

“Dying To Be Thin”

Learning Objectives

1. To identify poor nutrition as an important biological component in eating disorders.
2. To define hunger, appetite, taste, satiety, palatability.
3. To summarize homeostatic (set point) and non-homeostatic (behavioural) mechanisms of eating behaviour.
4. To describe the role of the hypothalamus in eating behaviour.
5. To summarize the role of leptin.
6. To identify components of energy expenditure.
7. To identify energy stores in the body and how they are used.
8. To summarize three important areas for nutritional assessment in a patient.
9. To list metabolic changes in starvation that result in malnutrition crisis.
10. To differentiate physical signs of anorexia nervosa in terms of starvation, malnutrition, and impairment of growth and development.

Eating Disorders

These are a group of syndromes with *biological*, *psychological* and *sociological* components. It is important that all three of these aspects are considered. The focus here is on biological aspects of eating /diet with a particular emphasis on the metabolic consequences of starvation. Nutrition concepts introduced here will be extended and developed in GI Nutrition Weeks.

Some Definitions

Hunger: sensation induced by a deficit in readily-metabolizable energy nutrients.

Appetite: sensory stimulation (desire) produced by the view, smell or memory of specific tastes / foods.

Taste: sensation produced by taste-buds on tongue: sweet, sour, salty, bitter.

Satiety: sensation of being satisfied and full after eating.

Palatability: subjective orosensory pleasantness of a food.

Eating Behaviour

‘Static’ theories assumes that our bodies sense a ‘set point’ and defends this using neurological and biochemical signals. For example, the glucostatic theory proposes that low blood glucose levels trigger hunger and feeding (short term) and that satiety is the result of high circulating glucose. Similarly, the lipostatic theory hypothesizes that the body tries to maintain steady lipid (fat) levels using central (hypothalamus) and peripheral (stomach, vagus nerve) mechanisms.

‘Dual-Center Theory’ proposed that the lateral hypothalamus (LH) was a ‘hunger center’ and the ventromedial nucleus of the hypothalamus (VMH) a ‘satiety center’. The original hypothesis did not hold up to experimentation but more detailed molecular information, however, does lend support to a hypothalamic mechanism (see below).

Non-homeostatic mechanisms may better explain eating behaviour in our modern environment where there is choice and food is plentiful and highly palatable. For example, the neuro-biological mechanisms that connect hunger, appetite and satiety involve both objective (unconditioned) and subjective (conditioned) components. We learn how our feeding behaviour is coupled to physiological events that we can sense directly from external stimuli as well as from physiological and neuro-endocrine responses.

This is a conditioned association. An example is the cephalic response of insulin secretion produced by the sight and smell of food. Taste aversions and preferences can be regarded as similar learned / reflex responses. Learning and social influences may similarly play a role in the anticipation of meals, palatability and self-control. Effectively such factors become important in the eating behaviour of societies where food is plentiful and cheap. Behavioural (non-homeostatic) mechanisms may also better explain why eating disorders (e.g. anorexia, obesity) are prevalent today.

Endocrine and neuroendocrine regulation: Many biochemicals are involved in the physiology of eating and satiety. Insulin promotes uses of glucose after feeding while glucagon, epinephrine and cortisol act to increase glucose availability and fatty acid release from adipose tissue during starvation (much more on this in Endocrine Weeks later in the year). Signals to terminate feeding include stomach stretch receptors, glucoreceptors receptors in the duodenum and cholecystokinin (CCK), a peptide neurotransmitter released by duodenum which binds to CCK receptors in the GI tract and brain. Many other GI peptides and hormones have effects: bombesin and somatostatin inhibit eating while neuropeptide Y and galanin increase feeding.

Hypothalamic regulation. The discovery of leptin has refocused attention on the hypothalamus as playing a critical role in maintaining energy homeostasis by integrating and coordinating metabolic, neuroendocrine and behavioral responses and arousal states. Leptin is the product of the *ob* gene which is only expressed by adipocytes (adipose tissue). Leptin decreases feeding by binding to receptors in the arcuate and dorsomedial nuclei of the hypothalamus (close to the VMH!) resulting in diminished secretion of neuropeptide Y, a potent stimulator of feeding behaviour. Many other neuropeptides and their receptors are the focus of current research (see figure).

Nutritional Background to Starvation

(N.B. Before continuing here, students will need to review the metabolism of carbohydrate, protein and fat in the Q1 notes. See 'Metabolic Compartments and Integration', particularly the section 'The Fasting State'.)

Question: What happens to someone who is not eating and who has no fat stores? Quick answer: "They die!"

The richest source of energy is fat (fatty acids) which provides 9 kcal/gram (dry). Carbohydrate (glucose) and protein (amino acids) each provide less than half of that by weight, 4 kcal/gram (dry). Energy is stored in the body as glycogen (liver and muscle) and as triglyceride (fat) in adipose tissue. In the short term, carbohydrate is the principal energy source while, in the long term, fat is the normal energy reserve. Normally, body proteins are broken down to amino acids and re-synthesized continuously. While gluconeogenesis from amino acids provides some circulating glucose when carbohydrate stores (glycogen) become depleted, the body inevitably switches to fat mobilization and oxidation to meet energy needs if food intake is restricted unduly. During very long periods of starvation, fat stores eventually become depleted, and amino acids (protein) are then used as a 'last resort' source of energy and body proteins are depleted. However this process cannot be continued for a long period as is not compatible with life. Why?

Nutrition Evaluation of a Patient

Broadly a physician can evaluate a patient by verifying three things: 1) that the food being eaten is adequate both in terms of *quantity* and *quality*; 2) that *intestinal absorption is intact* (not damaged or diseased); and 3) that the individual's *body metabolism is unimpaired* (no serious metabolic disease).

Growth and Development

Initially, an individual not eating enough calories (carbohydrate, fat and protein) will be unable to gain weight! In children and adolescents this critically results in impaired growth and development. Such a situation can also develop in an individual consuming some calories but whose physical activities exceed this energy input, resulting in an overall negative energy balance (weight loss). Secondly, an individual eating a diet which lacks variety (different kinds of foods) is at risk to developing micronutrient (vitamin and mineral) deficiency. Patient with eating disorders are at risk to both kinds of malnutrition.

Malnutrition Crisis

It has been said that victims of eating disorders are “dying to be thin”. Literally. Growth and development become impaired and life can be threatened. So much lean tissue is lost that the body’s BMR slows and physical performance is impaired. Digestion becomes inefficient (pancreatic impairment and thinning of the intestinal mucosa) causing diarrhea if food is eaten. Constipation is also general due to little food and poor GI transit. Body temperature drops, brain electrical activity becomes abnormal (insomnia is common) and hormonal secretion becomes impaired - both females and males lose their sex drives. Patients may die from heart problems. What kinds of problems and why do they occur? A list of physical signs noted in anorexia nervosa follows. Try to explain the etiology of each sign in terms of your understanding of malnutrition.

Physical Signs of Anorexia Nervosa

(from Mahan LK, Rees JM, *Nutrition in Adolescence*, Mosby, St. Louis, 1984)

Fat store depletion	Acrocyanosis
Muscle wasting	Postural hypotension
Amenorrhea	Dehydration
Cheilosis	Edema
Desquamation	Brachycardia
Dry skin	Bradypnea
Hirsutism	Hypothermia
Thin, dry, brittle hair	Constipation
Alopecia	Sleep disturbance
Degradation of fingernails	

Website reference

Jane Rees, an expert in the nutritional problems of adolescent eating disorders, maintains a detailed website for health care professionals, students and educators:

<http://faculty.washington.edu/jrees/adolescentnutrition1.html>

Red Blood Cell

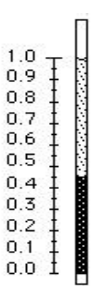
Learning Objectives

1. To summarize the life cycle of the erythrocyte (red blood cell).
2. To identify a mature red blood cell as having no nucleus, mitochondria or ribosomes and using the glycolytic and phosphogluconate pathways for metabolic energy.
3. To define anemia as a >10% lowering of red cell mass (hematocrit or hemoglobin)
4. To summarize the use of the mean cell volume (MCV) in differentiating microcytic, macrocytic

and normocytic anemias.

The red blood cell is born in the marrow as a stem cell. This is a common precursor for red and white cells. Stem cells differentiate (under the influence of erythropoietin, a hormone secreted by kidney) within marrow into erythroblasts, normoblasts, reticulocytes and, finally, erythrocytes.

During this progression, the size of the cells decreases, mitotic competence is lost as the cells differentiate, the size of the nucleus decreases and disappears completely in mature red cells. Mitochondria and ribosomes are still present in reticulocytes (polychromasia) but even these are absent in RBCs. RBCs generate energy through the glycolytic and phosphogluconate pathways. Mature RBCs are biconcave discs (see figure on right).



Erythrocytes exist in the circulation for about 120 days. Eventually they are removed by the mononuclear phagocyte system where they are broken down. The products of this catabolism are amino acids and Fe, both of which are reutilized, and bilirubin which is excreted in bile. A number of measurements are used to assess red blood cell status. A measure of the red cell mass is hematocrit or the packed cell volume (PCV) when a blood sample is centrifuged (see figure at left of microhematocrit tube). The wide availability of electronic counters allows the calculation of the mean cell volume (MCV). $MCV = PCV / RBC$ (the red blood cell count). Hemoglobin concentration can also be measured easily colorimetrically. A Complete Blood cell Count (CBC) gives all this information - it is one of the commonest tests ordered on patients.

Complete Blood Count (numbers are NOT examinable)

<u>Item</u>	<u>Abbrev.</u>	<u>Reference Range (Units)</u>
White Blood cell Count	WBC	4.0 - 10.0 ($10^9/L$)
Red Blood cell Count	RBC	4.0 - 5.6 ($10^{12}/L$)
Hemoglobin conc.	HB	115 - 160 (g/L)
Hematocrit	HCT	0.34 - 0.45 (L/L)
Mean Cell Volume	MCV	79 - 97 (fL)
Mean Cell Hb Conc	MCHC	320 - 370 (g/L)
Red cell Distribution Width	RDW	12.0 - 15.0
Platelet count	PLT	150 - 450 ($10^9/L$)
Lymphocyte count	LYMPH#	0.6 - 4.1 ($10^9/L$)
Monocyte count	MONO#	0.08 - 1.5 ($10^9/L$)
Granulocyte count	GRAN#	1.6 - 7.5 ($10^9/L$)
Percent lymphocytes	LYMPH%	0.14 - 0.41
Percent monocytes	MONO%	0.02 - 0.1
Percent granulocytes	GRAN%	1.6 - 7.5

Anemia is defined as a "significant" (>10%) reduction in total circulating red cell mass which is usually diagnosed by decreased blood hemoglobin concentration. Based on the hemoglobin concentration, anemias can be **hypochromic** or **normochromic**. Anemia may be due to: a) blood loss; b) underproduction of blood cells; or c) accelerated destruction of blood cells (hemolysis). Use of the MCV provides a useful classification of the underproduction anemias: a) **Microcytic** anemias have a lowered MCV. They are generally caused by an under production of one of the major components of hemoglobin, either globin chains (thalassemia), heme (sideroblastic anemia) or iron (iron-deficiency anemia);

- b) **Macrocytic** anemias have an increased MCV. These anemias are due to maturation problems in the marrow (classically, folate and cobalamin deficiencies);
- c) **Normocytic** are characterized by a normal MCV but a lowered RBC. Underproduction of cells can be due to a variety of secondary disease.

Iron

Learning Objectives

1. To identify biochemical and metabolic functions of iron.
2. To identify the functions of transferrin and ferritin in iron metabolism.
3. To summarize clinical measures of iron status in iron deficiency and iron overload.
4. To summarize factors influencing the absorption of dietary iron and explain why iron deficiency is common.
5. To summarize body iron metabolism and iron homeostasis.
6. To identify that excessive amounts of iron in the body are toxic and can result in damage to liver, heart and pancreas.
7. To identify abnormal iron absorption as the cause of genetic hemochromatosis.
8. To recognize that genetic hemochromatosis is inherited as an autosomal recessive mutation and that the disease frequency is approximately 3 per 1000 of the population.
9. To identify thalassemia (defective globin chain production) and sideroblastic anemia (defective heme synthesis) as important causes of secondary iron overload.
10. To identify phlebotomy as the treatment of choice in genetic hemochromatosis whereas patients with thalassemia and sideroblastic anemia may need to be transfused.

Iron Essentiality

Iron is essential for several separate mechanisms that involve the metabolism of oxygen or oxidation/reduction: a) transport of O₂; b) transport of electrons in the cytochromes of the respiratory chain of mitochondria; and c) as an essential cofactor in ribonucleotide reductase, a fundamentally important regulator of DNA synthesis (remember?). In biochemical terms, iron uses one of its 6 coordinate bond in a) and its ability to change valence states in b) and c).

Iron Proteins and Tests

Hemoglobin is the functional molecule in erythrocytes responsible for binding oxygen in the lung and delivering it to tissues.

Transferrin is a serum protein which delivers iron to the marrow for the synthesis of new RBCs.

Ferritin is (largely) a cellular protein (in the cytoplasm of all cells). It stores iron.

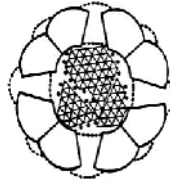
There are several tests of iron metabolism:

serum iron measures the iron concentration in serum (all bound to transferrin). The two Fe-binding sites in transferrin are normally < 50% saturated with iron.

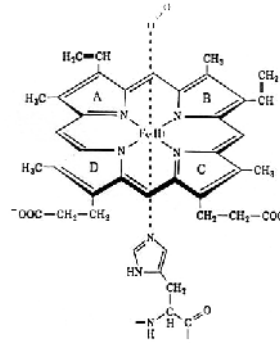
total iron binding capacity (TIBC) is the amount of iron capable of being bound by a person's circulating transferrin level, i.e. if it was completely saturated by iron.



TRANSFERRIN



FERRITIN



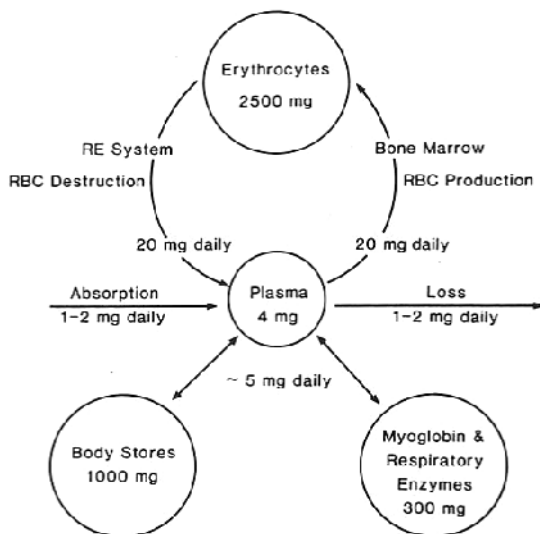
HEME

(Transferrin is a bilobular protein; the Fe atoms are highlighted. Ferritin is composed of 24 subunits and the Fe is crystallized lattice at the core of the protein. In heme the Fe is coordinated at the center of the protoporphyrin)

transferrin saturation is given by serum Fe/TIBC. Transferrin saturation is the best measure of Fe delivery to the erythron (growing red cell mass) - better than serum Fe or TIBC alone.

serum ferritin. Although ferritin is a cellular protein, tiny amounts of it leak into the circulation and are present in serum. This is very useful clinically to determine iron deficiency as the amount of serum ferritin is proportional to amount of ferritin in the body and is therefore a measure of iron stores.

hemosiderin. In a tissue sample (liver biopsy, marrow sample) iron stores can be visually estimated by a Prussian Blue stain. This stains for hemosiderin which is an ill-defined complex of ferritin with some protein removed. Hemosiderin is not water-soluble and is the most concentrated form of storage iron.



Iron Regulation

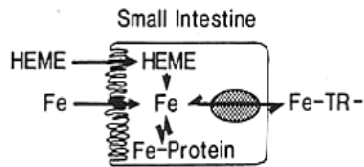
The iron content of the body is approximately 4g; body Fe stores (ferritin and hemosiderin) are approx. 1g. Fe loss from the body is small (1mg/day) and obligatory. There is no excretion mechanism for iron. Somehow the intestinal absorption of iron is regulated by the size of body iron stores. When iron stores drop, absorption increases to correct the deficit. The nature of this "communication" is not completely understood although it involves the metabolism of the transferrin receptor and other proteins at the basolateral membrane of intestinal absorptive cells. Iron deficiency. If iron stores are lowered but hemoglobin is still normal, a person may be iron-deficient without being anemic. Iron deficiency anemia only occurs when iron stores are exhausted.

Intestinal Iron Absorption

Iron is not easily absorbed in the duodenum, probably as a

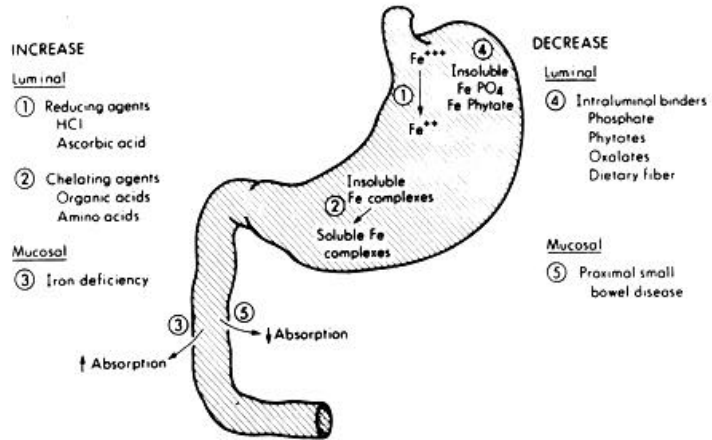
protection against iron toxicity. The absorption of iron from food depends on: a) the chemical form of iron - heme Fe or inorganic Fe (ferrous iron is more soluble than ferric); b) binding components in the diet (prevent iron release and solubility); c) good stomach function (acid solubilizes iron); d) the absorptive capacity of the upper intestine (the duodenum is where iron is absorbed).

Iron in food can be divided into heme Fe (broadly meat! Why?) and non-heme Fe (ferritin and ionic iron). Each is absorbed by separate mechanisms but non-heme Fe is



subjected to many intraluminal interactions (see figure). Most dietary iron is non-heme and inefficiently absorbed. Iron deficiency is a worldwide problem because many forms of vegetable and cereal iron are unavailable, because meat is not present in many and because many 3rd World populations are subjected to parasites which cause intestinal bleeding.

Phytate (*myo*-inositol hexaphosphate) is an example of a vegetable component which binds iron and renders it insoluble.



Iron Overload

While iron deficiency is a prevalent world-wide problem, iron overload, causing iron toxicity, occurs in some specific diseases.

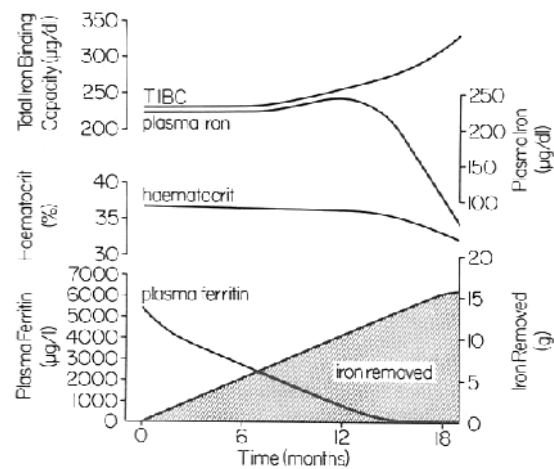
hemochromatosis is an autosomal recessive genetic disease and is said to cause primary iron overload by increasing the intestinal absorption of iron.

secondary iron overload occurs in diseases such as thalassemia or sideroblastic anemia because the iron accumulates secondary to a faulty synthesis of globin or heme, respectively, mainly because of therapeutic blood transfusion.

hemosiderosis is the term used to describe the presence of stainable Fe in tissues. Iron toxicity to cells may be due to disruption of Fe-laden lysosomes and lipid peroxidation of organelles by excess iron. Tissue iron overload from whatever cause leading to fibrosis and organ failure.

Hemochromatosis

Genetic HC is not a rare disease and is frequently missed as a primary diagnosis in patients with liver, pancreas or heart failure who experience organ damage due to iron toxicity. It is an 'autosomal recessive' disease and in Caucasian populations of Celtic descent the gene frequency is 5%. Therefore the heterozygote (carrier) frequency is 10% and the disease (homozygote) frequency is about 0.3%. The



genetic defect has led to the identification of a previously-unknown membrane protein in iron metabolism with similarities in structure to HLA (Human Leukocyte Antigen) proteins. Several mutations in this have been identified which could prevent the normal inhibitory effect the protein has on iron absorption. Further information can be viewed here:

<http://www.hosprract.com/genetics/9908/mmcpres.htm>

This 'silent' disease is expressed symptomatically 5-10 times more frequently in males than females (why?). Once diagnosed, iron is easily removed from the body by weekly phlebotomy of 500ml (200-250mg Fe). Phlebotomy is continued until the serum iron and ferritin return to normal values. Obviously patients with genetic HC do not have anemia since there is no problem with heme or globin production and iron is plentiful.

Sideroblastic anemia

Sideroblastic anemia is caused by defective heme synthesis. Sideroblastic anemia (SA) consists of a group of diverse disorders characterized by ringed sideroblasts in the nucleated red cell population of marrow. SA occurs in about 30% of hospitalized alcoholics. Ringed sideroblasts disappear within several days of cessation from alcohol. Occasionally, large doses of vitamin B₆ result in at least a partial correction of the anemia in some patients with an hereditary form of the disease due to defective δ -aminolevulinic acid synthetase, the first and rate limiting enzyme of heme synthesis. Fe overload is secondary, from increased Fe absorption as well as Fe from blood transfusions (treatment).

Thalassemia

The term covers a variety of diseases where globin synthesis is genetically defective. Fe overload is secondary, increased Fe absorption as well as Fe from blood transfusions (treatment). The severity of anemia relates to how severe the globin defect is (how many globin chains are affected). Many patients whose ethnic background is from the Mediterranean countries have δ -thalassemia which caused by mutations in both δ globin genes (Cooley's Anemia). Thalassemias resulting from defective α globin chain synthesis include, Hydrops Fetalis (no functional α genes) and Hb H Disease (three defective α globin genes). Patients that are heterozygous for defective α or δ globin genes exhibit thalassemia trait (or Thalassemia Minor) with mild clinical symptoms.

In all thalassemias, the severity of clinical disease depends on the degree to which intact functional hemoglobin is impaired. For example in δ thalassemia, patients appear normal hematologically at birth because of normal (α globin and Hb H formation. Normally Hb H switches to Hb A in the first year of life and this is when initial problems arise (severe anemia, pallor, poor growth and food intake, hepatosplenomegaly). Because untreated severe δ thalassemia causes gross expansion of marrow spaces in the skull, such children can become grossly deformed. Transfusion of red cells prevents this. It is now recognized that blood transfusions that can maintain a normal circulating hemoglobin level also will eliminate all the clinical signs of the disease (severe anemia, growth retardation, bony abnormalities, hepatosplenomegaly). Patients with δ thalassemia that require blood transfusions as said to have Thalassemia Major whereas those than can maintain some functional hemoglobin concentration, by virtue of high Hb F for example, are classified as having Thalassemia Intermedia.

Chelation therapy

Because survival depends on regular blood transfusion, thalassemia patients accumulate iron. One unit of blood (500 ml) contains 200 - 250 mg of iron. Many thalassemia patients, even in their teenage years, may have accumulated 25-30 g of iron. This must be removed by chelation therapy. A chelating agent, frequently deferoxamine, is administered subcutaneously. By using a small pump to deliver the drug

continuously and slowly for long periods, 20-30 mg of iron can be removed on a daily basis. Effective chelation and removal of iron from these patients is essential to prevent the morbidity and mortality due to iron toxicity.

Folic Acid

Learning Objectives

1. *To define a vitamin, vitamin 'B' and the term coenzyme.*
2. *Identify folate as a vitamin essential to several important biochemical reactions in 'one-carbon' metabolism.*
3. *To identify tetrahydrofolate as the coenzyme of the vitamin folacin and that tetrahydrofolate can exist in several metabolically-active forms which are specific for certain biochemical reactions.*
4. *To understand that the megaloblasts present in the circulation of people with folate deficiency arise because of defects in DNA synthesis due to defects in purine and thymidine synthesis.*
5. *To identify methyltetrahydrofolate as the most abundant form of folate and the circulating form in body fluids and formed by an irreversible reaction.*
6. *To recognize that folacin deficiency can occur through dietary insufficiency, impaired absorption, defective utilization and through competitive interactions with several kinds of drugs.*
7. *To recognize pregnant women and alcoholics as two populations at risk to developing folate deficiency.*
8. *To recognize that a prophylactic use of folate supplementation in preventing birth defects, and in reducing cancer and coronary heart disease risk, is supported by epidemiological evidence.*

Vitamins

A vitamin is an organic micronutrient which is required in the diet on an ongoing basis. Some vitamins (niacin, vitamin D) can be synthesized in the body but not always in amounts that are required.

Vitamin "B"

There is not one 'vitamin B' but eight!: Thiamin (B₁), Riboflavin (B₂), Niacin (B₃), Panthothenate (B₅), Pyridoxin (B₆), Cobalamin (B₁₂), Folate and Biotin. All are micronutrients (recommended intake <100mg/day), not stored appreciably (except B₁₂) and act biochemically in compounds called coenzymes. A coenzyme is a prosthetic group (non-amino-acid) which when bound to its apoenzyme gives a holoenzyme (a functional enzyme). In this way, coenzymes have an essential role in many biochemical reactions in metabolism. Coenzymes often are co-substrates in which the vitamin cofactor acts as an acceptor or donor of functional groups that are removed or added to the actual substrate (Exercise: pick any B vitamin; identify its coenzyme and an enzyme which uses the coenzyme; identify the substrate; what is being added or removed?).

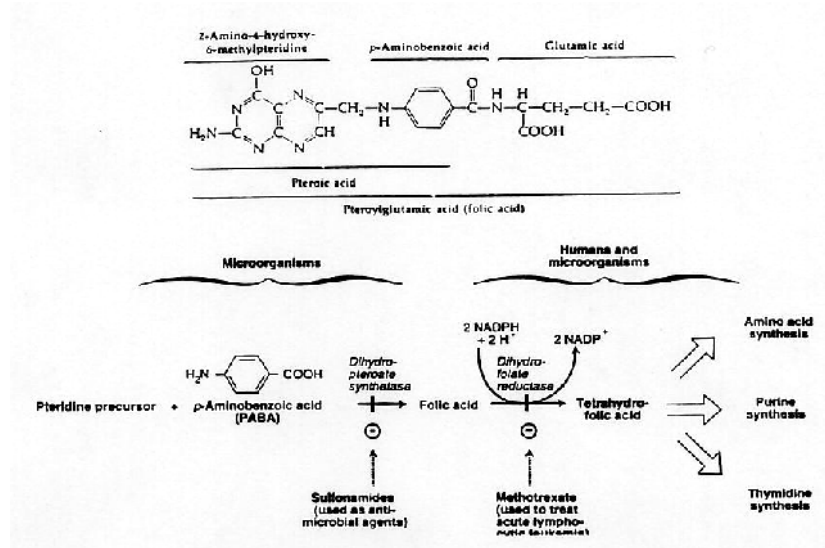
Fortification

Flour and pasta sold in Canada and the U.S. ('enriched') is supplemented with thiamin, riboflavin, niacin, folate and iron (and in some cases calcium) as a public health measure. Fortification programs have played an important role in public health nutrition for some 80 years - added iodine and vitamin D have essentially eliminated goitre and rickets.

Folacin (Folate, Folic Acid)

Named from the Latin word for "leaf" after it was found in high concentrations in spinach leaves. Though present in high amounts in leafy vegetables, it is widely distributed in plant and animal foods. The Dietary Reference Intake range for folate is 120-330: g/day, depending on sex and age. An additional 200: g/day is recommended periconceptually (all women of childbearing age should consume at least 400: g/day). Up to 40% of hospitalized alcoholics are folate deficient

because of poor diet and because alcohol impairs the absorption of folate. Since folate supplements are associated with a reduced risk of birth defects and with lowering blood homocysteine levels (see below), the fortification level in flour and cereal products has been recently increased to 140: g/100g.

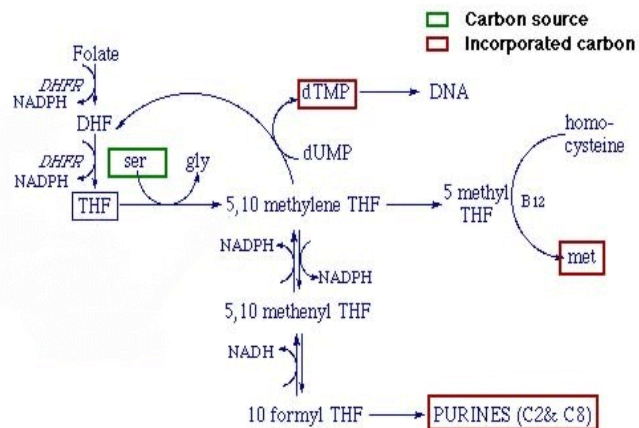


Poor Absorption

Folate contains a pterin, p-aminobenzoic acid and usually several molecules of glutamic acid ('polyglutamates'). The reaction between the pterin and p-aminobenzoate can be carried out by microorganisms but not animals. This reaction is inhibited by sulfonamides which are drugs that do not affect humans who require the intact vitamin (see figure). Polyglutamates have to be hydrolysed by intestinal polyglutamate hydrolase, folate conjugase, to the monoglutamyl residue in order for folate to be absorbed. Some food substances can inhibit this enzyme whose activity can also be impaired by intestinal disease. Moreover, defective absorption and utilization of folate may occur in subjects taking a variety of drugs including, dilantin (anticonvulsant), barbiturates (sedatives), quinine derivatives (antimalarials), methotrexate and aminopterin (chemotherapeutic agents) and sulfasalazine (anti-inflammatory). Therefore signs of overt folate deficiency, megaloblastic anemia, leukopenia and pancytopenia, can appear in a variety of clinical settings.

Coenzyme Forms

The coenzyme form of folacin is tetrahydrofolic acid (THF). THF is a cofactor in several important reactions where 1C fragments are added to or removed from molecules. 1C transfers are involved in the conversion of glycine to serine, the synthesis of thymine from DNA synthesis, the synthesis of purines for both DNA and RNA synthesis and the transfer of methyl groups to vitamin B₁₂ in methionine synthesis. Several forms of the cofactor are used: 1) methyl THF, the most abundant form of TFA, is required for the



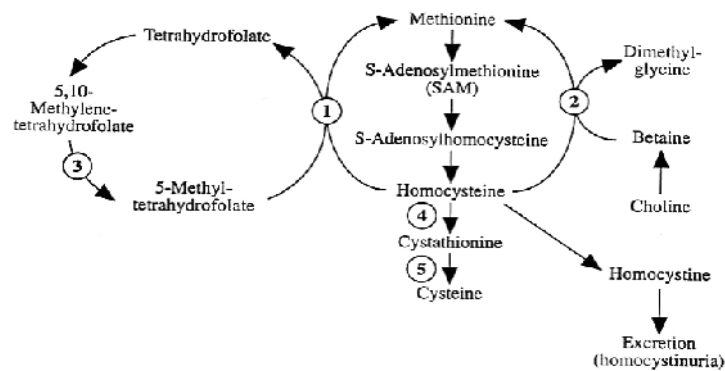
conversion of homocysteine to methionine (itself an important methyl donor); 2) *methenyl* and *formyl* TFA are employed in purine synthesis; and 3) *methylene* TFA is required for the conversion of deoxyuridylate to thymidylate, a rate-limiting reaction in DNA synthesis. Folate deficiency results in a critical impairment of DNA synthesis due to defective operation of the second and third reactions. The RNA/DNA ratio in the macrocytes is approx. 0.85, instead of the normal ratio of 0.3. Methionine synthesis, employing *methyl* TFA, also requires vitamin B₁₂ and provides the link between B₁₂ and folate metabolism. In B₁₂ deficiency, TFA is trapped as *methyl* TFA. TFA is not regenerated and the other forms of TFA become unavailable for nucleic acid synthesis, particularly that of DNA. Therefore B₁₂ deficiency effectively causes folate deficiency by impairing the recycling of various TFA forms.

Folate and Birth Defects

Much research has demonstrated a connection between poor maternal vitamin status, particularly of folic acid, and the incidence of neural tube defects (NTD) in the offspring. The incidence is greater in lower socioeconomic groups and in children conceived in spring when fresh foods are less available. Periconceptual supplementation with multivitamins lowered the risk of NTD in high-risk women as well as in women with no history of NTD. Compromised folate status also has been found to increase the risk of several adverse pregnancy outcomes including cleft palate/lip, congenital malformations and miscarriages.

Folate and Cancer

Epidemiological studies show that inadequate folate intake is associated with increased risk of cell dysplasia (change in cell size, shape and organization) and of developing certain cancers. Conversely, intervention trials of adequate or increased intake of folate have demonstrated a lowered risk of developing cell dysplasia in the cervix, colon, lung and esophagus. Folate is important in regulating the supply of methyl groups for DNA methylation. Methylation affects histone binding to DNA, chromosome structure and, inevitably, gene transcription. Folate appears to play an important role in the prevention of chromosome breaks which are inherent to several oncogenic processes. For this reason folate might be considered a cancer preventative.



(The enzymes in the figure are: 1. Methionine Synthase; 2. Betaine Homocysteine Methyltransferase; 3. Methylene-Tetrahydrofolate Reductase (MTHFR); 4. Cystathionine β -Synthase; 5. (-Cystathionase)

Folate and Cardiovascular Disease

Recent attention has focused on a connection between folate (and Vitamins B₁₂ and B₆) and cardiovascular disease that is mediated through blood homocysteine levels. The connection was initially suggested by occurrence of thrombotic complications in patients with the rare genetic disease of homocystinuria. Subsequently an association between hyperhomocyst(e)inemia with coronary artery disease, cerebrovascular disease and peripheral vascular disease was demonstrated. Excessive homocysteine may be directly toxic to the endothelium, may cause desquamation of endothelial cells, lipoprotein oxidation or increase the adhesion of monocytes to the vessel wall.

The metabolism of homocysteine is affected by several enzymes which use the vitamins folate, B₁₂ and B₆ (see the figure). These include methionine synthase (folate, B₁₂), methylene tetrahydrofolate reductase - MTHFR (folate), cystathionine β -synthase (B₆) and γ -cystathionase (B₆). An interruption of these transsulfuration or remethylation pathways can lead to hyperhomocyst(e)inemia. While there are very rare genetic traits in one or other of the enzymes in Figure that can lead to homocystinuria (cystathionine β -synthase, for example), of much greater general clinical interest is the high frequency of polymorphism in MTHFR. This results in a thermolabile variant of MTHFR that is inherited as an autosomal recessive trait at a frequency of 5%. This implies that a high proportion of the general population is heterozygous for this trait. It has been shown that the metabolic block caused by this trait can be corrected with oral folate supplements. While no studies have directly related daily intake of folate to reduced cardiovascular risk, there are studies linking folate intake to reduced plasma homocysteine and others relating elevated homocysteine levels with increased cardiovascular risk.

Cobalamin

Learning Objectives

1. To recognize B₁₂ as a water-soluble vitamin of animal food origin and that it is unique in being stored significantly in the body.
2. To describe B₁₂ absorption in the ileum as being dependent on binding to a glycoprotein, intrinsic factor, secreted by the stomach.
3. To recognize pernicious anemia as a form of B₁₂ malabsorption and caused by defective secretion of intrinsic factor.
4. To identify two important biochemical reactions involving B₁₂ are: i) the conversion of homocysteine to methionine; and ii) the conversion of methylmalonic acid to succinic acid.
5. Given that vitamin B₁₂ is involved in the recycling of tetrahydrofolate cofactors, to recognize that deficiency of B₁₂ can cause the symptoms of folate deficiency.
6. To recognize that folate is 'trapped' as methyl folate in B₁₂ deficiency.
7. To recognize that the neurological symptoms of B₁₂ deficiency likely result from problems with the metabolism of methyl groups and/or methylmalonate.
8. To recognize that folate administration for the correction of megaloblastic anemia can mask an underlying B₁₂ deficiency which may proceed to neurologic dysfunction.

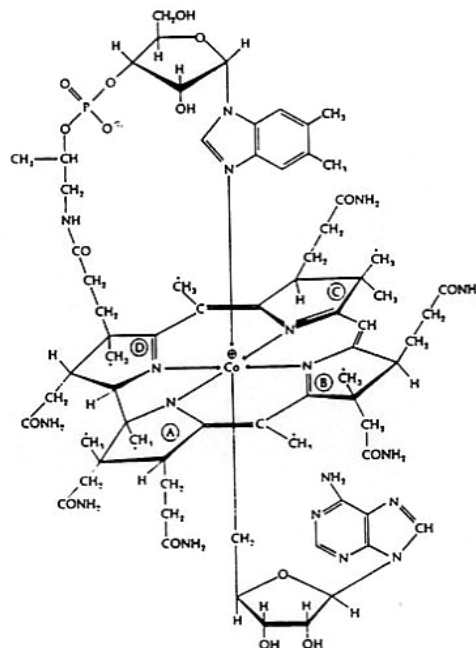
Cobalamin (Vitamin B₁₂)

Cobalamins (methyl-, 5'-deoxyadenosyl-, cyano-) are characterized by a corrin ring structure and a central Co atom. This represents the only known biochemical function of cobalt in humans and represents the entire essentiality for this element. There are only two known human biochemical reactions with a B₁₂ requirement: a) the conversion of homocysteine to methionine; and b) the conversion of methylmalonyl CoA to succinyl CoA. The first reaction also requires folate and, as mentioned previously in B₁₂ deficiency, usable folate becomes trapped as methyl TFA. This prevents the

other forms of TFA being available for nucleic acid synthesis, and especially DNA synthesis. This causes a megaloblastic anemia.

Vitamin B₁₂ Absorption

A megaloblastic anemia associated with neurological deterioration was inevitably fatal until, in the 1920s, it was found to be curable with extracts of liver. Subsequent work showed the need for an extrinsic factor present in liver (B₁₂) and an intrinsic factor which is a protein secreted by the stomach. Vitamin B₁₂ is widely present in foods of animal origin and the liver, which contains >80% of the body content, can store a 5-year supply. The RNI is 1: g/day. In food vitamin B₁₂ is usually freed from bound protein by the acid hydrolysis in the stomach or trypsin digestion in the small intestine. It is then bound to intrinsic factor which carries it to the ileum (the distal part of the small intestine) where it is absorbed. Inappropriate absorption of B₁₂ results in pernicious anemia. This is most often related to autoimmune destruction of parietal cells in the stomach, the cells responsible for intrinsic factor synthesis.



B₁₂ Deficiency Causes Neurological Damage

While increasing epidemiological evidence supports a role for vitamin B₁₂ deficiency in a variety of neurological disease, the precise mechanism of this is unclear. There are several possibilities: 1) neurones are particularly sensitive to decreases in methylation because they require ongoing maintenance of heavily methylated phospholipids in myelin; 2) homocysteine itself may be toxic to neurones or to neurological function; or 3) accumulation of methylmalonyl CoA in B₁₂ deficiency may be responsible for the neurological degeneration. Methylmalonyl CoA is a competitive inhibitor of malonyl CoA in fatty acid biosynthesis. Because the myelin sheath is continually turning over, an inhibition of fatty acid synthesis results in degeneration of myelin. The accumulation of methylmalonyl CoA may also result in branched-chain fatty acids being incorporated into membranes leading to disruptions of normal membrane structure.

Caution should be exercised in using folate supplements to correct megaloblastic anemia. While a block in DNA synthesis caused by B₁₂ deficiency can be overcome by giving extra folate (which can be converted to methenyl, formyl and methylene TFA), this will not correct an accumulation of methylmalonyl CoA due to B₁₂ deficiency and neurological symptoms may result. As a result, continuing food (folate) fortification programs have generated some controversy because they may increase the potential of neurological damage.

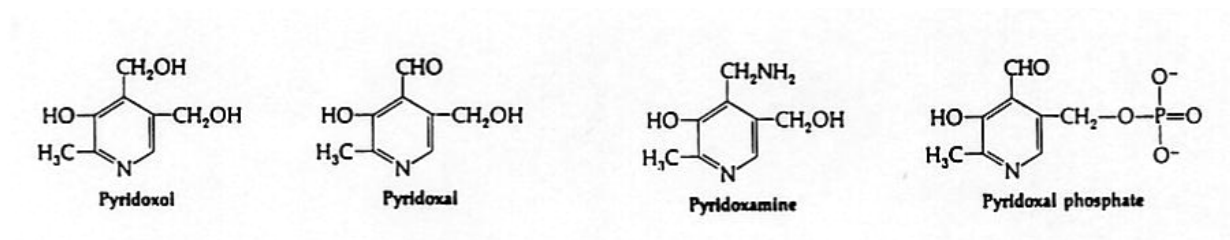
Pyridoxin

Learning Objectives

1. To identify B₆ as a vitamin involved in many reactions of amino acid conversion and

- catabolism.*
2. To identify pyridoxal phosphate as the coenzyme formed from vitamin B₆.
 3. To identify transamination as a crucial vitamin B₆-dependent reaction for conversion of protein to carbohydrate.
 4. To recognize that the neurological symptoms of B₆ deficiency arise from an impairment of (–)-aminobutyric acid synthesis.
 5. To identify sideroblastic anemia as a possible consequence of B₆ deficiency caused by defective heme synthesis.
 6. To identify B₆ as an example of a water-soluble vitamin with documented evidence of toxicity due to overdosing.

Pyridoxin (Vitamin B₆)



The requirement for B₆ is roughly in proportion to the protein content of the diet. Ask why when you know its function! The daily requirement may be altered by ingestion of drugs such as contraceptives isoniazid (isonicotinic acid hydrazide) and penicillamine. Good sources include whole grains, liver, green vegetables. The metabolic role of B₆ is in amino acid conversion and catabolism, e.g. transamination (aminotransferases), decarboxylation, dehydration and de sulfhydration. For these reactions pyridoxin must be converted to the active coenzyme form pyridoxal phosphate. The biochemical role of pyridoxal phosphate as a cofactor in several important enzymes of synthesis and amino acid metabolism. If all If a diet contained no vitamin B₆, all amino acids would become essential. Why? B₆ deficiency can occur in infants and is also commonly found in chronic alcoholism. Severe deficiency can cause convulsions and peripheral neuropathy. Seizures are probably due to brain depletion of (–)-aminobutyric acid (GABA), an inhibitory neurotransmitter that requires pyridoxal phosphate for its synthesis. It is likely that this is also responsible for the milder symptoms of irritability, nervousness and depression seen in mild B₆ deficiency. Pyridoxal phosphate is also required for the synthesis of (–)-aminolevulinic acid, a precursor of heme. Deficiency can therefore cause sideroblastic anemia where immature iron-loaded red cells accumulate. Vitamin B₆ is also required for the conversion of homocysteine to cysteine and hyperhomocyst(e)inemia can result from B₆ deficiency.

Pyridoxin toxicity

It is often stated in the popular press that large doses of *water-soluble* vitamins are harmless because the excess is easily excreted. Pyridoxin megadosing provides an example contrary to this popular view. B₆ is popularly advised for tingling, muscle spasms, numbness in the hands and for edema (a study on premenstrual syndrome showed some benefit). Megadosing is dangerous in view of the demonstration of peripheral neurological damage with overdoses (2-6g/day). Some of the changes were irreversible. The toxic effects are probably caused by "starvation amid plenty". The excess pyridoxin may overload the conversion mechanism to the active coenzyme and uselessly occupy sites on enzymes reserved for pyridoxal or pyridoxamine phosphate (the coenzyme). The structure of pyridoxin prevents it from being a coenzyme in these reactions and deficiency symptoms may result.

Objective: The student will be able to describe a systematic molecular view of classic inherited diseases that lead to premature erythrocyte destruction

A physician picks up the chart for the next patient, and recalls sending this 21 y.o. ♂ for an abdominal ultrasound while following up on complaints of epigastric and right upper quadrant crampy pain especially after fatty meals. The report reads, "gallstones; no significant dilation of bile duct; enlarged spleen". The doctor remembers the old adage of *fat/female/forty* as the classic gall bladder attack victim, and wonders if something unusual is going on in this patient (and splenomegaly is always of interest). The patient looks healthy enough perhaps a bit pale no H_x of previous ill health other than the usual childhood diseases ... on no medications ... no recent illness. Physical exam is unremarkable except for mild jaundice and the fact that, now he knows it is enlarged, he can feel a spleen tip. The patient is afebrile. There is a slight (grade I/VI) cardiac flow murmur. The doctor is particularly interested in the patient's CBC, serum amylase, liver enzymes (alkaline phosphatase, ALT, AST); and liver function tests such as total serum bilirubin, total protein, serum albumin, maybe INR. The CBC shows a mild anemia with normal size RBC, presence of spherocytes, and ↑ reticulocytes (newly-made erythrocytes). [actually, depending upon the lab doing the CBC, comments on the presence of spherocytes, and other comments on cell shape and general appearance, might have to be specifically requested: this could be done by asking for a "peripheral blood smear" or a "peripheral blood film"] [if the *smear* is normal, one most likely is not dealing with a hemolytic problem]. [There is a serum protein called *haptoglobin*, whose job is to bind free hemoglobin that happens to have been released into the bloodstream and carry it to the liver for destruction. When RBC are breaking in the bloodstream, serum haptoglobin is quickly depleted: thus you could ask for a measurement of serum haptoglobin]. Serum bilirubin was found to be elevated, but otherwise liver function seemed normal.

Normally, erythrocytes survive about 120 days in the circulation (being constantly exposed to shear forces and repeated deformation within tiny capillaries, oxidative damage, metabolic processes, proteases) before being recognized as 'old' and ready for phagocytosis by the reticuloendothelial system.

In *Hemolytic Anemia* one gets *excessive* Red Blood Cell destruction, with resultant:

anemia = ↓ RBC (or ↓ hemoglobin) per liter of blood.

RBC destruction may be increased by:

1) abnormalities *outside* the RBC (eg. infection, runaway clotting, prosthetic heart valves, autoimmune attack, hypersplenism) we will ignore these important causes today, except to note that a "Coombs test" or a "Direct Antibody Test" would examine a sample of the patient's blood for the presence of the patient's own antibodies or complement bound to their own erythrocytes (ie. looking for an autoimmune hemolytic process)

2) abnormalities *of* the RBC: we will focus on these

- defective hemoglobin
- defective enzymes
- defective membranes



Hemoglobinopathies

- over 100 defective primary hemoglobin chains have been identified typically substitution mutants in which a single amino acid is incorrectly specified by a gene locus
- of these, some abnormal hemoglobins precipitate too easily from solution, resulting in RBC membrane deformation and weakness .. this leads in turn to increased likelihood of lysis due to the high shear forces in capillaries, and to earlier recognition and phagocytosis by macrophages in the spleen (part of the *reticuloendothelial system*).

- the most famous is *sickle cell disease*, in which homozygotes have a hydrophobic valine replacing a charged glutamate at residue # 6 in all β -chains such high concentrations of HbS aggregate as a result of their hydrophobic surfaces fleeing exposure to H₂O by sticking together. The exposure of the hydrophobic surface is especially marked in the *deoxy* form (thus low-oxygen environments put the patient at risk of a *sickle crisis*). A similar common hemoglobinopathy in negroid people is replacement of the same glutamate by a lysine in β -chains = *hemoglobin C disease*. Interestingly, heterozygotes for hemoglobin S are not symptomatic (unless they happen to also be heterozygous for hemoglobin C).

Then there are the *Thalassemias*. Usually, normal hemoglobin chains are produced in thalassemia - but in insufficient quantities (ie. the genetic defect is not within the region of the gene that actually codes for the hemoglobin chains, but there may be errors in production or processing of globin mRNA due to abnormalities in the joining segments or in the promoter sequence etc.). " α -thalassemia" gives too few α -chains; " β -thalassemia" gives too few β -chains: so not enough chains are produced, and the ratios of the different chain types are wrong. Often precipitable forms of assembled hemoglobin are produced *via* abnormal combinations of the chains that are made, and these deform and weaken the RBC membranes. Heterozygotes may well be symptomatic.

- *hemoglobin electrophoresis* is a common test that would pick up abnormal chains or abnormal chain ratios if one suspected a hemoglobinopathy

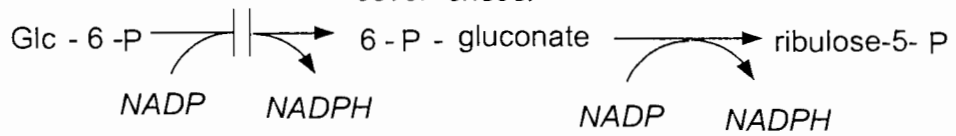
Defective (RBC) Enzymes

The RBC relies on the *glycolytic pathway* to generate the ATP energy it uses to preserve its structural integrity (it has no mitochondria). Thus genetically-defective glycolytic enzymes within the RBC lead to poor general RBC health - eg. not enough ATP to run membrane ion pumps optimally. As a result, one sees \uparrow RBC destruction in the spleen and bloodstream. By far the most common defect in glycolysis occurs within an RBC isoenzyme of *pyruvate kinase* (but even *this* defect is *rare*). It is called ***pyruvate kinase deficiency***.

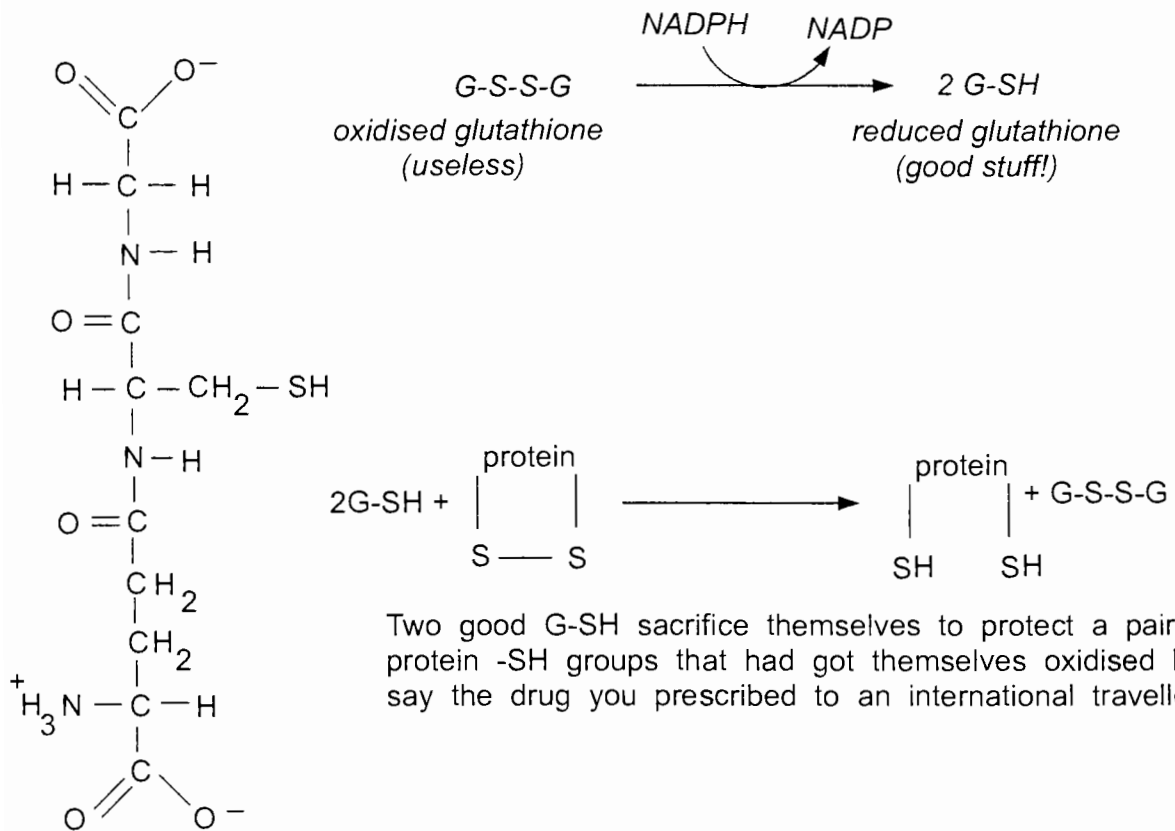
A much more clinically important aspect is that the RBC relies on the *pentose phosphate shunt* pathway to produce NADPH. The major need for NADPH in RBC is to maintain the important intracellular reductant, glutathione, in its *reduced* (-SH) vs *oxidized* (-S-S-) state. Clinically, the important (and fairly common) problem is a genetic defect or deficiency in the enzyme, glucose-6-PO₄-dehydrogenase, with resultant \downarrow levels of NADPH. It is called ***glucose-6-PO₄-dehydrogenase deficiency***, and is X-linked recessive (thus generally seen in σ^{r}).

- when reducing power within the RBC is compromised (ie. lack of NADPH or lack of reduced glutathione) the RBC contents, especially Hb, are subject to oxidative damage

With a (partial) block in the pentose phosphate shunt.
This pathway won't produce enough NADPH to cover crises.

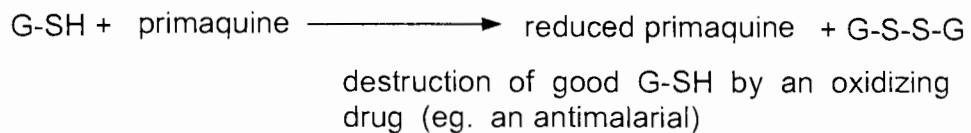


REGENERATION OF GLUTATHIONE



Two good G-SH sacrifice themselves to protect a pair of protein -SH groups that had got themselves oxidised by say the drug you prescribed to an international traveller

Reduced glutathione (G-SH)
(it's the -SH that counts!)



and denaturation/precipitation. "Heinze bodies" is a classic term used in the 'smear' report to refer to precipitates of damaged hemoglobin which are seen within the RBC, deforming the cell and leading to lysis or phagocytic recognition.

- most patients manage well enough until they present acutely with a *hemolytic crisis*, eg. after their doctor administers an oxidizing drug such as antimalarials, aspirin, or sulfa drugs. When this happens, the delicate balance of 'just enough antioxidant' is lost and there is a sudden crosslinking of -SH groups with resultant hemolysis, flank pain, red urine, renal failure due to plugged renal glomeruli and tubules. 'Normal' individuals have excess reducing capacity and can handle the extra strain of oxidizing drugs. *Hemolytic crisis* in G-6-P-dehydrogenase deficiency is of variable severity.

- some patients are sensitive to Fava beans.
- many *screening* tests exist; but the best D_x is demonstration of an enzyme abnormality

Defective membrane

- in today's patient, the diagnosis was made under the microscope (from the RBC appearance) as *hereditary spherocytosis* (fairly rare). Most such patients have a family H_x of the disease.

- RBC in the circulation are subject to considerable shear forces as they are pumped through capillaries repeatedly ... their normal biconcave disc shape and membrane proteins allow deformation without damage. In *spherocytosis* a membrane 'scaffolding' protein called *spectrin* may be abnormal. The result is that the cells are less deformable and more subject to damage and uptake in the spleen. One result is splenic hypertrophy; and splenectomy is the treatment (apparently abnormal RBC induce the splenic filter to become even larger and more selective in a vicious cycle).

- an even rarer, but similar, disease is hereditary elliptocytosis of erythrocytes.
- The D_x in such patients would often be made by having microscopy of the RBC done in an experienced lab (a blood *smear*). Also the lab could check for membrane fragility by measuring the ease with which RBC lyse in a test tube).

In all hemolytic anemias the body tries to compensate by an erythropoietin-induced stepping-up of the rate of RBC production ... so you see \uparrow numbers of reticulocytes (young RBCs that still have residual RNA). This should show up on the "CBC" that you ordered. *Chronic* hemolysis will lead to chronically excessive loads of bilirubin excreted in the bile. {Bilirubin is the coloured breakdown product of the heme group; and is normally got rid of by conjugation of sugar groups to solubilize it in the liver, with subsequent excretion of the conjugated material in the bile}. Chronically-excessive loads can lead to precipitation as calcium bilirubinate salts in the gall bladder (which are x-ray opaque) (often called "pigment stones" because of the *bilirubin* colour) ... as in this patient. Note that, in distinct contrast, "*bile salts*" refers to charged salts of *cholesterol* which function as digestive tract detergent.

Objectives for Hemostasis Rounds:

The student will be able to develop a systematic molecular picture of the clinically relevant events surrounding hemostasis, in terms of factor names or numbers; and will be familiar with common tests of clotting and common 'anticoagulants'. The widespread existence of relevant natural extracellular proteases should be appreciated.

A 9 day old ♂ was referred to the pediatrics service after presenting in the emergency department with a bleeding circumcision site. He had been circumcised by his family doctor at 4 days and bled since then - sometimes to the extent of soaking his diaper. He had been given intramuscular vitamin K several days previously, and had even had 2 stitches in emergency at 8 days. There was no H_x of umbilical stump bleeding, vomiting, diarrhea, fever, or blood at other sites. He fed well from the breast. Term pregnancy, uncomplicated vaginal delivery, normal weight. Mother claims she has always bled for a long time if cut, but no other family H_x of similar problems.

Patient looked well. However the circ site looked inflamed, oozing blood, 2 sutures in place. Exam otherwise normal.

There was some fear of infection causing bleed (although no evidence for it, other than local redness). Hence swabs of the site were cultured, blood cultures were taken, and the patient was put on IV antibiotics. We did BUN/Cr (partly to have a record of renal function before antibiotics), CBC and diff, urine cultures and microscopy, bilirubin, INR (PT in old days), PTT, test for D-dimers. The only abnormal test was PTT 3x normal.

Infection is obviously a major clinical consideration; but here we will only note in passing that often an infant can be badly infected while having few signs of such (maybe just lethargy), so one must be suspicious.

Faced with a bleeding disorder, physicians tend to consider that the defect might be: (1) in the vessel wall (normally vessels constrict immediately in response to injury and are tough enough to withstand minor trauma).

(2) in the platelets (normal platelets can quickly plug a small hole in a vessel by adhering {via their receptors} to exposed collagen and forming aggregates, especially when stimulated by ADP and thromboxane A₂ released by other activated platelets).

(3) in the clotting factor function (coagulation).

(4) in the controlled shutdown of clotting after the job is done.

Steps 2-4 involve much modern relevant biochemistry. For purposes of maintaining sanity, they are studied as separate entities - but their final functions overlap importantly.

Testing vessel wall 'health' would be a bit unusual; but elderly people and people who are malnourished (esp. vitamin C) tend to bruise more easily. I suppose one might consider that the old "Bleeding Time" test partly measures this: you make a scratch through the skin with a sterile needle .. blot away oozing blood so coagulation is minimized .. and measure time to cessation of bleed. Probably though it better tests the process of *platelet plug formation*. Some people are getting quite interested in tests of platelet plug formation looking for von Willebrand's disease; since women may end up chronically affected without being aware. A platelet count to see if their numbers are too low is a very important early

test (and this is part of the CBC). Platelet # was normal in this patient and Bleeding Time was not done.

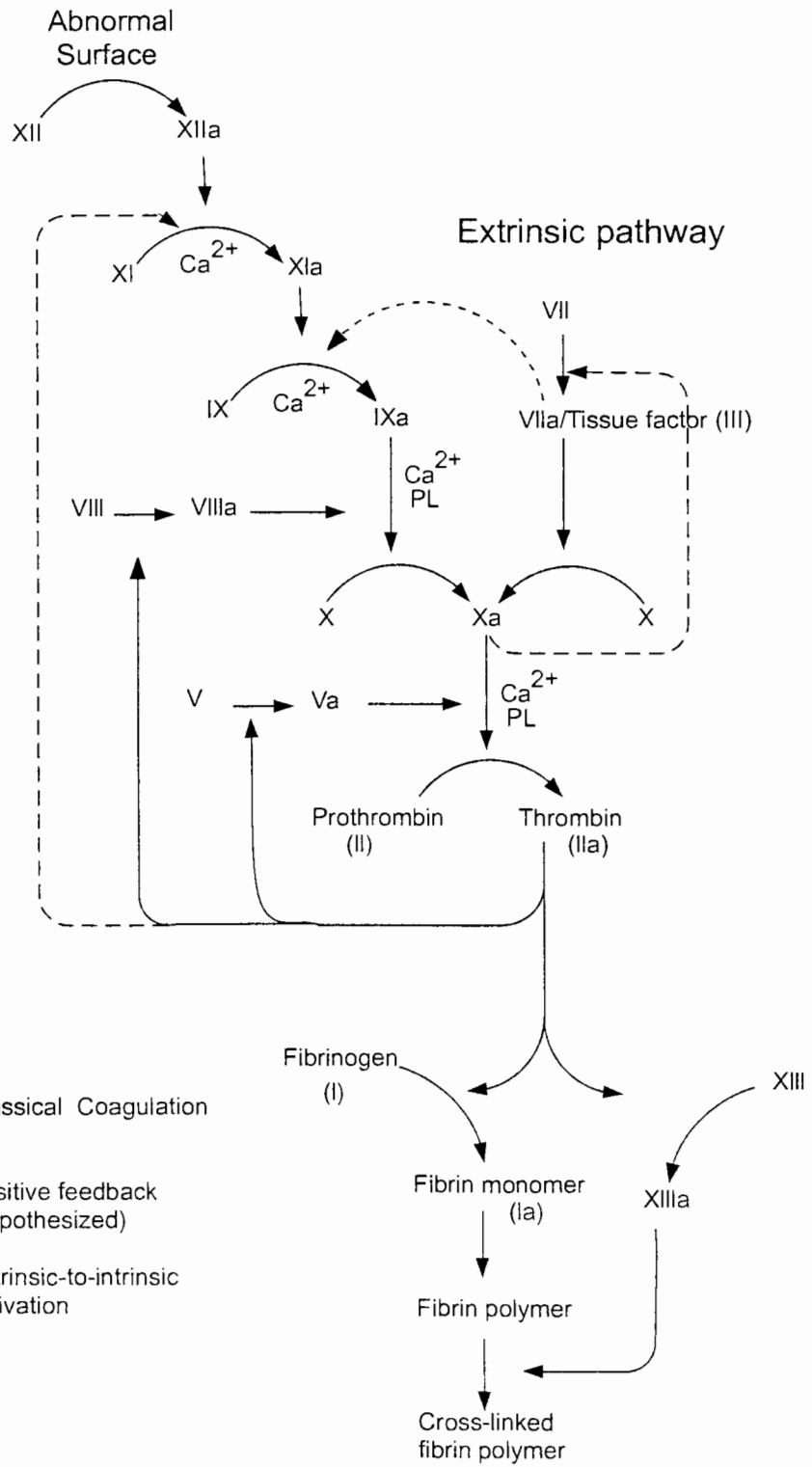
Platelets play a central role in clotting by forming a plug ("platelet thrombus") and by releasing ADP, thromboxane A_2 , and other factors that help initiate the coagulation cascade. They *can* be present in normal # but have abnormal function, eg. in von Willebrand's disease. The mechanism of platelet activation might be best viewed as a form of signal transduction such as that seen subsequent to binding of a peptide hormone to its cell membrane receptor. The platelet cell surface receptor would be a transmembrane glycoprotein. It peacefully minds its own business as the platelet trundles through the blood vessels ... until there is a sudden lock-and-key contact with an abnormal surface (eg. a collagen molecule exposed in a plaque of atherosclerosis). Then all heck breaks loose. The platelet jerks to an abrupt halt in the rushing blood flow. The cytoplasmic end of the receptor glycoprotein wakes with a start, and causes a cascade of molecular rearrangements within the platelet cytoplasm. One cytoplasmic result is activation of a molecular contractile machinery related to muscle actin/myosin. As a result, the platelet's surface receptors are rearranged into more adhesive groupings (eg. which can now bind fibrinogen) ... also granules of chemical signals such as serotonin are released from the cell interior, also ADP, which make neighbouring platelets more adhesive. Passing platelets are recruited to a *platelet plug* that can seal small tears in the vessel wall. A popular current statement would be that, 'after the ~ 100 mechanisms of platelet activation have occurred, a final key result is rearrangement of the platelet membrane glycoprotein receptor IIb/IIIa, which binds fibrinogen and allows thrombus formation' (expensive drugs are now available to block this receptor).

We mentioned that one studies the blood clotting components in isolation for simplicity: the above is 'the platelet story'. However, we will see that the clump of platelets with physically rearranged membrane proteins acts as a surface upon which later coagulation events occur (especially prothrombin conversion to thrombin - see below). The platelet membrane also has receptor glycoproteins for fibrin and thrombin formed by later events in coagulation, which signal for even greater platelet activation.

Step (3) above may be viewed as involving the coagulation ("clotting") factors ... an extremely complex system with many interrelating controls ... 13 clotting factors have been given Roman numerals, but #VI has been found not to exist. In order to be prepared for a vascular emergency, the body keeps a goodly stockpile of many clotting factors circulating in the blood. Yet clearly one does not want coagulation to go on at a high rate in the absence of a wound. So they circulate in 'inactive' forms. By tradition, the 'active' forms are denoted by a lower case 'a' (for *active*). Classically there are two pathways (*Intrinsic* & *Extrinsic*), each of which culminates in the *Final Common Pathway*. The final common pathway causes enzymatic cleavage of small charged peptides from *fibrinogen* ("factor I", which is soluble) which then forms a tangled precipitate of insoluble *fibrin* (factor I_a) (ie. a *clot*) ... as follows.

The protease, *thrombin* (factor II_a){from *prothrombin* = factor II}, that activates *fibrinogen* (factor I) to *fibrin* is central to the final common pathway. {As a non-examinable aside, it is highly analogous to the digestive enzyme, trypsin, which activates the pancreatic zymogens trypsinogen, chymotrypsinogen, proelastase and procarboxypeptidase}. Thrombin itself was formed from the inactive zymogen, *prothrombin* (factor II), on platelet

Intrinsic pathway



membrane surfaces where it bound in proximity to a protease, factor X_a , and the non-enzymatic modifier protein, factor V_a (which accelerates the proteolytic action of X_a by 10^4).

The Intrinsic and Extrinsic pathways have different methods of activating X to X_a though. The Intrinsic pathway uses the enzyme factor IX_a which needs the modifier protein, factor $VIII_a$. The classical concept is that factor XI_a activates factor IX by proteolytic cleavage after itself being activated by factor XII_a in concert with other species ... and factor XII_a comes from the factor XII which is activated by contact with an abnormal surface (eg. exposed strands of collagen in a damaged blood-vessel wall).

As in the Intrinsic pathway, the end result of the Extrinsic pathway is activation of factor X to X_a . But it begins with *tissue factor* (factor III). Factor III is unusual in that it does not circulate in the blood normally: it is a cell membrane glycoprotein of solid tissues which is released into the bloodstream after tissue trauma. Factor III binds to factor VII, deforming it so that it becomes cleaved by normal blood proteases into VII_a . VII_a then cleaves factor X.

Congenital lack of a factor (or, more likely, a genetic defect in one) can be a problem in this patient factor VIII proved to be defective (*Hemophilia A*) in *Hemophilia B* it is factor IX ("Christmas factor") which is the problem. Defects in the Intrinsic Pathway show up as prolonged "partial thromboplastin time" (PTT)(actually one probably should use the term, "aPTT" ["activated PTT"]; but people use them interchangeably). The (a)PTT is the time for the patient's plasma to clot (~30 sec) in the presence of a reagent mixture that includes a foreign surface, "partial thromboplastin" (which is phospholipid), and Ca^{2+} . Defects in the Extrinsic Pathway give a prolonged "prothrombin time" (PT). PT is (was) the time for plasma to clot (~12 sec) when a vast excess of *tissue factor* (factor III) is added as well (hence "complete thromboplastin"). PT should now be replaced with the *INR (International Normalized Ratio)*, which is essentially the unitless ratio of patient PT to the 'normal' value, and also multiplies by a number to correct for lab reagent variability. The presence of detectable "D-dimer" in the patient's blood (a degraded fragment of fibrinogen), suggests that clotting has been going on within the blood vessels; which can happen if there is infection-related "disseminated intravascular coagulation (DIC)". Clinicians alternatively ask that the blood be tested for "Fibrin Degradation Products" or "Fibrin Split Products" for the same reason.

The mother of this baby probably has ~ ½ genetically normal factor VIII and ½ defective, since she has 1 good and 1 bad X-chromosome. So she never got into serious bleeding trouble herself. In this patient we gave intravenous fresh frozen plasma to stop bleeding. Then we retested for defective factors when crisis was past to ensure diagnosis.

Once a clot has formed, factor $XIII_a$ stabilizes and contracts it by chemically crosslinking fibrin. As in a number of important clotting steps, Ca^{2+} (factor IV) is required. The absolute requirement for Ca^{2+} is reflected in a day-to-day clinical reality: when you take blood from a patient, if you want it to remain liquid in the tube you have to "anticoagulate" it ... often by adding citrate or EDTA whose carboxyl groups chelate Ca^{2+} . The manufacturers of the tubes know this and have added such agents to the vacuum tube you will use ... but you have to be sure you select tubes with anti-clotting agents that will not interfere with the tests you wish to have performed (this is done by checking the colour of

the tube top).

Note that the clotting cascade has some beautiful biochemistry: the concept of zymogen activation by proteolysis, the concept of signalling across membranes by a specific lock-and-key contact, the concept of increasing a catalyst (enzyme) action *via* binding to some other protein or surface. Note too that, when you turn on one single enzyme molecule, you activate many other molecules: amplification. Also, there is stimulatory feedback amplification: thrombin not only activates factor I, it also exerts proteolytic activation upon XI, VIII, and V. To complicate things, the extrinsic pathway activates the intrinsic pathway because VII_a activates IX.

The 4th step, natural limitation of the clotting process, will be examined in the next Round.

A 57 y.o. ♂ was in hospital on the chest service. He had been admitted 2 weeks previously after an upper respiratory tract infection exacerbated his chronic emphysema (secondary to a 60-pack-year history of smoking). He had seemed to be much improved and was to be discharged shortly. The on-call clerk was phoned at night by a nurse who reported that the patient seemed anxious and had been breathing rapidly for several hours, and now was complaining of sharp chest pain worsened by deep inspiration. The sleepy clerk found the patient somewhat distressed, respiratory rate 25 per minute. Breathing shallow, colour was good. The patient was afebrile, BP normal, HR 90, chest sounds wheezy but not wet, air entry seemed adequate in all lung fields, heart sounds normal.

The patient's left calf was somewhat tender to squeezing, although it was not noticeably red. He had been very inactive during his stay, but mentioned that this leg had felt "heavy" the last few days when he was up. On measurement, it was 3 cm larger in circumference than the other. The clerk agreed with the nurse that O₂ by mask to keep the patient's hemoglobin saturation of ≥90% would be nice. He ordered an ECG, and a chest X-ray; then called the resident to say he suspected a pulmonary embolus from a clot in the leg vein. Blood was sent for blood gas determination, PT (INR these days) and PTT determination, and for measurement of cardiac muscle proteins in the patient's serum (although these might not have risen yet even if the patient had had an acute MI). The resident ordered complete bed rest with foot of bed elevated, and IV heparin after seeing the patient. She also ordered a ventilation/perfusion scan of the lungs to be done in the morning, along with a repeat measurement of cardiac proteins in the patient's serum.

The ventilation/perfusion scan showed small areas of poor blood perfusion in both lower lung fields, while their ventilation by air was normal. Chest X-ray was normal and the patient felt better 12 hours later. Heparin was continued IV, and plans were made to start coumadin by mouth. Venous ultrasound (Doppler) indicated a clot in the left deep calf vein. Clerk and resident tentatively concluded that the pulmonary embolus was secondary to bed rest, and the clerk made a mental note that in future he would encourage bedridden patients to move their legs, and to keep them elevated to avoid stasis of venous blood ... venous stasis can predispose to clot formation, especially if the vessels are damaged or distorted.

Immediately after heparin bolus IV the patient's PTT rose from 25 s (within reference range) to 60 s. His PT/INR began to climb 12 hrs after oral warfarin (Coumadin) was begun. The patient was discharged a week later on warfarin only, and with his PT/INR and PTT both elevated. These values had to be followed frequently, and the patient was warned to watch for bleeding from any orifice and inappropriate bruising, as the most serious side effect of anticoagulants is overdoing them.

As advertised, we now consider the 4th step in hemostasis - turning it off.

- firstly, activated factors have short half lives: they are subject to uptake and degradation by cells of the reticuloendothelial system and liver; and also are more subject to proteolytic degradation in the bloodstream.
- specific inhibitors exist for key clotting pathway steps: especially *antithrombin III* - a plasma protein that binds to thrombin, blocking its active site, (it also inhibits other proteases eg. IX_a, X_a, XI_a, XII_a). Another protease 'inhibitor' is "Protein C" which becomes

an active enzyme when cleaved by thrombin; and its job is to chew up various clotting factors with the help of a modifier protein called "Protein S". There are other protective anti-proteinases (protease inhibitors) in the body whose jobs are to limit the activity of natural proteases.

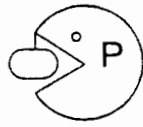
- *fibrinolysis* (clot destruction) is another control: *plasminogen* is an inactive proteolytic enzyme always present in the circulation. It binds to fibrin and becomes incorporated into clots. It is activated to *plasmin* via proteolytic cleavage by the proteolytic enzyme, tPA (*tissue plasminogen activator*), made by endothelial cells. Like factor III, tPA is released in response to tissue damage. tPA is active when bound to fibrin. The result of plasmin on fibrin is "Fibrin Degradation Products" (small peptides), including one called "D-dimer". You can assay for plasma concentrations of these and for fibrinogen if you think coagulation may have run wild.

A tasteful diagram of the tPA logic is included.

The common anticoagulant, *heparin*, is a naturally-occurring 8,000-15,000 MW (-)-charged polysaccharide found in the body on the surface of endothelial cells. It acts by binding to and accelerating the action of proteins that inactivate clotting factors (esp. antithrombin III that binds to and reversibly inactivates thrombin and other factors). Intravenous heparin begins to lengthen PTT immediately. You can perhaps rationalize heparin's relatively *selective* effect on PTT (ie. not PT) by invoking antithrombin's effect on IX_a, X_a, XI_a, XII_a. In recent years, lower-molecular-weight fractions of heparin have been produced in the lab by cleaving heparin: these are now very important replacements for heparin [but confusingly, they don't prolong the PTT; so PTT is not a useful indicator of whether you have given enough of these new heparins ... but fortunately they are so dose-predictable that you usually don't have to worry]. Clinically, you can immediately reverse the effect of heparin overdose by injecting the (+)-charged peptide, *protamine* (which binds to the (-)-charged heparin). An amazing number of new drugs are being developed that interfere with hemostasis in fancy biochemical ways: platelet thrombus formation and clotting are at the core of stroke and MI damage.

We gave heparin IV to this patient in order to immediately reduce his tendency to clot further (although it will not reverse the existing clot). Warfarin has the advantage that it can be given by mouth. It has a structure similar to vitamin K. It acts by inhibition of a vitamin K-dependent enzyme system used in clotting factor synthesis in the liver. Vitamin K is especially needed for synthesis of fully functional factors II, VII, IX, X: this is why infants get vitamin K injection at birth. {keep in mind that most clotting factors are synthesized in the liver ... therefore alcoholics can bleed} The role of vitamin K is to act as a coenzyme in reactions that put a second -COOH group on glutamic acid residues in certain clotting factors. This makes them look chemically a bit like EDTA - and able to bind Ca²⁺, like EDTA. Ca²⁺-binding is terribly important to their conformation and attachment (eg. to platelets). PT/INR is a clotting test that is particularly sensitive to the presence of factor VII, WARFARIN, and to the absence of liver function. But from the above, you would correctly expect warfarin to lengthen PTT as well.

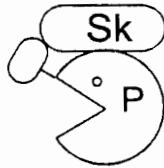
Streptokinase (a *bacterial* protein) or r-tPA ("recombinant human Tissue Plasminogen Activator" made by genetic engineering) are often injected intravenously in



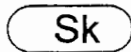
PLASMINOGEN (MOUTH PLUGGED ... INACTIVE)



TISSUE PLASMINOGEN ACTIVATOR



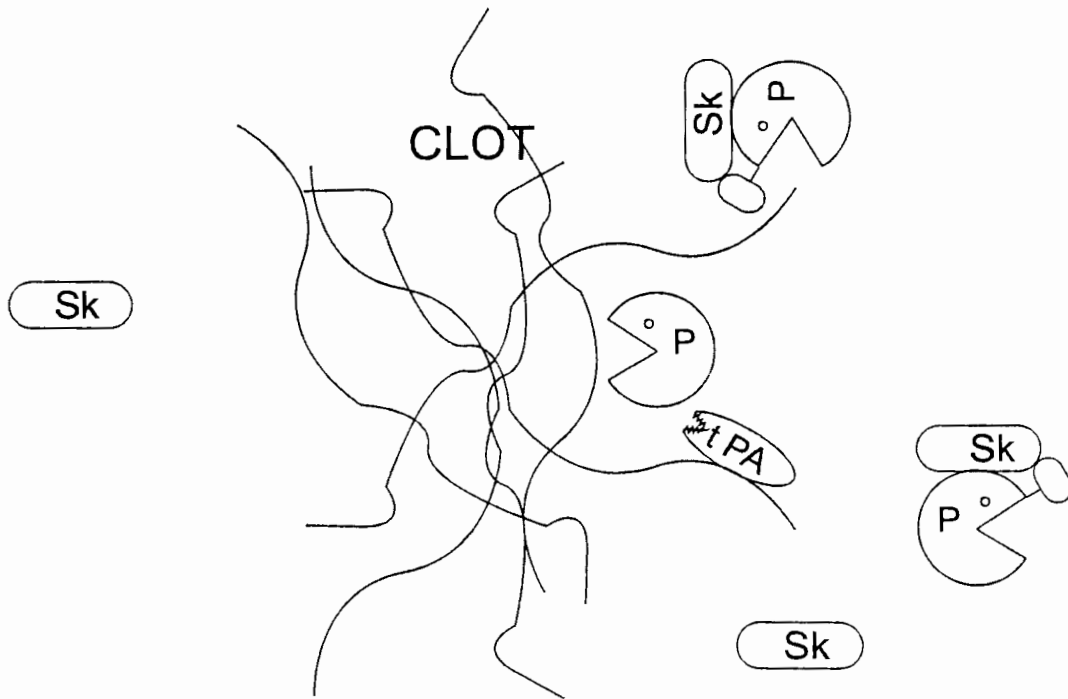
ACTIVE PLASMINOGEN
(PLUG PULLED ASIDE BY STREPTOKINASE)



STREPTOKINASE



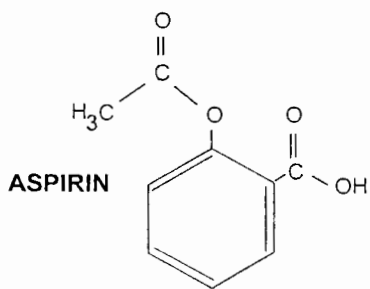
PLASMIN (PLUG NIBBLED OUT BY ACTIVE tPA)



very early acute MI (and in some life-threatening pulmonary embolus or stroke situations) to lyse clots in a patient's vessels. Streptokinase binds to plasminogen and makes it active at converting other plasminogen molecules to plasmin. r-tPA behaves just like natural tPA (and is 'natural' to the body). Both can induce generalized bleeding as an undesirable side effect.

Finally, why did this patient get a clot when others in the same situation do not? We saw that the newborn of the previous case *bled* excessively due to a defective clotting factor. Theoretically, defects in antithrombin III, protein C, or protein S could increase clotting ... but these account for less than 10% of cases. Yet there is a rather high familial tendency to thrombosis. There has been much interest in inherited "resistance to activated protein C": a fairly common point mutation in the gene for factor V causes one amino acid to be switched, making factor V resistant to proteolytic degradation by protein C! If a patient is unlucky enough to be homozygous for this, or say, combines it with an unrelated defect in protein S ... you can see the complex possibilities. These days, one could ask for a test for "resistance to activated protein C" in some patients (eg. a young woman with a family Hx of thromboembolism who was pondering the birth control pill). It is getting fairly common for people to assay for excessive fibrinogen levels and excessive serum homocysteine as risk factors in people with unexplained excessive clotting.

Clearly having even a small damaged area in an artery (an atherosclerotic plaque) can trip the cascade of platelet plug formation and coagulation. For instance physical exertion may increase blood flow, rip off a bit of plaque, and expose some collagen strands ... resulting in a sudden development of impediment to blood flow within that vessel. A huge area these days is use of low dose ASA to reduce platelet aggregation by acetylation of the platelet cyclooxygenase enzyme needed for thromboxane A₂ production within platelets ... 1 daily ASA reduces strokes and heart attacks in certain risk groups.



Structural formula of the repeating disaccharide unit of HEPARIN

