The liver as a storage organ:
Ferritin, vitamins, blood clotting factors.

GI / LIVER CORE GROUP MODULE

Dr. D. Mangnall

NOTE: This document contains some additional material to that presented in the lecture, which I hope will be of use in furthering understanding. The diagrams and tables referred to in this text are on the lecture handout.

AIMS
The aim of this lecture is to consider the role of the liver as a storage organ, particularly with respect to ferritin for the storage of iron, vitamins A, B12, folate, and vitamin K, and blood clotting factors. The role of the liver as a glycogen store, (which is probably a major storage role of the liver if you're a biochemist) will be considered in other lectures.

Iron storage

IRON STORAGE AND FERRITIN
Firstly, a brief background to the subject of iron in the body. The average 70kg man contains 3-4g of iron, 68% of which is found in the haemoglobin of the red blood cells, and about 27% is stored as ferritin within tissues, mainly within the liver.
(refer to Table 1).
Iron is important to mammals because it is intimately linked to the transport and participation of oxygen in a variety of biochemical processes. Iron can bind to a variety of macromolecules and influence their structure and function, with deleterious consequences for the organism. To protect against these events there are several iron-binding proteins which function specifically to store and transport iron. These proteins have a high affinity for iron, and in the normal physiological state have incompletely filled iron binding sites, ie. they are not normally fully saturated with iron. The interaction of iron with its ligand has been well worked out for some proteins (eg haemoglobin and myoglobin), but is still being established for others such as transferrin which transports iron in the blood. A major area of ignorance is how iron is transported from one macromolecule to another.

The route of entry of iron onto the body and the general transport around the body is outlined in Figure 1.

Iron is tightly conserved in a nearly closed system where each iron atom cycles
repeatedly from plasma and extracellular fluid to the bone marrow where it is incorporated into haemoglobin. It travels around the peripheral blood in the erythrocytes for about 4 months before phagocytosis in the reticuloendothelial system resulting in the degradation of the haem and the release of the iron into the plasma, where the cycle continues. Within each cycle a small proportion of the iron is transferred to storage sites, (the largest of which is in the liver), where it is incorporated into ferritin and haemosiderin, a small proportion of the stored iron is released into the plasma, a small proportion is lost as faeces, sweat, urine or blood, and an equivalent small amount is absorbed from the intestinal tract. A small amount (about 10%) of newly formed erythrocytes is destroyed within the bone marrow, and the iron released, bypassing the circulating blood part of the cycle. The numbers in the upper part of Fig 1 represent approximate amounts of iron (in mg) that enters and leaves each of these compartments each day.

IRON CONTAINING PROTEINS.
These can be haem containing (eg. haemoglobin, for transport of oxygen, myoglobin for storage, and some enzymes eg tryptophan pyrrolase, have haem as part of a prosthetic group), or non-haem proteins such as transferrin and ferritin, plus a variety of redox enzymes that have iron at their active sites, and iron-sulphur proteins.

Transferrin.
This is a 78kD glycoprotein synthesised by the liver and is the major transport protein for iron in plasma. It will bind iron in the ferric (Fe³⁺) state, but not in the ferrous (Fe²⁺) state at two iron binding sites. The binding of iron is dependent on the binding of an anion, usually carbonate in the normal physiological state, at the same time. Usually approx. one ninth of the circulating transferrin molecules are saturated with iron at both sites, about four ninths have iron at only one site, and about four ninths are free of iron. The iron is transferred into cells by the transferrin binding to a specific transferrin receptor on the surface of cells which mediates the uptake of the transferrin and its iron. The receptor is a transmembrane protein of two 90kD proteins linked by disulphide bonds, and each subunit can bind a transferrin molecule, but binding favours the diferric form of transferrin. Internalisation of the transferrin-receptor complex requires receptor phosphorylation by a Ca++-calmodulin dependent protein kinase. The internalised transferrin-receptor complex is acted upon by lysosomes, and the iron released into the interior of the cell. The apo-transferrin-receptor complex is returned to the cell surface where the apo-transferrin is released to the plasma for re-use.

Ferritin.
This is the major protein of iron storage. It has an outer protein shell 130 A in diameter, with a central core of ferric hydroxide and ferric phosphate some 60 A across. The outer protein shell consists of 24 subunits made of a varying mixture of H chains (which are 21kD) and L chains (which are 19kD). The ratio of iron to protein is not constant since the protein has to be able to bind and release the iron according to physiological need. The apoferritin has the capacity to bind 4500 atoms of iron, but usually contains less than 3000. When iron is in excess, the storage capacity of the ferritin may be exceeded, and iron
is deposited on the outside of the ferritin spheres which aggregate into a form which can be seen histologically called haemosiderin.

**INTESTINAL ABSORPTION OF IRON.**
The major site of absorption is the duodenum. Iron enters the mucosal cell either as free iron or as haem, in the latter case the iron is split off from the porphyrin ring in the cytosol of the mucosal cell. Whatever the requirements of the host for iron, if an adequate amount of iron is ingested, a substantial amount will be taken into the mucosa. Transfer of the iron into the capillary bed is subject to some form of regulation. In the normal state some 1 to 2 mg of iron per day will be transferred across the mucosal cell wall into the plasma. In an iron deficient state the amount of transfer is increased, and in iron overload transfer is reduced. One mechanism involved in this transfer regulation involves the synthesis of apoferritin by the mucosal cell. When the host has iron overload and no further iron uptake is needed, the mucosal cells synthesise a large amount of apoferritin, which binds the iron and becomes holo-ferritin, and stays within the mucosal cell, effectively trapping the iron and preventing transfer to the capillary bed. As the cells turn over (within a week), their contents are extruded back into the lumen of the intestine without absorption occurring. In situations where the host is iron deficient, virtually no apoferritin is synthesised so as not to compete against transfer into the capillaries. Note that there are additional, poorly understood mechanisms, which operate to increase the rate of iron absorption in the iron deficient state. Iron transferred to the capillaries is trapped almost entirely by transferrin.

**Molecular regulation of iron utilisation.**
Resting, non-proliferating cells have a modest number of transferrin receptors on the cell surface. Proliferating cells have a variable number of receptors depending on the iron content of the cell. A high iron content reduces the number to a basal level, a low iron content can increase the number up to 7 fold. Two iron dependent mechanisms act to regulate transferrin receptor synthesis. (see Fig 3) One acts at the DNA level, where a 5' flanking sequence of the receptor gene responds to iron deprivation by increasing gene transcription to RNA 2 to 3 fold. A second mechanism acts at the level of mRNA where low iron levels lead to stabilisation of mRNA for the receptor. A 3' untranslated region of the mRNA contains 5 stem-loop motifs which constitute and iron responsive element (IRE). In the iron deprived state, this region binds a 90kD regulatory protein which stabilises the mRNA, leading to reduced mRNA turnover and increased synthesis of receptor protein.

Hepatocytes are a major site for ferritin subunit synthesis, which is again regulated by the iron content of the cell. High iron content stimulates synthesis whilst low iron content decreases synthesis, and the regulation occurs at the mRNA level. The 5’ untranslated region of ferritin mRNA contains a single stem loop structure similar to that in the 3’ end of the transferrin-receptor mRNA, which is again an iron-resonse element which binds the 90kD protein. However, in this case binding leads to a decreased rate of translation and hence results in decreased levels of apo ferritin in the cell Low iron concentrations lead to activation of the 90kD regulatory protein, which then binds to the
regulatory regions of the transferrin receptor and ferritin mRNAs. As a result, there is increased synthesis of the transferrin receptor and decreased synthesis of apo-ferritin. The net result is that in proliferating cells there is an increased uptake of iron via the transferrin receptor, and its increased utilisation within the cell rather than its storage since there is a reduced capacity to bind the internalised iron to ferritin. Thus there is an increased utilisation of iron by the proliferating cells. High levels of iron lead to inactivation of the regulatory protein, and a resulting shift of iron from proliferating cells to storage by the liver.

Iron excreted in faeces is primarily exogenous. ie, dietary iron that has not been absorbed into the circulation.

Plasma ferritin is closely related to body stores, whereas plasma [iron] only becomes abnormal in the presence of gross abnormalities of iron storage, a low plasma [ferritin] (less than 20 µg/l) indicates depleted iron stores. Increased plasma [ferritin] is found in iron overload, irrespective of cause.

Transferrin has much longer half life than plasma iron or ferritin, and plasma [transferrin] shows fewer short term fluctuations.

Clinical iron disorders

CLINICAL IRON DISORDERS.
These consist of 2 major states; (a) iron deficiency, which is one of the most prevalent nutritional disorders in the world, and (b) iron overload, which is related to 2 main genetic disorders, thalassaemia and genetic haemochromatosis, both of which are health problems on a world wide scale.

( Thalassaemia is an inherited condition in which there is a total or incomplete synthesis of the alpha or beta chains of haemoglobin. Haemochromatosis is a hereditary defect in iron metabolism characterised by deposits of iron in many tissues, with resulting tissue damage. Haemosiderosis is iron overload characterised by excessive deposits of haemosiderin, a normal iron storage protein).

Ingestion of massive amounts of iron can cause death within hours. The second most common cause of death from accidental poisoning in small children is from ingestion of iron supplements or vitamins with iron. As few as 6 to 12 iron tablets can cause death in a child.

The liver plays a major role in whole body iron homeostasis by serving as the major iron storage organ and the principal site of transferrin synthesis. Increases in hepatic iron storage are brought about by sequestration of iron as the nontoxic form ferritin, and as requirements for the synthesis of haem are increased at non-hepatic sites iron is released from the ferritin to the free binding sites on plasma transferrin.
VITAMIN STORAGE.
The major vitamins stored by the liver are vitamins A, B12, Folic acid and K (and possibly D, and E although there seems to be some conflict about this in the text books ). (A and K are lipid soluble and absorbed from the GI tract incorporated into chylomicrons)

Vitamin A.
Vitamin A is a generic term for retinoids based on retinol.

Metabolic roles of Vitamin A
Vitamin A has 3 metabolic roles;

i) as the prosthetic group of the visual pigments

ii) as a carrier of mannosyl units in the synthesis of hydrophobic glycoproteins

iii) less well defined role in regulation of cell growth and differentiation. In addition it is fairly clear that vitamin A deficiency leads to increased sensitivity to viral, bacterial and protazoal infections, although how this is mediated is unclear.

Clinical significance of vitamin A.
Deficiency of vitamin A only occurs after prolonged lack in the diet. The earliest symptoms are night blindness, followed by follicular hyperkeratinosis, increased susceptibility to infection and cancer and anemia. Prolonged lack of vitamin A leads to deterioration of the eye tissue through progressive keratinisation of the cornea, a condition known as xerophthalmia, as well as keratinisation in the lung and intestinal mucosa. The biochemical basis for the keratinisation may relate to the need for retinol as a sugar carrier in glycosylation reactions. The increased risk of cancer in vitamin A deficiency is thought to be due to the depletion of beta-carotene, which is a highly effective anti-oxidant thought to reduce the risk of cancers initiated by the production of free radicals. Increased beta-carotene has been suggested to reduce the risk of lung cancer in smokers. However, caution is needed when increasing the intake of any lipid soluble vitamin, excess accumulation of vitamin A in the liver leads to toxicity which manifests itself as hepatoatsplenomegaly, nausea and diarrhoea

Digestion and absorption. Vitamin A and carotinoids are released from proteins in the diet during proteolysis in the stomach. They then aggregate with lipids to form globules which pass into the small intestine where emulsification and hydrolysis with bile salts and pancreatic enzymes occurs. The resultant micelles are absorbed into the intestinal cells.

Transport in chylomicra. Within the intestinal cells newly formed chylomicra contain the vitamin A as retinol or retinyl esters. The chylomicra are released into the circulation and during passage through the circulation become chylomicron remnants as the triglyceride part of the chylomicron is removed. The chylomicron remnants are thus relatively enriched in vitamin A.

Uptake and storage by the liver. The chylomicron remnants interact with hepatic cell surface receptors for apo- protein E, and possibly also apo-B, and the remnants are taken into the liver by a process of receptor-mediated
endocytosis. Retinol esters are hydrolysed from the remnant and bind to a cellular binding protein (CRBP) in the hepatocyte cytosol, and can then be subject to a number of alternative metabolic fates. Storage occurs by reforming the retinyl ester, usually with palmitate and then storing the ester in lipid droplets in the hepatocyte. Alternatively, the retinol may be transferred to Stellate cells (also called lipocytes, Ito cells or fat-storing cells) within the liver, where esterification also occurs with storage of the vitamin A in lipid globules. Under normal conditions about 80% of the stored vitamin A is in the stellate cells and about 20% in the hepatocytes. The stored retinyl ester is readily mobilised and used.

**Release from the liver.** The hepatocyte retinol binding protein (apo-RBP) is a 21kD single chain protein of 182 aas, which bind retinol in a 1:1 ratio to form holo RBP. This is then transported via the Golgi apparatus into the plasma. The route of release from the stellate cells is unclear, but may be by 2 possible routes.

(i) transfer back to the parenchymal cells followed by RBP complexing and subsequent release, or

(ii) direct release as an RBP complex into plasma. Route (i) has not been shown experimentally, and whether stellate cells can synthesise RBP is not clear. Whether either or both of these routes occurs is uncertain. Besides storage of vitamin A stellate cells synthesise and secrete collagen, and they proliferate in fibrotic livers. They are also present in many non-hepatic tissues, and may have several as yet undefined physiological roles.

Although vitamin A is stored primarily in liver, all tissues contain some vitamin A. Many non-hepatic tissues (kidney, adipose tissue, bone marrow and lacrimal gland) have been shown to express the mRNA for RBP, although the physiological role of RBP in these tissues is unclear. In plasma, 90% of the RBP is holo-RBP ie, bound to retinol, and it is the holo-RBP which binds the receptors for RBP on the surface of target tissue cells, most of which are epithelial. After binding the retinol is taken into the cytosol of the target cell, whilst the apo-RBP which is left is modified in its conformation and released. This modified apo-RBP can no longer bind retinol and is ultimately degraded, mainly by the kidney.

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### folate

**Folic acid and Vitamin B12.**

(Figure 5 Structures of Folic Acid and Vitamin B12)

Folate (also known as pteroylglutamic acid PGA) is presented to the body in the diet largely in the bound form, where bound means that the folate is attached to a string of glutamic acid residues (polyglutamate). However, the absorption of folate by the intestine functions best with the mono-glutamic acid derivative (ie. only one attached glutamate residue) and with folate with a methyl group attached. Enzymes on the intestinal cell surface hydolysie the polyglutamate and may attach a methyl group, and specific transport systems then absorb the mono glutamate form, with or without the added methyl group, and both travel freely in the blood. On arrival at the liver these 2 forms are
treated in different ways. To the non-methylated form the liver adds further glutamate residues and forms polyglutamate again, and this form is stored. The methyl form is secreted into bile and transported to the gall bladder, from where it returns to the intestine again. so that there is an enteric circulation of the methyl form from gut to liver to gall bladder and back to gut. If the need arises to utilise the methyl form elsewhere in the body the methyl group has to be removed, and the enzyme that does this requires vitamin B12 as cofactor. Thus lack of B12 may lead to folate deficiency at a metabolic level, the so-called 'methyl-folate trap'. Tissues other than the liver are also capable of trapping folate from the blood as the polyglutamate form, and hydrolysing it to release free glutamate if needed. About half of the bodies reserve of folate is in the liver, the total body reserve being 5-10mg in the normal state.

Since the folate is cycled between the gut and the liver, anything that interferes with absorbtion - such as injury to the cells of the GI tract by alcohol abuse, impairs folate absorbtion and can lead to folate deficiency which, since the folate is involved in the activity of enzymes involved in cell division (both folate and vitamin B12 serve as cofactors for enzymes involved in the synthesis of thymidate and hence DNA. The methyl group from the storage form of folate ie. N5-methyl tetrahydofolate is removed by a vitamin B12 dependent enzyme. The THF produced is then used to make N5-N10 methylene which acts to convert dUMP to dTMP via thmidylate synthetase and generates dihydrofolate which can be reconverted onto THF for reuse. The dTMP is used in DNA synthesis. Vitamin B12 (as adenosyl cobalamin) is a cofactor for methyl malonyl CoA mutase, yielding succinyl CoA, and methylcobalamin is a cofactor for methyl tetrahydofolate- homocysteine methyltrasferase which yields methionine), and the cells lining the GI tract are among the most rapidly renewed in the body, the folate deficiency impairs the GI tract further. Without the ability to make new cells the tract rapidly deteriorates . Since blood cells also turn over rapidly they need nucleic acids for their synthesis and are also vulnerable to folate deficiency which leads to megablastic anemia.

**Folate deficiency.** A too low intake is theoretically possible with a diet low in folate- eg babies fed goat milk. Impaired absorbtion or increased unusual metabolic need may lead to a need for the vitamin. Folate deficiency impairs cell division and protein synthesis. In folate deficiency replacement of red blood cells and GI tract cells is reduced, and the first symptoms of folate deficiency are a type of anemia and GI tract deterioration.

**Folate toxicity.** High doses of folate may have adverse effects but the literature is conflicting. One possible danger with high folate arises from the relationship with vitamin B12. Without enough vitamin B12, folate may be trapped inside the cells as methyl tetrahydrofolate, and as such is unavailable for the bodies use. (The Me group has to be removed , by a reaction requiring a Vit B12 dependent enzyme, and the Me group is transfered to homocyteine to yield methionine and tetrahydrofolate)
B12
Vitamin B12. (also called cobalamin)
Vitamin B12 is not found in plants at all, and this can lead to dietary problems in strict vegetarians.
Vitamin B12 and folate are linked in that the vitamin B12 is a cofactor for the enzyme that removes the methyl group from methyl folate. Vitamin B12 is also important as cofactor for methyl malonyl CoA mutase, involved in the biosynthesis of lipids in the myelin sheath around nerves. The 'methyl folate trap' hypothesis explains why haematological symptoms of B12 deficiency (ie. megaloblastic anemia in which giant haemopoietic cells accumulate in the bone marrow, since they are unable to divide due to inhibition of DNA synthesis) responds to folate treatment, but the neurological effects of B12 do not.

Absorption of B12 requires a protein known as intrinsic factor produced by the stomach. The intrinsic factor attaches to the vitamin B12 and the complex moves to the intestine where absorption occurs. Loss of intrinsic factor (which occurs with some specific genetic defects in later life, or if the stomach is injured or following gastrectomy) means absorption is defective and injections of B12 may be needed. The vitamin B12 deficiency due to lack of intrinsic factor is known as pernicious anemia.

Vitamin B12 is transported around in the blood attached to specific binding proteins and uptake into cells is via receptors for the binding protein-vitamin complex. Body stores of vitamin B12 are from 1 to 10mg, with 50-90% of the stored vitamin being in the liver.

K
Vitamin K and blood clotting factors.
The synthesis of the clotting factors occurs largely in the liver, refer to Table 2,
and the liver acts as a reserve of clotting factors. For some of these factors there is an essential involvement of vitamin K.

(Fig 6 Vitamin K structure and role in carboxylation of glutamyl residues)

Vitamin K acts as part of a membrane-bound carboxylase system that participates in the posttranslational carboxylation of a number of vitamin K-dependent proteins involved in blood clotting. As yet however, the vitamin K-dependent gamma-glutamylcarboxylase has not been isolated and its reaction mechanism is currently unclear. Vitamin K does have roles beyond its involvement in blood coagulation. Body stores of Vitamin K are small, but take several weeks to deplete.

All compounds with vitamin K activity are based on the 2-methyl-1,4-napthoquinone nucleus, Vitamin K1 (from plants) and vitamin K2 from animal tissues differ in the nature of the attached side chain and are lipid soluble due to the aliphatic nature of the side chain, whilst vitamin K3 without the side chain is water soluble. Vitamin K2 can be synthesised by intestinal bacteria so that deficiency of the vitamin in adults is rare, but can result from long term antibiotic
treatment. In the new born infant the intestine is sterile and so vitamin K deficiency is possible if the early diet lacks vitamin K. The primary symptom of deficiency in infants is a haemorrhagic syndrome.

The only well defined role for vitamin K is its role as cofactor for a microsomal carboxylase, although it may well have other roles as yet undefined. The carboxylase is involved in the formation of gamma-carboxyglutamate, which occurs at several points in prothrombin and blood clotting factors VII, IX and X, and in protein C and protein S which act as anticoagulators. The carboxylase introduces a second carboxyl group onto the gamma carbon of glutamic acid residues in these proteins, and these residues are involved in formation of the Ca2+ binding sites of these proteins. This protein modification is a posttranslational event, and in the process vitamin K is oxidised and regenerated by reduction with lipoic acid or NADPH. The regeneration process is inhibited by analogues of vitamin K such as warfarin and dicoumarol, which are used as inhibitors of blood clotting in the treatment of thrombosis. By inhibiting the gamma-carboxyglutamate formation they reduce the Ca2+ binding of the affected factors and thus inhibit the clotting cascade.

Vitamins K1 and K2 are absorbed from the small bowel incorporated into chylomicrons and appear in the lymph. Vitamin K is transferred via the liver to beta-lipoproteins. No specific carrier for vitamin K in plasma has been identified. The whole body pool of vitamin K is quite small, and vitamin K is found in several other tissues as well as liver. The vitamin K in the liver is not really a store in the sense that this is mobilised to the other tissues, but vitamin K does have this special role in the synthesis of the blood clotting factors, which are stored in the liver.

**Deficiency of vitamin K**, (due to malabsorption, obstructive jaundice, antibiotic therapy or antagonists such as warfarin), is associated with a marked decrease in prothrombin, factor VII, IX and X activities and an increased tendency to bleed. As anticipated, the prothrombin and other proteins produced in the liver in vitamin K deficiency have a reduced amount of gamma-carboxyglutamate and reduced capacity to bind calcium and function normally.