. The pernicious anemic vitamin B₁₂ results are grouped with the healthy vitamin B₁₂ results. The same was done with the healthy and pernicious anemic Hct and MCV results.

NORMAL and ABNORMAL FEMALE Hct VALUES



⊕ Abnormal Hct

* Normal Hct

FIGURE 5-4-1. Graphic presentation depicting the comparison of Hct values between pernicious anemic females and healthy reference females.

NORMAL and ABNORMAL FEMALE MCV VALUES

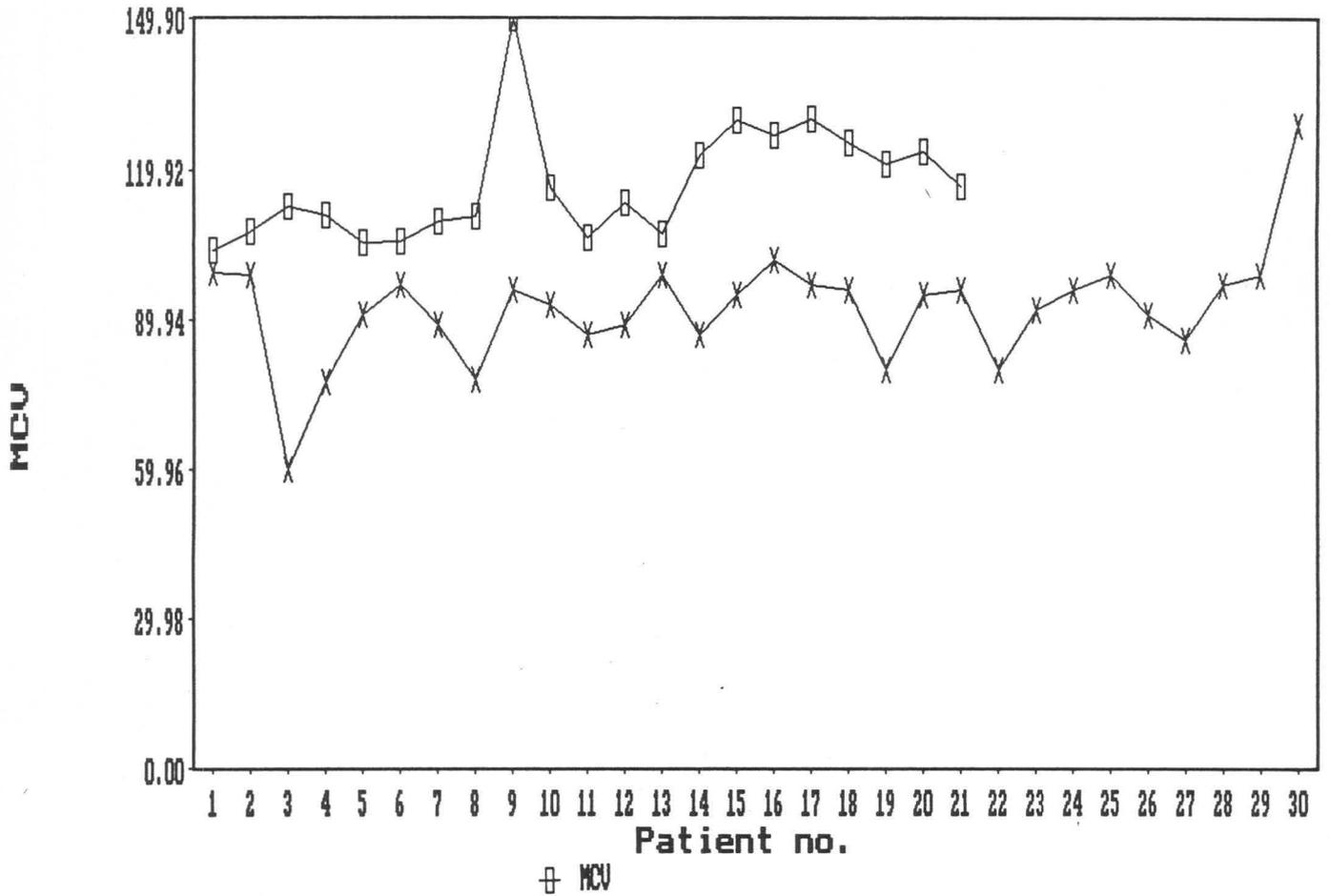


FIGURE 5-4-2. Graphic presentation depicting the comparison of MCV values between pernicious anemic females and healthy reference females.

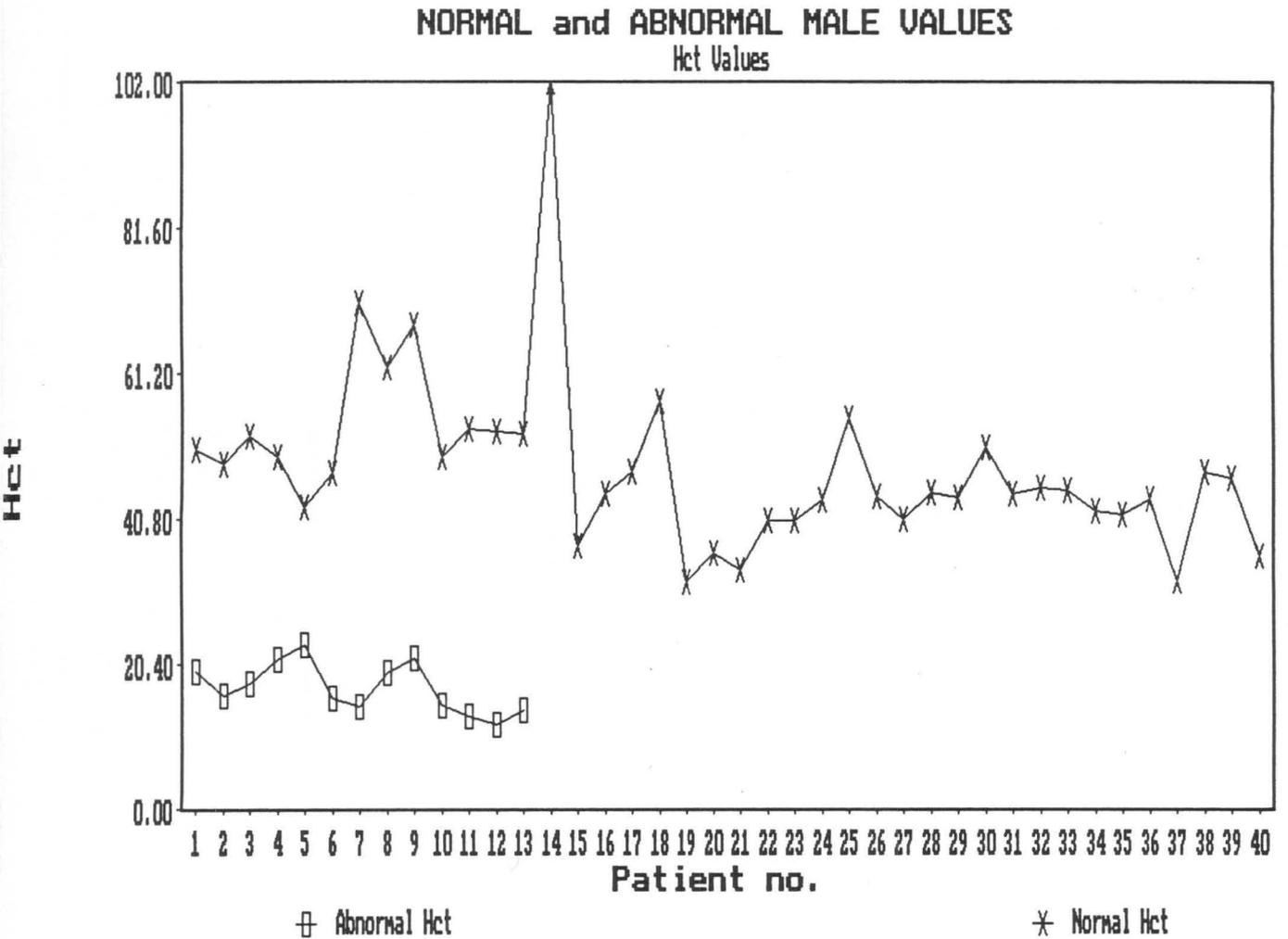


FIGURE 5-4-4. Graphic presentation depicting the comparison of Hct values between pernicious anemic males and healthy reference males.

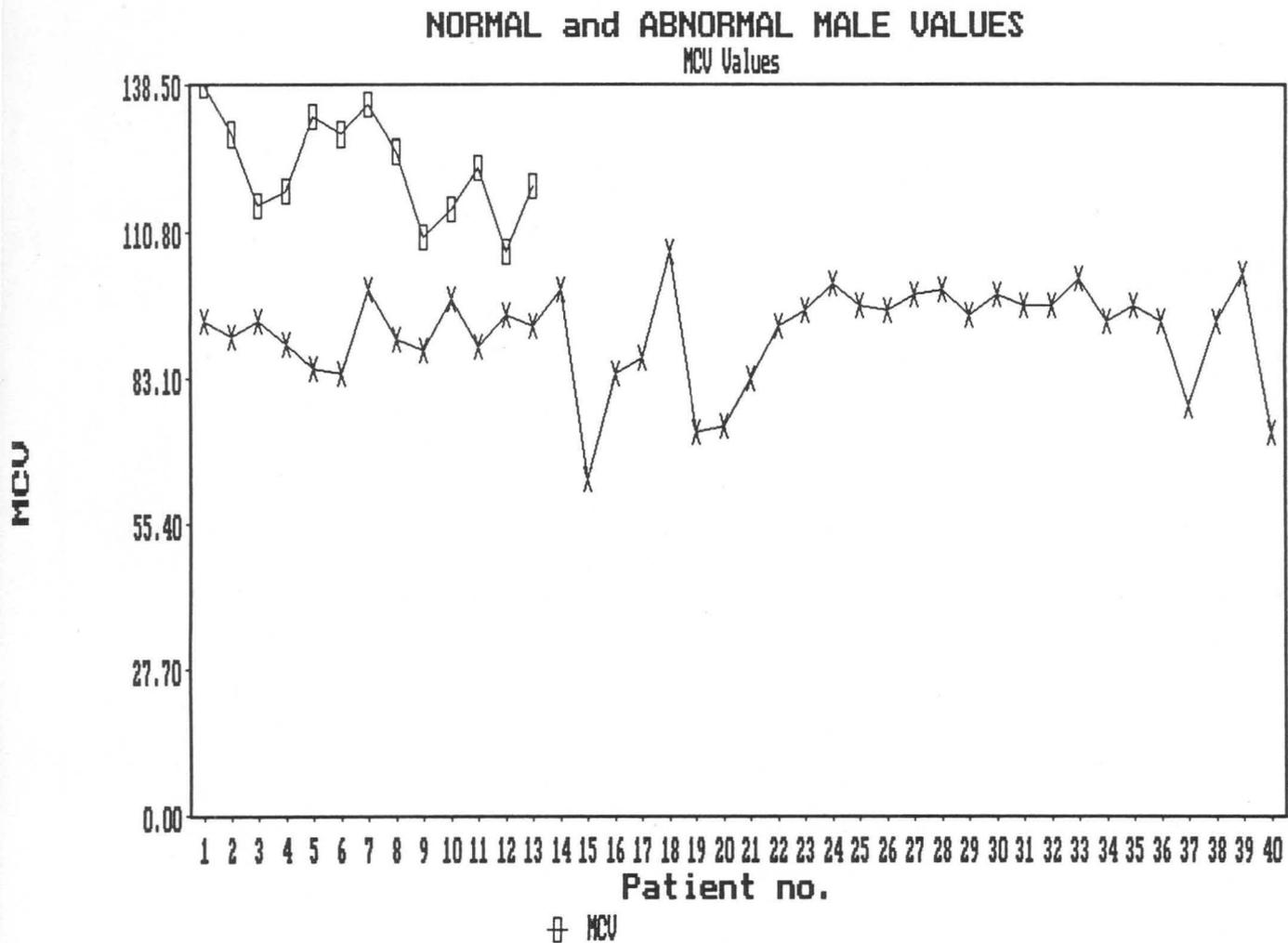


FIGURE 5-4-5. Graphic presentation depicting the comparison of MCV values between pernicious anemic males and healthy reference males.

Vitamin B12

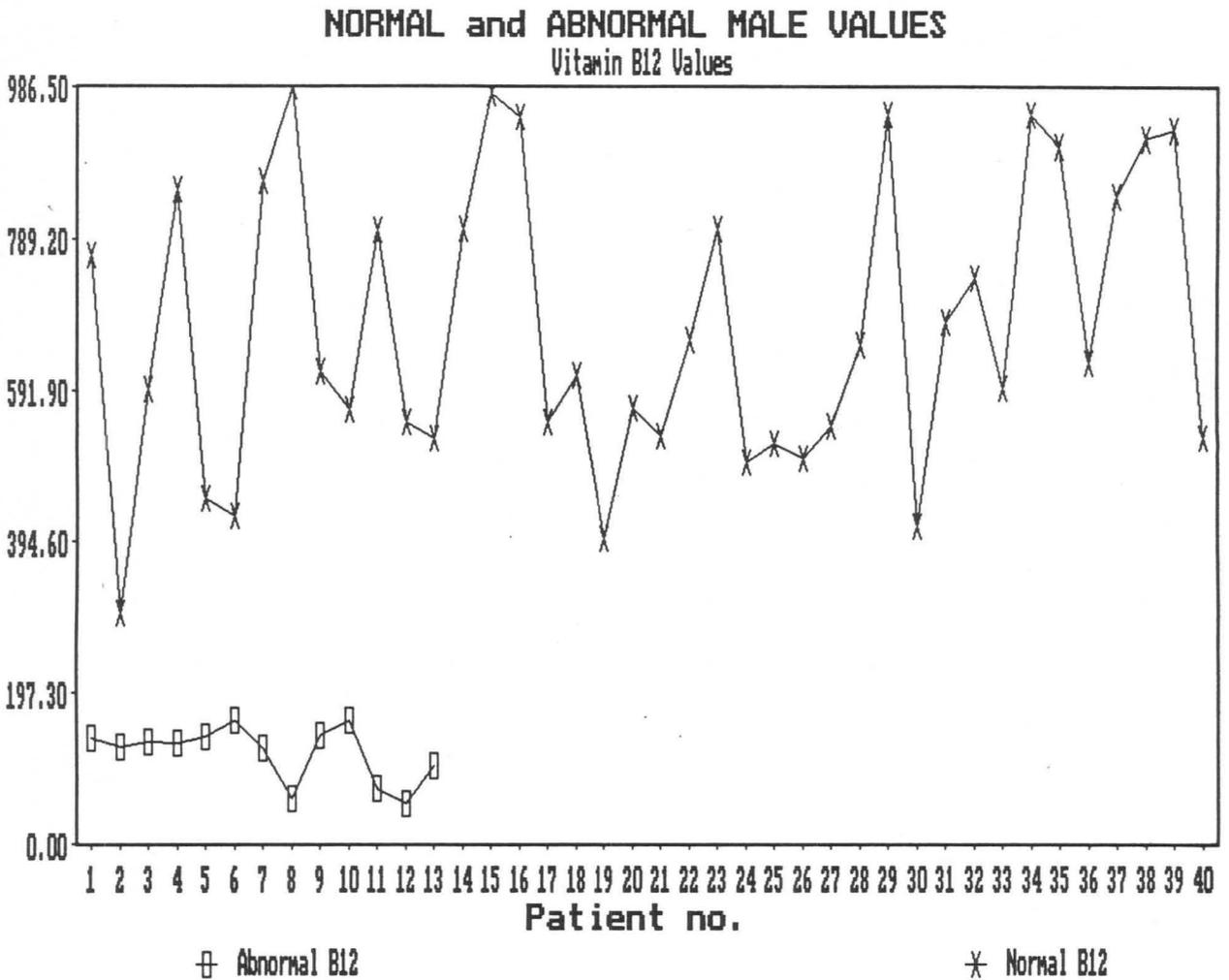


FIGURE 5-4-6. Graphic presentation depicting the comparison of vitamin B₁₂ values between pernicious anemic males and healthy reference males.

From Figures 5-4-1 and 5-4-4 it can easily be seen that the Hct and vitamin B₁₂ results for the male and female patients suffering from pernicious anemia are below the results obtained for normal healthy patients. From Figures 5-4-2 and 5-4-5 it is clear that the pernicious anemic male and female MCV readings are above normal. Figures 5-4-1, 5-4-2, 5-4-3, 5-4-4, 5-4-5, 5-4-6 clearly show the graphical difference between the normal and pernicious anemic hematological values typically found in pernicious anemia. With the help of these graphs the comparison between normal and pernicious anemic values can easily be compared and evaluated.

If the table results and the graphs are taken together for the pernicious anemic males and females and compared to the table results and graphs of healthy males and females, a clearer picture of the effects of pernicious anemia on anemic patients can be seen.

Part 2

5.5. COMPARATIVE DISCUSSION OF PHOTOMICROGRAPHS OF HEALTHY AND PERNICIOUS ANEMIC BLOOD AND BONE MARROW SMEARS

To show the differences between healthy and megaloblastic blood, a comparison between normal and pernicious anemic blood and bone marrow smears is discussed.

In order to understand the interpretation of smears from bone marrow and peripheral blood, a few terms will first be explained. What follows is an explanation of terms specific to pernicious and megaloblastic anemias.

Macrocytosis is a condition in which the erythrocytes have a diameter greater than $9\ \mu$ and frequently have a mean corpuscular volume (MCV) greater than $95\ \mu$ to $100\ \mu$ (Platt, 1979). Macrocytic cells can indicate the presence of more hemoglobin than normal. This is found in vitamin B₁₂ and folic acid deficiencies i.e. pernicious anemia and other macrocytic anemias (Platt, 1979).

Anisocytosis is a condition where an abnormality in size of the erythrocyte outside the normal or normocytic range of $6,2\ \mu$ to $8,2\ \mu$, where the average is $7,2\ \mu$, is present. This is a common and important erythrocyte morphologic variation (Platt, 1979).

Poikilocytosis describes the condition in which there are major variations in the shape of the erythrocyte, which is usually that of a biconcave disk. Crenated erythrocytes fall under this category. These are erythrocytes that possess a crinkled, serrated periphery (Platt, 1979). They are not clinically diagnostic and only reflect changes produced by the exposure of erythrocytes to dehydration, hypertonic agents and lytic agents.

Megaloblasts are nucleated erythrocytes found in the bone marrow (Platt, 1979). These cells are $15\ \mu$ to $22\ \mu$ in diameter and are associated with vitamin B₁₂ and folic acid deficiencies. The cell is larger than its normal counterpart (the basophilic normoblast), the nuclear chromatin is finer and the cytoplasm is lighter staining and larger in amount (Platt, 1979).

Leukopenia is the reduction of the total number of leukocytes below 4×10^9 /l. In most situations, this decrease is due to a decrease in the number of cells in the granulocyte series (Platt, 1979).

Thrombocytopenia is the decrease in number of circulating blood platelets of which the average count is 300 000 / μ l (Platt, 1979; Ganong, 1987).

5.5.1. General Aspects of Megaloblastic Anemias:

Megaloblastic anemias are characterized by distinctive cytological and functional abnormalities in bone marrow and peripheral blood due to impaired DNA synthesis. These anemias are deficiency diseases caused by the lack of either vitamin B₁₂ or folate, both of which are vital for the normal development of erythrocytic cells. The most common causes for deficiencies are:

- vitamin B₁₂ deficiency due to lack of IF
- nutritional folate or vitamin B₁₂ deficiency
- deficiency due to malabsorption
- infestation with the fish tape worm

(*Diphyllobothrium latum*)

The name megaloblastic anemia is derived from the characteristic appearance of morphologically abnormally large nucleated erythrocyte precursors in bone marrow (Cronje, 1987). These cells are abnormal in function as well as in appearance. This results in the formation of mature erythrocytes showing abnormalities in size and shape.

5-5-2. Description of Peripheral Blood and Bone Marrow Smears from Healthy Volunteers and Pernicious Anemic Patients:

Blood and bone marrow was collected and stained as described in Chapter 3. In pernicious anemia the bone marrow is red, voluminous and hypercellular with hyperplasia of all the elements, especially the erythrocyte precursors. These precursors do not mature properly because of a disordered synthesis of deoxyribonucleic acid (DNA). An erythroid hyperplasia is present in macrocytic anemia. This means that the developing megaloblastic normoblasts are predominant over the developing granulocytes. The development of the granulocytes is suppressed, while there are more normoblasts present. In photomicrograph 6 the abnormal shapes of the erythrocytes can be seen in this overall view of a macrocytic blood smear. There is even an abnormally late macrocyte visible still with its nucleus.

Nucleated erythrocytes may incorporate 30 to 50 percent of all nucleated cells. There is a disruption in the normal myeloid : erythrocyte (M : E) ratio. Normally there is one nucleated erythrocyte to four myeloid type cells i.e. neutrophils, etc. (Platt, 1979). This is referred to as the M : E ratio or myeloid : erythrocyte ratio. This ratio is obtained by dividing the number of myeloid cells (myeloblasts, promyelocytes, myelocytes, metamyelocytes and polymorphonuclear neutrophils) by the total number of erythroid cells (pronormoblasts and normoblasts).

If a peripheral blood smear of macrocytic anemia is studied under a microscope the striking feature is the large megaloblastic normoblasts. The granulocytes are fewer than in a normal blood smear.

The condition of macrocytic normochromic anemia is pronounced when the MCV is 94 to 160 fl and the MCHC is 32 to 36 g/100ml (Platt, 1979). The bone marrow shows abnormal (megaloblastic) erythrocyte maturation. If photomicrograph 25 (normal bone marrow) is compared to photomicrographs 13 and 17 (abnormal marrow), it can be seen that normoblast development is not normal. The large pronormoblast seen in photomicrograph 25 is far too large for normal. The nucleus is too light in colour and the chromatin is spread out. This indicates the abnormal development of the chromatin material. The nucleus of a normal normoblast, as shown in photomicrograph 17, is much darker and smaller. In other words the chromatin

material is concentrated closer together. The normal late normoblast in photomicrograph 13 is smaller than the megaloblastic late normoblast in photomicrograph 25. This leads to the hemoglobin level being lower as well as a low erythrocyte count and hematocrit.

Macrocytosis, anisocytosis and poikilocytosis can be observed in bone marrow and peripheral blood smears. These features can be seen in photomicrographs 9, 12 and 8 in particular. If the erythrocytes are compared to the size of the lymphocytes in photomicrographs 9 and 12, the abnormally large size of the erythrocytes are evident. Photomicrographs 4 and 5 are of normal peripheral blood and compared to the above mentioned photomicrographs, the irregular size and shape of the erythrocytes can be seen. The macrocytic nature of the erythrocytes can be seen when compared to the lymphocytes in photomicrographs 9 and 12. In photomicrographs 4 and 5 the uniformity of the erythrocytes are clearly seen and they are all smaller than the lymphocytes.

Leukopenia is present with large macropolycytic (hypersegmented) polymorphonuclear leukocytes and occasional immature leukocytes (Platt, 1979; Cronje, 1987). On the average, normal granular polymorphonuclear neutrophils have 2,5 lobes to their nucleus. In macrocytic peripheral blood and bone marrow smears the mature polymorphs have many more lobes, as many as five to six. The 5-lobed neutrophil in photomicrograph 10 clearly shows the hypersegmentation found in the polymorphs of peripheral blood of a macrocytic patient. Photomicrograph 23 shows the hypersegmentation of abnormal neutrophils found in bone marrow. If the neutrophil in photomicrograph 23 is compared to the normal neutrophils of peripheral blood in photomicrographs 1, 2 and 3, the abnormal hypersegmentation of the polymorph in photomicrograph 23 is evident. In macrocytic bone marrow doughnut shaped neutrophilic myelocytes are found as shown in photomicrograph 22. The nuclei of these cells differ from normal myelocytes in that they are rounder. In photomicrograph 16 normal juvenile neutrophilic myelocytes are shown. The chromatin of these doughnut shaped myelocytes is also lighter than compared to normal chromatin as seen in photomicrograph 16. The difference in chromatin in photomicrograph 22 shows the interference in DNA synthesis due to in a lack of vitamin B₁₂ and folate.

A slight thrombocytopenia can also be observed with bizarre platelet forms including giant types (Platt, 1979). In photomicrographs 11 and 12, of macrocytic peripheral blood, big platelets can be seen. Photomicrograph 7 shows an even larger platelet present in a smear of macrocytic blood. The abnormal oval shapes of the erythrocytes in photomicrograph 7 are indicators of macrocytic anemia if compared to the normal erythrocyte present in photomicrograph 5. If the platelets of photomicrographs 11 and 12 are compared to the size of the platelets of normal peripheral blood in photomicrographs 1 and 4, the abnormal size of these platelets can be seen. The platelets are also fewer in number in macrocytic blood than in normal healthy blood. Photomicrograph 1 shows a group of platelets in healthy blood. Finding a large group of platelets in macrocytic blood is very difficult and rare. Platelets in macrocytic blood are therefore few and large in size.

The formation of megaloblasts is characterized by large cell size, bizarre fine nuclear chromatin with dense lumpy basophilic cytoplasm and occasionally binucleated forms. If nucleoli are present, they are generally very sharply outlined (Platt, 1979). When a smear of macrocytic bone marrow is studied, the most striking feature is the large megaloblastic normoblasts ($10\ \mu$ to $27\ \mu$) present as in photomicrographs 24, 25 and 26. These cells have large nuclei with fine grainy spread out chromatin. The cytoplasm is very basophilic. Normal normoblasts, as seen in photomicrographs 14, 15, 16 and 17, are smaller ($8\ \mu$ to $19\ \mu$) and possess a darker and more dense nucleus. The cytoplasm is much less than in megaloblasts and also not as basophilic. The cytoplasm of megaloblastic normoblasts are more in relation to the nucleus and lighter staining areas around the nucleus can be seen, as in photomicrograph 25. The binucleated megaloblastic normoblast in photomicrograph 20 is another sign of the abnormal DNA synthesis. Many of the cells in macrocytic bone marrow show signs of division as shown in photomicrographs 19 and 21. This indicates the late stage at which megaloblastic cells still divide due to the disruption of DNA synthesis caused by a vitamin B₁₂ and folate shortage which leads to a slower cell development. The cell in photomicrograph 21 is a megaloblastic normoblast and in photomicrograph 19 a myelocyte is dividing. This shows that both the granulocyte and erythrocyte cell series are affected by the shortage of vitamins. Even late megaloblastic normoblasts as in photomicrographs 21, 24 and 25 show multiple nuclei. This feature of the appearance of binucleated normoblasts in macrocytic anemia is called diserythropoiesis. In pernicious anemia there are numerical and

morphological chromosomal aberrations. These include chromatid breaks, gaps and giant chromosomes which all disappear after treatment (Platt, 1979).

Although nuclear maturation is defective, the cytoplasmic development and hemoglobin synthesis proceeds normally. This results in asynchrony in which large cells containing young looking nuclei and mature orthochromic or eosinophilic cytoplasm are formed (Platt, 1979). An example of where this difference in development can be seen are in photomicrographs 18 and 24. The cytoplasm is in both cases fully or almost completely hemoglobinized (compare to neighbouring cells) and the nuclei should have been extruded by this stage. The difference in cytoplasmic and nuclear development is caused by the shortage or lack of vitamin B₁₂ and/or folate deficiency. This leads to interruption of DNA synthesis causing the slow down in nuclear development.

In pernicious and megaloblastic anemia the erythrocytes may contain abnormal inclusions (Platt, 1979). These inclusions are cytoplasmic blemishes often indicating disease. An example of this are Howell-Jolly bodies which are single or double eccentrically placed small, round nuclear fragments. These Howell-Jolly bodies can be seen in photomicrographs 9, 12 and 26. In photomicrograph 26 the nuclear fragment is visible together with the nucleus. They appear when abnormal mitosis of the orthochromic-normoblast occurs.

Deviant nuclear chromatin and cell enlargement are also found in the granulocyte series with giant metamyelocytes, giant band forms (doughnut shapes), macropolycytes and giant megakaryocytes and platelets (Platt, 1979).

The peripheral blood in pernicious anemia usually shows macrocytosis, anisocytosis, poikilocytosis, diffuse or punctuate basophilia, Howell-Jolly bodies, polychromasia, macropolycytosis of polymorphonuclear neutrophil nuclei, occasionally giant metamyelocytes and myelocytes that are poorly granulated. Occasionally eosinophilia is found and leukopenia is fairly common as well as thrombocytopenia with bizarre giant platelets (Platt, 1979).

Bone marrow studies show that 30 to 50 percent of nucleated cells in the erythrocyte series are present, especially megaloblasts, which are larger than normoblasts and show early hemoglobinization. This early hemoglobinization can be seen in the medium-sized cell in photomicrograph 25. The medium-sized megaloblast in photomicrograph 25 is still relatively early in its development but already hemoglobin is being produced in the cytoplasm. A scroll-like chromatin network in the nuclei and a deeply basophilic cytoplasm are also visible. Promegaloblasts can be seen. Polychromatophilic and acidophilic megaloblasts can be seen in groups of three to six, many showing signs of mitosis, with reticulocytes (Platt, 1979). Giant metamyelocytes are visible containing doughnut nuclei with vacuoles. The cytoplasm is light blue to basophilic and relatively agranular. The megakaryocytes are reduced or possibly abnormal.

5-6. CONCLUSION

The importance of vitamin B₁₂ has been shown in this workpiece. With the use of techniques and certain instruments, the effects of a shortage of vitamin B₁₂ has been shown. Analyses of the blood from normal healthy patients was compared to that of patients suffering from pernicious anemia. Though fewer anemic patients were investigated than healthy patients, it became clear that the effect of a shortage of vitamin B₁₂ on a person is dramatic. This effect can visually be seen in photomicrographs taken of anemic blood as well as in the comparative graphs between reference healthy and pernicious anemic patients. The dramatic effect is clearly shown in the bone marrow by the abnormal development of the erythrocytic precursors and the formation and maturation of abnormally large erythrocytes in the peripheral blood. A drop in erythropoiesis was also noted which was indicated by the drop in the erythrocyte count.

Due to previous dedicated researchers pernicious anemia can be diagnosed at an early stage and therefore minimized. This is clearly shown by the rare occurrence of the condition and the few patients that are hospitalized to receive treatment. Research into nutrition began in the nineteenth century and was active in the 1920s, when papers were published that are still important today. The literature available today has led to extensive insight into topics that are of considerable medical importance. How will understanding grow in the future? Perhaps there are some leads ahead and gaps to be filled that can be extrapolated from the present known literature.

SUMMARY OF THESIS

**THE PHYSIOLOGICAL EFFECT OF VITAMIN B₁₂ DEFICIENCY
IN HUMAN BLOOD**

by

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SUMMARY

The earliest reported case of pernicious anemia was in 1822. Then in 1948 vitamin B₁₂ was isolated for the first time. Vitamin B₁₂ and folic acid are vitally important for the normal development of the body and its cells. The most well known feature of a deficiency in these vitamins is the effect on the production and maturation of the erythrocytes in the bone marrow. Large or megaloblastic erythrocytes are formed and found in the blood. These cells are called macrocytes and have a shorter life span than healthy erythrocytes. Fewer erythrocytes are also produced and therefore the erythrocyte count in blood is lower than normal.

The search for vitamin B₁₂ began in 1926 when Minot and Murphy discovered the ability of liver to treat pernicious anemia. Castle and his associates (1929) demonstrated that an intrinsic factor found in human gastric juice bound an extrinsic factor found in animal protein. It was later discovered that vitamin B₁₂ was the extrinsic factor. In 1948 two groups of investigators isolated a compound from liver that produced hematologic remissions in patients with pernicious anemia.

The next important contributing discovery was by Barker and associates in the discovery of the coenzyme forms of vitamin B₁₂. Up to then the coenzymes had been overlooked as they are easily broken down by light.

The structure of the vitamin B₁₂ molecule was described by Lenhert and Hodgkin (1961) using an X-ray crystallographic analyses method. The distinguishing features of the molecule is its complexity and the fact that it is the only vitamin to contain a cobalt atom.

The vitamin B₁₂ molecule has a molecular mass of 1355 d and consists of two main parts. These are a corrin ring and a nucleotide. The corrin ring is composed of four pyrrole rings linked to a centrally located cobalt atom. The nucleotide consists of a base, 5,6-dimethylbenzimidazole. The nucleotide is attached to

the corrin ring by ribose-3-phosphate and 1-amino-2-propanol. It is also directly bonded to the cobalt atom. The cobalt atom can bind with further side groups to form the various vitamin B₁₂ analogues.

In the human there are three groups of vitamin B₁₂ carriers. They are intrinsic factor (IF), transcobalamin II (TC II) and cobalophilin, which includes transcobalamin I (TC I) and transcobalamin III (TC III). IF is secreted by the parietal cells of the stomach and binds to vitamin B₁₂ in the duodenum and transports the vitamin to the ileum where it is absorbed. TC II is mainly produced in the liver and binds to vitamin B₁₂ after absorption of the vitamin. TC II then carries the vitamin to the body cells where it is either used or stored. The role of TC I and TC III is not quite clear yet. What is known is that TC I and TC III bind the vitamin exclusively for the transport to the liver for discard into the bile. The binding of TC I and TC III to vitamin B₁₂ also prevents the uptake of the vitamin by intestinal bacteria.

The only source of vitamin B₁₂ for man is any product of animal origin. Vitamin B₁₂ is only produced by micro-organisms i.e. bacteria that are found in the soil, water or the gastro-intestinal tract. Rich sources of the vitamin are the organ meats such as the liver, heart, kidney and bivalves i.e. clams and oysters. Thus for human intake the main sources of vitamin B₁₂ are meat and dairy products such as milk and cheese.

The nutritional need for vitamin B₁₂ is very little. As little as 0,1 µg daily is enough to produce a hematological response in patients with uncomplicated vitamin B₁₂ deficiency. The Recommended Daily Allowance (RDA) for adults has been set at 3 µg per day and this will sustain a normal body store content of 1 mg to 10 mg. In pregnancy a heightened vitamin B₁₂ intake of 4 µg daily is needed to compensate for fetal drainage on the maternal stores.

The absorption of vitamin B₁₂ takes place along two pathways. In the one path, also called the pharmacological path, absorption is along the entire small intestine and takes place by diffusion. This path is not dependant on IF for vitamin transport. About 1% of the free vitamin B₁₂ is absorbed via this path. The second pathway, also called the physiological path, is more complex in that it incorporates carriers for the transport of the vitamin to the site of absorption in the ileum via receptors.

In the mouth, vitamin B₁₂ is split from ingested food by salivary enzymes and then binds to a cobalophilin. The cobalophilin is also found in the saliva. The vitamin B₁₂-cobalophilin complex is swallowed and enters the stomach. In the stomach further vitamin B₁₂ is set free from food by the action of the stomach enzymes and acid. This free vitamin B₁₂ binds to more cobalophilin. In the duodenum, the vitamin B₁₂-cobalophilin complex comes in contact with an alkali surrounding. This is brought about by pancreatic juice which also contains enzymes that split or break down the cobalophilin from the vitamin B₁₂. The alkali medium is needed for the binding of IF to the vitamin B₁₂ which now takes place for the first time. For this, a pH above 6 is needed. The IF-vitamin B₁₂ complex is then transported to the ileum for absorption. In the ileum are receptors which bind the IF-vitamin B₁₂ complex. A pH above 6 is needed again as well as Ca²⁺.

After absorption, the vitamin B₁₂ binds to TC II and is transported to the body cells for utilization or storage.

Excess vitamin B₁₂ is stored in the body tissues. The total human body store ranges between 1 and 10 mg. The liver is the main storage organ with further smaller storage amounts in muscle, skin, bone, lungs, kidneys and spleen. The main storage form of vitamin B₁₂ is coenzyme B₁₂.

The average daily loss of vitamin B₁₂ is about 1,3 µg which is roughly equally divided between urine and feces.

In the human body, vitamin B₁₂ is directly involved in only two reactions. They are:

- a) the conversion of methylmalonyl CoA to succinyl CoA and
- b) the generation of methionine from homocysteine.

Through these reactions vitamin B₁₂ is linked to nucleic acid metabolism, fat metabolism, deoxyribonucleic acid (DNA) synthesis, carbohydrate metabolism and protein synthesis. The biologically active forms of the vitamin in man are coenzyme B₁₂ (5'-deoxyadenosylcobalamin) and methylcobalamin. There are many more reactions in which vitamin B₁₂ is involved, but these all take place in bacteria.

Vitamin B₁₂ deficiency is diagnosed when the serum level of the vitamin drops below 160 pg/ml. To look at the role vitamin B₁₂ has in the normal functioning of the human body, the effects of a vitamin B₁₂ deficiency can be looked at.

Illnesses attributed to folic acid deficiency have been reported as early as 1851. In 1937 Dr. L. Wills successfully treated patients with folic acid deficiency. The substance used for the treatment of the deficiency was named folic acid in 1941 and the structure of the vitamin was described in 1945. In the same year folic acid was found to cure megaloblastic anemia present in pregnancy.

The complete chemical name for folic acid is pteroylmonoglutamic acid which is usually shortened to pteroylglutamic acid or PGA. The folic acid molecule has a molecular mass of 454 d.

The folates are widely distributed in animal and plant foodstuffs. The highest concentrations are found in green vegetables, liver, yeast and nuts.

The Recommended Daily Allowance (RDA) for folic acid is 400 µg for adults. Normal total-body folate stores are 5 - 10 mg of which approximately half is in the liver. The folate stores are sufficient for only 3-5 months and therefore a folate deficiency can develop rapidly. THFA in conjunction with vitamin B₁₂ is essential for DNA synthesis and therefore essential for normal erythropoiesis.

Folic acid deficiency is usually a result of inadequate dietary intake or is secondary to disease. Deficiency produces an anemia in which the erythrocytes are abnormally large and their numbers are reduced. Most of the symptoms of folate deficiency mimic those of vitamin B₁₂ deficiency. The exception is that folate deficiency does not cause neurological damage. With a deficiency, the serum folate level is reduced and there is a change in erythrocyte production in the bone marrow. The diagnosis for folate deficiency is

usually based on serum and/or erythrocyte concentrations of folate. Values for serum folate equal to or greater than 6 ng/ml are generally regarded as normal.

A patient with folate deficiency is treated with 100 μg of folic acid orally or parenterally daily. The administration of folic acid to patients with megaloblastic anemia causes dramatic reversal of the abnormal changes in bone marrow. The erythrocytes become normal in size, their number increases, the total hemoglobin increases and leucocyte levels return to normal.

Pernicious anemia is characterized by a low erythrocyte count, Hct, Hb and vitamin B₁₂ levels together with a higher MCH and MCV. In severe cases of pernicious anemia these levels are extremely high or low as the case may be. Together with these values, the investigation of pernicious anemic blood and bone marrow smears reveal abnormally large erythrocyte precursors and fewer leucocytes. Abnormally shaped cells are also seen due to the disruption in DNA synthesis caused by the vitamin B₁₂ deficiency.

This condition occurs when there is a lack of vitamin B₁₂ and/or folic acid in the diet or when these substances cannot be absorbed. Megaloblastic anemia can be suspected from the presence of macrocytes, oval erythrocytes, pear-shaped poikilocytes and polymorphonuclear neutrophils with hypersegmented nuclei in a blood smear. This diagnosis can be confirmed by finding megaloblasts and giant metamyelocytes in the bone marrow and the assay of serum vitamin B₁₂ and folate can provide additional evidence for a firm diagnosis.

Abnormal vitamin B₁₂ radio isotope assay (RIA) readings were obtained from Tygerberg Hospital, as well as the results from a male patient suffering from pernicious anemia.

Normal values in hematology are important. From these normal values in can be determined whether a person is suffering from some or other disease or disorder.

Reference values against which vitamin B₁₂ and folate deficiency can be diagnosed were found to be:

Vitamin B ₁₂	-	males: 391,6 to 1136,8 pg/ml
		females: 307,9 to 821,9 pg/ml
Folate (serum)	-	males: 1,2 to 8,8 ng/ml
		females: 1,7 to 6,5 ng/ml
Erythrocyte count	-	males: 335 to 648,6 x 10 ¹⁰ /l
		females: 293,8 to 595,4 x 10 ¹⁰ /l
Hemoglobin	-	males: 13 to 17,4 g/dl
		females: 9,3 to 15,9 g/dl
Hematocrit	-	males: 23,7 to 72,1 %
		females: 26,3 to 58,3 %
MCV	-	males: 73,3 to 110,1 fl
		females: 70,2 to 114,6 fl
MCH	-	males: 24,9 to 37,7 g/dl
		females: 22,7 to 36,3 g/dl
MCHC	-	males: 22 to 43,6 g/dl
		females: 21,5 to 40,7 g/dl

In a typical case of pernicious anemia these above mentioned values will change as follows:

Vitamin B ₁₂	-	< 160 pg/ml
Folate (serum)	-	< 1,5 ng/ml
Erythrocyte	-	< 380 x 10 ¹⁰ /l
Hemoglobin	-	< 9 g/dl
Hematocrit	-	< 37 %
MCV	-	> 96 fl
MCH	-	> 33 pg
MCHC	-	not above 36 g/dl

In this workpiece the results from a patient suffering from pernicious anemia were compared to normal reference patient values. These analyses were done with the aid of a radio-isotope assay (RIA) kit for vitamin B₁₂, a Coulter Counter and by the visual means of photographs from microscopic preparations.

From the results obtained with the RIA kit the difference between the reference values and the abnormal values were clearly noted. The difference was also apparent when the readings for the Coulter Counter were done. With the help of photographs the effect of the deficiency in vitamin B₁₂ were seen on the formation of the erythrocytes in bone marrow and in the peripheral blood. Graphs were used to compare the results from anemic and normal reference blood. The graphs showed a good correlation between expected values and known normal values.

The microscopic investigation of abnormal blood and bone marrow smears were found to contain the following picture:

Clinical Features of Peripheral Blood:

Investigation of an abnormal blood smear reveals characteristic macrocytes, poikilocytes, polychromasia, anisocytes and Howell-Jolly bodies. Leukopenia can be seen with hypersegmentation of the polymorphonuclear neutrophils. Also present are giant metamyelocytes, myelocytes, thrombocytopenia and giant platelets.

Clinical Features of Bone Marrow Smears:

Thirty to fifty percent of the nucleated erythrocyte series are megaloblastic with the nuclei having a sieve-like appearance, bead-like chromatin strands and parachromatin areas. Promegaloblasts are also present with deeply basophilic cytoplasm. Polychromatophilic and acidophilic megaloblasts can be seen in groups showing signs of mitosis. Giant metamyelocytes are present with doughnut-shaped vacuolated nuclei. The cytoplasm is a poorly stained light blue to basophilic and relatively agranular. The megakaryocytes are reduced and abnormal in pattern by being multilobular.

Graphically, the results obtained clearly show the difference between normal patients and patients suffering from pernicious anemia.

There are many different methods of obtaining blood and bone marrow. There are also different stains which can be used to stain blood and marrow specimens. The methods as described in Chapter 3 were found to be satisfactory and gave good results.

Not only did this study produce a set of hematological reference values, but a significant drop in serum vitamin B₁₂ values during pernicious anemia was illustrated. The hematological effect was noted in the Coulter Counter blood analysis results and the microscopic examination revealed the presence of megaloblastic erythrocytes during vitamin B₁₂ deficiency.

OPSOMMING VAN TESIS

**DIE FISILOGIESE EFFEK VAN 'n VITAMIEN B₁₂ TEKORT
IN MENSLIKE BLOED**

deur

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OPSOMMING

Die eerste geval van pernisiëuse anemie was in 1822 gerapporteer. In 1948 was vitamien B₁₂ vir die eerste keer geïsoleer. Vitamien B₁₂ en folaat is baie belangrik vir die normale ontwikkeling van die liggaam en sy selle. Die bekendste kenmerk van 'n tekort aan hierdie vitamienes is die effek op die produksie en maturasie van die eritrosiete in die beenmurg. Groot of megaloblastiese eritrosiete word gevorm en in die bloed gevind. Hierdie selle word makrosiete genoem en het 'n korter leeftyd as normale eritrosiete. Minder eritrosiete word ook geproduseer en dus is die eritrosiet getal in die bloed laer as normaal.

Die soektog na vitamien B₁₂ is in 1926 begin toe daar ontdek was dat lewer vir die behandeling van pernisiëuse anemie gebruik kan word. Castle en medewerkers (1929) het gedemonstreer dat 'n intrinsieke faktor vanaf menslike maagsap aan 'n ekstrinsieke faktor wat in diere proteïene teenwoordig is gebind het. Vitamien B₁₂ was later as die ekstrinsieke faktor geïdentifiseer. In 1948 het twee groepe werkers 'n substans vanaf lewer, wat 'n hematologiese respons op persone met pernisiëuse anemie gehad het, geïsoleer.

Die volgende belangrike ontdekking was toe Barker en medewerkers die ko-ensiem vorms van vitamien B₁₂ ontdek het. Tot op daardie tydstip was die koensieme nog altyd mis gekyk omdat hulle maklik deur lig af gebreek is.

Lenhert en Hodgkin (1961) het die struktuur van vitamien B₁₂ beskryf deur gebruik te maak van 'n X-straal kristallografiese metode. Die uitstaande kenmerk van die molekule is sy kompleksiteit en die feit dat dit die enigste vitamien is wat 'n kobalt atoom bevat.

Die vitamien B₁₂ molekule het 'n molekulêre massa van 1355 dalton (d) en bestaan uit twee hoof dele, naamlik 'n korrin ring en 'n nukleotied. Die korrin ring bestaan uit vier pirroolringe wat aan 'n sentraal gelêe kobalt atoom gebind is. Die nukleotied bestaan uit die basis 5,6-dimetielbezimidazool. Die nukleotied is gebind aan die korrin ring deur ribose-3-fosfaat en 1-amino-2-propanol. Dit is ook direk aan die

kobalt atoom gekoppel. Die kobalt atoom kan nog met verskeie ander substituentte bind om sodoende die verskeie vorms van vitamien B₁₂ te vorm.

In die mens kom daar drie groepe vitamien B₁₂ draers voor, naamlik intrinsieke faktor (IF), transcobalamin II (TCII) en cobalophilin, waarin transcobalamin I (TCI) en transcobalamin III (TCIII) ingesluit word. IF word deur die parietale selle van die maag gesekreter. IF bind met vitamien B₁₂ in die duodenum en vervoer dit na die ileum waar dit geabsorbeer word. TCII word hoofsaaklik in die lewer geproduseer en bind na absorpsie met vitamien B₁₂. TCII dra die vitamien dan na die liggaamsselle waar dit of gebruik of gestoor word. Die funksie van TCI en TCIII is nog nie heeltemaal duidelik nie. Dit is wel bekend dat TCI en TCIII met vitamien B₁₂ bind en na die lewer vervoer vir storting in die gal. Die binding van TCI en TCIII verhoed ook dat intestinale bakterië die vitamien absorbeer.

Die enigste bron van vitamien B₁₂ vir die mens is vanaf 'n dierlike oorsprong. Vitamien B₁₂ word net deur bakterië wat in die grond, water en gastrointestinale kanaal voorkom vervaardig. Die orgaan vleisse soos die lewer, hart, nier en mossels is ryke bronne van die vitamien. Die hoof bronne van vitamien B₁₂ vir die mens is vleis en suiwelprodukte soos kaas en melk.

Die liggaams aanvraag vir vitamien B₁₂ is baie min. 'n Daaglikse inname van selfs 0,1 µg is genoeg om 'n hematologiese respons teweeg te bring in persone met ongekompliseerde vitamien B₁₂ tekort. 'n Daaglikse inname van 3 µg vitamien B₁₂ word aanbeveel wat 'n normale liggaams stoor van 1 mg tot 10 mg sal onderhou. Gedurende swangerskap word 4 µg daaglik aanbeveel om te kompenseer vir die fetale onttrekking uit die moeder se store.

Die absorpsie van vitamien B₁₂ vind langs twee paaie plaas. In die een pad, ook die farmakologiese pad genoem, vind absorpsie deur diffusie langs die hele dunderm plaas. Hierdie pad is nie afhanklik van IF vir vervoer van die vitamien nie. Omtrent 1% van die vrye vitamien B₁₂ word op hierdie manier geabsorbeer. Die tweede pad word die fisiologies pad genoem en is meer kompleks omdat draers betrokke is met die vervoer van die vitamien na die ileum, die plek van absorpsie.

In die mond word vitamien B₁₂ van voedsel geskei deur speeksel ensieme. Die vrye vitamien B₁₂ bind dan aan 'n cobalophilin wat ook in speeksel voorkom. Die vitamien B₁₂-cobalophilin kompleks word gesluk en beland in die maag. In die maag word verdere vitamien B₁₂ vanaf voedsel geskei deur die werking van maagsap ensiemes en suur. Die vrye vitamien B₁₂ bind aan nog cobalophilin. In die duodenum kom die vitamien B₁₂-cobalophilin kompleks in kontak met 'n alkaliese omgewing wat veroorsaak word deur die pankreatiese sekresie. Die sekresie bevat ook ensieme wat die cobalophilin van vitamien B₁₂ skei of afbreek. Die alkaliese omgewing is nodig vir die binding van vitamien B₁₂ met IF wat nou vir die eerste keer plaasvind. Hiervoor is 'n pH groter as 6 nodig. Die IF-vitamien B₁₂ kompleks word dan na die ileum vervoer vir absorpsie. In die ileum kom daar reseptore voor waaraan die IF-vitamien B₁₂ kompleks bind. Hiervoor is Ca²⁺ nodig sowel as 'n pH groter as 6.

Na absorpsie bind vitamien B₁₂ aan TCII wat die vitamien vervoer na die liggaamsselle waar die vitamien of gebruik word of gestoor word. Oortollige vitamien B₁₂ word in die liggaamswefsel gestoor. Die totale liggaamsstoor by die mens is tussen 1 mg en 10 mg. Die meeste vitamien B₁₂ word in die lewer gestoor, maar klein hoeveelhede word ook in spierweefsel, vel, been, longe, niere en die milt geberg. Vitamien B₁₂ word hoofsaaklik in die koensiem B₁₂ vorm gestoor. Die gemiddelde daaglikse verlies van vitamien B₁₂ is 1,3 µg.

Vitamien B₁₂ is slegs aan twee reaksies direk by die mens gekoppel, naamlik:

- a) die omskakeling van metielmaloniel KoA na suksiniel KoA
- b) die produksie van metionien vanaf homosisteien.

Deur middel van hierdie twee reaksies is vitamien B₁₂ betrokke in nukleiensuur metabolisme, vet metabolisme, de-oksiribonukleïensuur (DNA) sintese, koolhidraat metabolisme en proteïen sintese. Die biologies aktiewe vorms van vitamien B₁₂ by die mens is koensiem B₁₂ (5'-deoksi-adenosielcobalamin) en metielcobalamin. Daar is nog baie ander reaksies waarby vitamien B₁₂ betrokke is, maar dit vind in bakterië plaas.

'n Vitamin B₁₂ tekort word gedefinieer wanneer die serum vlak van die vitamien onder 200 pg/ml is. Om die invloed van vitamien B₁₂ op die normale werking van die menslike liggaam te illustreer, kan die effek van 'n vitamien B₁₂ tekort ondersoek word.

Siektes deur folaat veroorsaak is sedert 1851 al gerapporteer. In 1937 het Dr. L. Wills pasiënte suksesvol vir folaat-tekort behandel. In 1941 was die substans wat vir die behandeling van 'n folaat-tekort gebruik was bekendgemaak en die struktuur was in 1945 beskryf. In dieselfde jaar is ontdek dat folaat ook megaloblastiese anemie in swangerskap genees.

Die volledige chemiese naam vir folaat is pteroylmonoglutamiensuur wat verkort word na pteroylglutamiensuur of PGA. Die folaat molekule het 'n molekulêre massa van 454 d.

Die folate is versprei in dier- en plant-voedsel. Die hoogste konsentrasie word in groen groentes, lewer, gis en neute gevind.

Die Voorgeskrewe Daaglikse Toelaag vir folaat is 400 µg vir volwassenes. Die normale totale liggaams folaat stoor bevat 5 mg tot 10 mg waarvan die helfte in die lewer is. Hierdie store is genoegsaam vir slegs 3 tot 5 maande en daarom kan 'n folaat tekort baie gou ontwikkel.

'n Folaat tekort is gewoonlik as gevolg van 'n lae dieët inname en is sekondêr tot siektes. 'n Tekort lei tot 'n anemie met 'n verlaagde getal abnormaal groot eritrosiete. Die meeste simptome van 'n folaat tekort is dieselfde as in 'n vitamien B₁₂ tekort, maar die verskil is dat 'n folaat tekort nie neurologiese beskadiging veroorsaak nie. Die serum folaat vlak by 'n tekort is laer en daar is 'n verandering in eritrosiet produksie in die beenmurg. Die diagnose vir 'n folaat tekort word gebaseer op serum en/of eritrosiet folaat konsentrasies. Die normale waardes vir serum folaat is gelyk aan of groter as 6 ng/ml.

'n Pasiënt met 'n folaat tekort word behandel met 100 µg folaat per dag. Die toediening van folaat op pasiënte met megaloblastiese anemie veroorsaak dat die abnormale veranderinge in die beenmurg ophou en

terugkeer na normaal. Die eritrosiet word normaal in grote, hul getal vermeerder, die totale hemoglobien verhoog en die leukosiet vlak styg na normaal.

Pernisieuse anemie word gekenmerk deur lae eritrosiet, hematokrit (Hct), hemoglobien (Hb) en vitamien B₁₂ vlakke tesame met hoër gemene korpuskulêre hemoglobien (GKH) en gemene korpuskulêre volume (GKV) lakke. Tesame met hierdie waardes wys die ondersoek van bloed en beenmurg in pernisieuse anemie abnormale groot eritrosiet voorgangers en minder leukosiete. Abnormale sel vorms word ook gesien a.g.v. die onderbreking in DNA sintese wat deur 'n vitamien B₁₂ tekort veroorsaak word.

Hierdie toestand vind plaas wanneer daar 'n vitamien B₁₂ en/of folaat tekort in die dieët is of wanneer hierdie vitamien nie geabsorbeer kan word nie. Die teenwoordigheid van makrosiete, ovaal eritrosiete, peervormige poikilosiete en polimorfonuklêre neutrofiele met hipergesegmenteerde kerne in bloedsmere dui op die toestand van megaloblastiese anemie. Hierdie diagnose kan ondersteun word deur die aanwesigheid van megaloblaste en reuse metamiëlosiete in die beenmurg. Die bepaling van vitamien B₁₂ en folaat vlakke in die bloed kan as addisionele bewysstukke vir 'n volledige diagnose dien.

Abnormale vitamien B₁₂ waardes was vanaf Tygerberg Hospitaal verkry, sowel as resultate van 'n manlike pasiënt wat aan pernieseuse anemie gely het.

Normal waardes in hematologie is baie belangrik. Vanaf hierdie waardes kan bepaal word of 'n persoon aan een of ander siekte lei.

Vergelykende waardes waarteen vitamien B₁₂ en folaat tekorte gediagnoseer kan word was:

Vitamien B ₁₂	- mans: 391,6 tot 1136,8 pg/ml
	vrouens: 307,9 tot 821,9 pg/ml
Folaat	- mans: 1,2 tot 8,8 ng/ml
	vrouens: 1,7 tot 6,5 ng/ml
Eritrosiet getal	- mans: 335 tot 648,6 x 10 ¹⁰ /l
	vrouens: 293,8 tot 595,4 x 10 ¹⁰ /l
Hemoglobien	- mans: 13 tot 17,4 g/dl
	vrouens: 9,3 tot 15,9 g/dl
Hematokrit	- mans: 23,7 tot 72,1 %
	vrouens: 26,3 tot 58,3 %
GKV	- mans: 73,3 tot 110,1 fl
	vrouens: 70,2 tot 114,6 fl
GKH	- mans: 24,9 tot 37,7 g/dl
	vrouens: 22,7 tot 36,3 g/dl
GKHK	- mans: 22 tot 43,6 g/dl
	vrouens: 21,5 tot 40,7 g/dl

In 'n tipiese geval van pernisiëuse anemie sal die bogenoemde waardes soos volgs verander:

Vitamien B ₁₂	- < 160 pg/ml
Folaat	- < 1,5 ng/ml
Eritrosiete	- < 380 x 10 ¹⁰ /l
Hemoglobien	- < 9 g/dl
Hematokrit	- < 37 %
GKV	- > 96 fl
GKH	- > 33 pg
GKHK	- nie hoër as 36 g/dl nie

In hierdie werkstuk was die resultate van 'n pasiënt wat aan pernisiëuse anemie gelyk het vergelyk met normale waardes. Hierdie analyses is gedoen met behulp van 'n RIA toets vir vitamien B₁₂, 'n Coulter teller en deur fotos van mikroskopiese preparate van periferebloed en beenmurg smere.

Die resultate het 'n duidelike verskil tussen normale vergelykende waardes en die abnormales getoon. Hierdie verskil was ook in die Coulter teller lesings te sien. Met behulp van die fotos was die effek van die vitamien B₁₂ tekort op die produisie van eritrosiete in die beenmurg sowel as in die perifere bloed smeer duidelik sigbaar. Grafieke is gebruik om die waardes van anemiese en normale vergelykende waardes aan te dui.

Die mikroskopiese ondersoek van die abnormale bloed en beenmurg smere het die volgende beeld gegee:

Kliniese Kenmerke van Perifere Bloed:

Die kenmerkende makrosiete, poikilosiete, polikromasie, anisosiete en Howell-Jolly liggaampies was teenwoordig. Leukopenie was ook teenwoordig met hipergesegmenteerde polimorfonukleêre neutrofiel. Daar was ook reuse metamiëlosiete, miëlosiete, trombosiete en reuse bloedplaatjies teenwoordig.

Kliniese Kenmerke van Beenmurg Smere:

Dertig tot vyftig persent van die nukleêre eritrosiete reeks was megaloblasties. Die kerne was lig en die chromatien uitgesprei. Promegaloblaste was ook teenwoordig met diep basofiel gekleurde sitoplasma. Polikromatofiele en asidofiele megaloblaste was in groepies sigbaar asook tekens van mitose in die kern. Reuse metamiëlosiete was teenwoordig met ringvormige kerne. Die sitoplasma was lig gekleur en relatief agranulêr. Die megakariosiete was minder en abnormaal in vorm.

Daar is verskeie metodes vir die verkryging van bloed en beenmurg. Verskeie kleurings metodes kan ook gebruik word vir die kleuring van bloed en beenmurg. Die metodes in Hoofstuk 3 beskryf was bevredigend en het goeie resultate gelewer.

Gedurende hierdie studie is daar nie alleen 'n stel hematologiese verwysingswaardes vasgestel nie, maar die studie het ook getoon dat daar 'n beduidende verlaging in serum vitamien B₁₂ waardes gedurende pernisiëuse anemie is. Die hematologiese effek in die Coulter teller se bloed analiese was duidelik waarneembaar en mikroskopiese ondersoeke het die teenwoordigheid van megaloblastiese eritrosiete tydens 'n tekort aan vitamien B₁₂ aangetoon.

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