Review

Choline deficiency

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Introduction

Choline is required to make the phospholipids phosphatidylcholine, lysophosphatidylcholine, choline plasmalogen, and sphingomyelin—essential components of all membranes. It is a precursor for the biosynthesis of the neurotransmitter acetylcholine and also is an important source of labile methyl groups.[1] Much attention has been given to the effects of supplemental choline upon brain function—it has been suggested that such treatments enhance acetylcholine synthesis and release.[2-7] Still unresolved, however, is the question of whether choline is normally required as part of the human diet.

Several lines of evidence suggest that choline is indeed an essential nutrient for humans:

1. Human cells grown in culture have an absolute requirement for choline.[8]
2. Healthy humans fed diets deficient in choline have decreased plasma choline concentrations (discussed later in this review).
3. Malnourished humans have diminished plasma or serum choline concentrations.[9,10]
4. Humans fed by vein with solutions containing little or no choline develop liver dysfunction that is similar to that seen in choline deficient animals.[9]
5. In other mammals, including the monkey, choline deficiency results in severe liver dysfunction.[1,11]

The major reasons choline is considered a dispensable nutrient for humans are:

1. There is an endogenous pathway for the *de novo* biosynthesis of choline moiety via the sequential methylation of phosphatidylethanolamine.[12]
2. It has been difficult to identify a choline deficiency syndrome in healthy humans because most common foods contain choline and because the demand for choline is modified by the rate of growth of an individual and by complex inter-relationships between choline and the nutrients methionine, folic acid and Vitamin B_{12} (lipotropes).[1]
3. No one has tried to experimentally induce choline deficiency in normal humans.

Obviously, the above arguments do not prove or disprove that humans require dietary choline. Diminished tissue levels of a nutrient associated with removal of the nutrient from the diet are suggestive of a nutrient requirement, but deficiency should be associated with deterioration of organ function if a nutrient is essential. The presence of a pathway for endogenous synthesis does not make a nutrient dispensable. Most mammals can synthesize choline moiety, yet they become severely (often fatally) ill if deprived of choline. Under certain circumstances vitamin D is an essential nutrient—deficiency is associated with organ dysfunction—yet endogenous pathways exist for the biosynthesis of vitamin D. In this review, I will discuss the expected biochemical and physiological uses for choline, the expected effects of choline deficiency and will present evidence that there is a requirement for choline in the human's diet.

Dietary sources of choline

Calculations of dietary choline intake are based upon estimates of the free choline and phosphatidylcholine content of foods.[13-17] Older assay procedures for choline were imprecise, making many of the available data unreliable. We have recently measured the choline, phosphatidylcholine and sphingomyelin contents of some foods using a gas chromatography/mass spectrometric assay (*Table 1*). Our own measurements of the lysophosphatidylcholine, glycerophosphocholine and phosphocholine contents of rat tissues,[18] show that these choline-containing compounds are present in high concentrations in many tissues (e.g., muscle concentrations of these three esters were approximately 100 nmol/g each). Thus, the foods eaten by humans probably also contain significant amounts of these esters of choline. Phosphatidylcholine is also often added to processed foods because it acts as an emulsifying agent or as an antioxidant.

Healthy humans in the United States probably in-

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Table 1 Choline content of some common foods

Food	Concentration (nmol/g)		
	Choline	Phosphatidylcholine	Sphingomyelin
Apple	27	280	15
Banana	240	37	20
Beef liver	5831	43500	1850
Beef steak	75	6030	506
Butter	42	1760	460
Cauliflower	1306	2770	183
Corn oil	3	12	5
Coffee	1010	15	23
Cucumber	218	76	27
Egg	42	52000	2250
Ginger ale	2	4	3
Grape juice	475	15	5
Iceberg Lettuce	2930	132	50
Margarine	30	450	15
Milk (bovine, whole)	150	148	82
Orange	200	490	24
Peanut butter	3895	3937	9
Peanuts	4546	4960	78
Potato	511	300	26
Tomato	430	52	32
Whole wheat bread	968	340	11

Choline, phosphatidylcholine, and sphingomyelin were measured using a gas chromatography/mass spectrometry assay[18] in foods prepared in the form that they would normally be consumed.

gest at least 6 g of phosphatidylcholine/day (100 mg/day of this amount deriving from addition to foods during processing). Total choline intake in the adult human (as free choline and the choline in phosphatidylcholine and other choline esters) probably is in excess of 600 to 1,000 mg/day. Consumption of choline will be higher in humans ingesting phosphatidylcholine (also called lecithin) a dietary "health-food" supplement. The capsules or granules of lecithin sold over the counter are usually impure, (only 35% phosphatidylcholine). In the adult human, serum choline concentrations fluctuate over an approximately 2-fold range when common choline-containing foods are ingested[19] (see *Figure 1*).

Milk is the first, and often the sole food for the human neonate. It contains approximately 200 nmol/ml each of free choline, phosphatidylcholine and sphingomyelin (colostrum and transitional milk have 3 to 4-fold higher free choline content; bovine milk and formulae derived from it are similar in choline content to mature human milk. Soy bean derived formulae can have 3 to 4-fold higher choline concentration. Mammary is capable of actively accumulating choline from maternal blood[20] (see discussion below) and of *de novo* synthesis of choline molecules[21] (see discussion below). For these reasons, human mammary can achieve choline concentrations in milk that are 60 times those found in maternal plasma, whereas artificial formulas may have a choline content differing greatly from that of mother's milk.[17] Neonatal animals and humans have exceptionally high blood choline concentrations.[22,23]

The extent to which dietary choline is bioavailable depends upon the efficiency of its absorption from the intestine. Some ingested choline is metabolized before it can be absorbed from the gut. Gut bacteria degrade it to form betaine and to make methylamines.[24-27] The free choline surviving these fates is absorbed all along the small intestine.[25,28,29] At this time, no other component of the diet has been identified as competing with choline for transport by intestinal carriers.

Both pancreatic secretions and intestinal mucosal cells contain enzymes capable of hydrolyzing phosphatidylcholine in the diet. Phospholipase A_2 (which cleaves the β-fatty acid moiety) is found in pancreatic juice and in the intestinal brush border.[30] Within the gut mucosal cell, phospholipase A_1 cleaves the α-fatty acid, and phospholipase B cleaves both fatty acids.[31] Quantitatively, digestion by pancreatic lipase is the most important process. The net result is that most ingested phosphatidylcholine is absorbed as lysophosphatidylcholine (deacylated in the β position).[32] Within the cells of the gut wall, lysophosphatidylcholine can be deacylated to form glycerophosphocholine, or it can be acylated to reconstitute phosphatidylcholine.[31,32] Two lysophosphatidylcholine molecules are converted to a phosphatidylcholine and a glycerophosphocholine molecule. For this reason, approximately twice as many phosphatidylcholine molecules are absorbed from the gut as are reconstituted and secreted from the mucosal cell into the lymphatic circulation.[32]

Glycerophosphocholine is also present in the diet and is formed from dietary phosphatidylcholine. Within the gut wall, glycerophosphocholine diesterase (L-3-glycerophosphocholine glycerophosphohydrolase) catalyzes the conversion of glycerophosphocholine to glycerophosphate and free choline. This

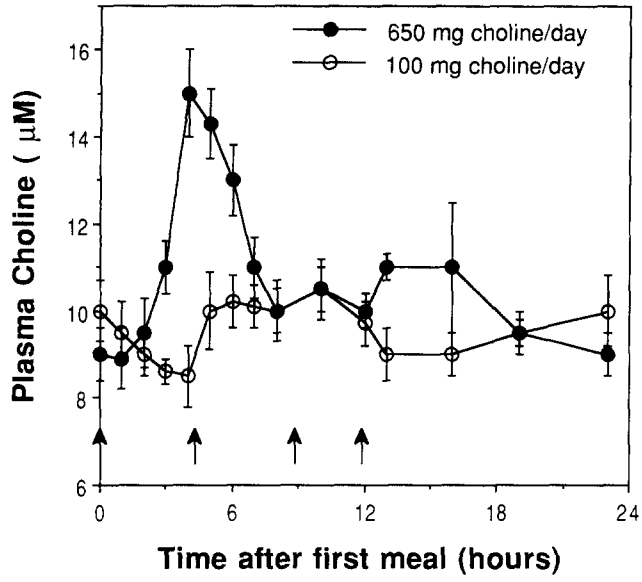

Figure 1 Plasma choline concentrations in normal humans. Six adult humans ingested diets of common foods designed to be low in choline content (100 mg choline/day) or normal in choline content (650 mg choline/day). Meal times are indicated by arrows. Breakfast in the normal choline diet included 150 g eggs, lunch included 90 g peanut butter—both are rich in choline content. Plasma was obtained at regular intervals and assayed for choline content using a radioenzyme assay. Data are presented as mean ± SEM (n = 6/point). From Zeisel *et al.*[19] with permission.

free choline enters the portal circulation of the liver.[32] Phosphocholine is also present in small amounts in the diet. It is rapidly degraded by intenstinal alkaline phosphatases, liberating free choline and inorganic phosphate.

The phosphatidylcholine absorbed and then reformed within gut mucosal cells enters the lymphatic circulation, and then enters the blood. Many tissues possess enzymes that are capable of degrading phosphatidylcholines and lysophosphatidylcholines. Although we are sure that blood choline concentration increases after humans eat phosphatidylcholine,[33] we do not know which organ or which enzyme activity is responsible for the liberation of most of the free choline seen.

Uptake of choline by tissues

All tissues accumulate choline, but uptake by liver, kidney, mammary gland, placenta and brain are of especial importance.[34-36] Most tissues take up choline by a combination of transport processes (diffusion and mediated transport) such as have been described in brain, liver, kidney, erythrocytes, placenta, and intestine.[28,35,37-41]

Choline is accumulated by liver via an active uptake system, and much of it is converted to betaine, phosphocholine and phosphatidylcholine.[35,36] Hepatectomy increases the half-life of choline and results in an increase in blood choline concentration. The rate at which liver takes up choline is sufficient to explain the rapid disappearance of choline injected systemically.

The kidney also accumulates choline.[40,42-45] Some of this choline appears in the urine unchanged, but most is oxidized within kidney to form betaine.[46] This betaine may serve as an important osmoprotectant within kidney.[47] Mean free choline concentration in the plasma of azotemic humans is several times greater than in normal controls and hemodialysis rapidly removes choline from the plasma.[48] Renal transplantation in humans lowers plasma choline from 30 μM in the azotemic patient to 15 μM within 1 day.[49]

Uptake of choline by mammary cells enables this tissue to concentrate choline almost 70-fold versus maternal blood.[20] Both mediated (energy dependent, sodium requiring process) and passive transport mechanisms contribute to choline uptake in the mammary epithelial cell. Mediated uptake predominates at choline concentrations below 100 μM (when intracellular choline concentrations were higher than those of the medium). Serum choline concentration in the rat or human is normally approximately 10 μM, and exceeds 50 μM only after pharmacologic doses of choline or choline-containing compounds have been administered.[33] Concentrations as high as 100 μM have never been reported. The metabolism of choline by mammary epithelial cells has not been carefully characterized. We have observed that choline is converted to betaine, phosphocholine and phosphatidylcholine, as expected (see below).[20] In addition, we observed that an unkown compound was formed that is not sarcosine, dimethylglycine, or any obvious metabolite of choline.

The placenta also actively accumulates choline.[37,50-52] There are specific transport systems for choline on both sides of the syncytiotrophoblast.[53] Much of this choline is used to make acetylcholine,[54] the function of which is unknown at this time, and to deliver choline to the fetus.

A specific carrier mechanism transports free choline across the blood-brain barrier.[55] This carrier has a low affinity for choline ($K_{m\ apparent}$ = 440 μM). Thus at physiologic concentrations of choline in serum (10 μM), this carrier is unsaturated and is able to carry choline into the brain at a rate that is proportional to serum choline concentration.[55] In the neonate this choline transporter has very high capacity.[56] The capacity for choline transport across the blood-brain barrier decreases as rats age ($V_{max\ apparent}$ was 50 fold lower in 24 month old than in 2 month old rats).[57] This may mean that the aged brain is much more susceptible to decreased availability of choline than is young brain. Choline is an important constitutent of brain, yet more unesterified choline leaves the brian, *in vivo*, than enters it when plasma choline concentration is less than 15 μM;[58-60] at higher plasma choline concentrations there is always net influx of choline into brain.[61] It is possible that esterified choline might also enter brain,[62] although there is minimal permeability of the blood-brain barrier to lysophosphatidylcholine and phosphatidylcholine.[60]

Choline metabolism

Choline can be acetylated, phosphorylated, and oxidized (*Figure 2*).

Acetylation of choline

Only a small fraction of dietary choline is acetylated, catalyzed by the activity of choline acetyltransferase (EC 2.3.1.6)[36,63] This enzyme is highly concentrated in the terminals of cholinergic neurons,[64] but it is also present in such non-nervous tissues as the placenta.[65] The availability of choline and acetyl-CoA influence choline acetyltransferase activity.[2-5] In brain it is unlikely that choline acetyltransferase is saturated with either of its substrates, so that choline (and possibly acetyl-CoA) availability determines the rate of acetylcholine synthesis.[66] Some investigators report that administration of choline or phosphatidylcholine results in the accumulation of acetylcholine within brain neurons,[2-5] whereas others observe that such acceleration of acetylcholine synthesis by choline administration can only be detected after pretreatments with agents that cause cholinergic neurons to fire rapidly.[6,67-71] Increased brain acetylcholine synthesis is associated with an augmented release into the synapse of this neurotransmitter. A temporal dissociation between choline administration and effects on brain acetylcholine synthesis and release has been observed.[68] Choline taken up by brain may first enter a storage pool (perhaps the phosphatidylcholine in membranes) before being converted to acetylcholine.

Oxidation of choline

A major use for choline is via irreversible oxidation forming betaine, an important methyl donor. Once betaine is formed it cannot be reduced to reform choline; however, it can donate a methyl group to homocysteine, thereby producing dimethylglycine and methionine. Dimethylglycine is converted to sarcosine and then to glycine, producing a 1-carbon fragment. Thus, the oxidation pathway acts to diminish the availability of choline to tissues while, at the same time, scavenging some methyl groups. Much greater amounts of choline are oxidized to form betaine (9 μmol/hr/g) than are phosphorylated to form phosphocholine (1 μmol/hr/g) by rat liver.[72] The metabolism of choline to form betaine is slower in the neonatal than in the older rat[23,72,73]; thus tissue choline has a longer half-life in the neonate. *In vivo*, the rate of betaine formation from administered radiolabelled choline was slower in 3-day old than in 10-day old rats (0.15 μmol/hr/rat at 3 days versus 0.69 μmol/hr/rat at 10 days).[23] The rate of phosphocholine formation was the same in 3 and 10 day old rats (3.3 μmol/hr/rat).[23] As measured *in vitro*, betaine formation (choline dehydrogenase and betaine aldehyde dehydrogenase activities) in liver, increased between birth and 40 days of age (from 0.5 μmol betaine formed/min/g liver on day 1, to 5 μmol/min/g liver on day 40).

Betaine is formed from choline via the intermediate betaine aldehyde. Choline dehydrogenase (EC 1.1.99.1) catalyzes the conversion of choline to betaine aldehyde and uses molecular oxygen as the electron

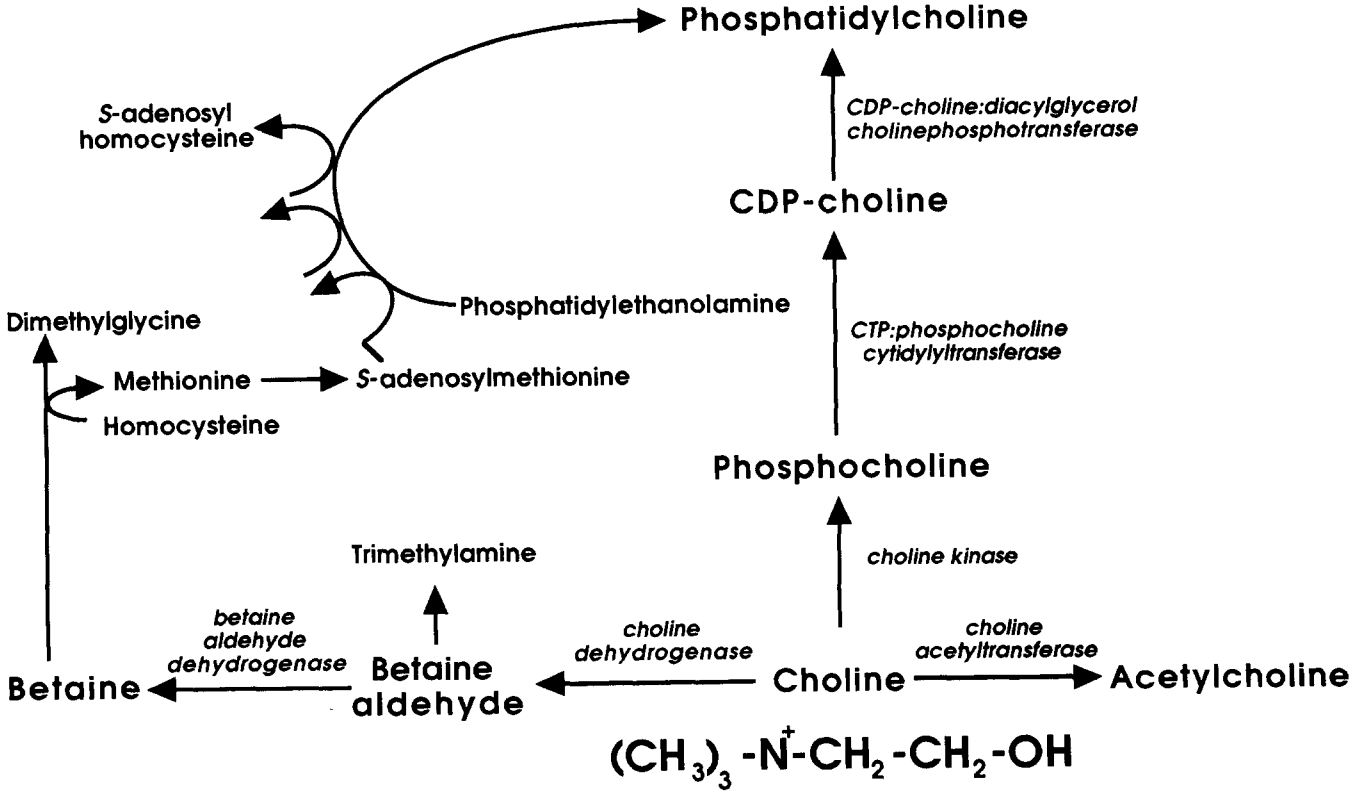

Figure 2 Metabolism of choline.

acceptor; this activity also is capable of converting betaine aldehyde to betaine in the presence of NAD.[74] Choline dehydrogenase in mammalian liver and kidney is mitochondrial, located on the matrix side of the inner membrane.[73,75,76] There is another enzyme, betaine aldehyde dehydrogenase (EC 1.2.1.8), which also catalyzes conversion of betaine aldehyde to betaine. This enzyme requires NAD+, and is found in both mitochondria (this mitochondrial enzyme may be identical to choline dehydrogenase) and cytosol.[76] Choline dehydrogenase activity is present in rat liver > kidney > brain > lung and is not detected in muscle.[77] Activities in rat liver and kidney are 100 fold higher than in other organs.[77] Human liver and kidney have activity (kidney 7-fold more than liver) but less than that measured in the rat.[77] In the kidney, choline dehydrogenase activity is located in the inner medulla and proximal tubules.[47] Mitochondria extracted with *n*-pentane lose choline dehydrogenase activity, which can be restored by addition of ubiquinone[78] or coenzyme Q_2.[74] It has been suggested that pyrroloquinoline quinone (PQQ) is the endogenous cofactor.[79,80]

The mitochondrial choline oxidation system consists of the following steps:

1. choline transporter from the cytosol,
2. oxidation of choline to betaine aldehyde by choline dehydrogenase,
3. oxidation of betaine aldehyde to betaine by betaine aldehyde dehydrogenase, and
4. release of betaine from the matrix into the cytosol.

Since large concentrations of choline have been found in the mitochondrial matrix, and choline uptake from the medium to the matrix occurs at rates faster than the maximal rates of choline oxidation, it is believed that the oxidation per se is rate limiting in the overall process.[81-83] Regulation of choline oxidation could occur at any of these four steps. Betaine aldehyde is an inhibitor of choline dehydrogenase (0.1 mM betaine aldehyde diminished activity by 50%.[74,82] Choline uptake into mitochondria occurs against a concentration gradient,[83] betaine leaves via a special transport mechanism[81] but it has been reported that betaine aldehyde only leaves in permeablized mitochondria.[82] Choline dehydrogenase activity decreased when animals were fed a choline deficient diet.[84]

We have observed that liver mitochondria can form trimethylamine (TMA) from choline via betaine aldehyde.[85] Our data suggest that betaine aldehyde may be an intermediate formed during conversion of choline to TMA. Studies on the metabolism of organic arsenic compounds also suggest that TMA may be formed from betaine aldehyde. Arsenocholine, found in fish and crustaceans, is converted to arsenobetaine aldehyde and to trimethylarsenine oxide and trimethylarsenine by rat liver.[86,87] This activity is localized in the mitochondria.[87] These recent observations suggest that we must consider changes in TMA formation from betaine aldehyde as potential means for regulation of flux through choline oxidation pathways. Once betaine aldehyde is generated it may be further oxidized to betaine, or it may be converted to TMA and acetaldehyde (this latter compound is a postulated second product of this reaction; we are currently investigating its chemical identity).

During the oxidation of choline, NADH is generated which can be oxidized resulting in the generation of ATP. ATP decreases the V_{max} of choline dehydrogenase, while AMP increases it.[82]

Phosphorylation of choline

The phosphorylation of choline is catalyzed by choline kinase (EC 2.7.1.32) using Mg^{2+}-ATP.[88-90] This enzyme is widely distributed in mammalian tissues including the liver, brain, kidney, and lung.[88-92] It is a cytosolic enzyme[88] and in liver is present as three isozymes.[91,93] Choline kinase has a pH optimum of 8 to 9 and a $K_{m\ apparent}$ for ATP of 2 mM and for choline of 30 μM.[91] Purified enzyme from rat kidney exists as a dimer with two 42,000 kDa units.[94] Choline kinase activity in liver is induced by treatment with choline, carbon tetrachloride and insulin.[93] Adenosine inhibits choline kinase.[95] Phosphorylation of choline is the first step in the major pathway for phosphatidylcholine synthesis.[91,96] CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) catalyzes the synthesis of CDP-choline from CTP and phosphocholine. This enzyme's activity is rate limiting for the pathway, and is present in both cytosolic and membrane bound fractions.[91] The membrane associated cytidylyltransferase interacts with phospholipids and is activated, while the cytosolic form is an inactive reservoir of the enzyme.[97] Translocation of the enzyme from cytosol to membrane is regulated by three mechanisms: hydrophobic interactions with membranes, the phosphatidylcholine content of membranes, and phosphorylation of the enzyme. Fatty acids and diacylglycerol cause cytidylyltransferase to bind to membranes, probably by creating a hydrophobic complex.[98] The phosphatidylcholine content of the endoplasmic reticular membranes influences the ability of cytidylyltransferase to bind to this membrane—when phosphatidylcholine content of membranes is high the enzyme disassociates from the membrane and becomes inactive.[99] Cytidylyltransferase is also inactivated and released from the membrane when it is phosphorylated by a cAMP dependent kinase.[93,100,101] A cytosolic phosphatase removes the phosphorus and makes the enzyme more likely to translocate when fatty acids are present.[93,101] In choline deficient hepatocytes most cytidylyltransferase is associated with membranes of the endoplasmic reticulum, and therefore is activated.[101] We observed that, during choline deficiency, whatever choline was available was converted to phosphatidylcholine. We suggest that when choline supplies are limited phosphatidylcholine synthesis takes precedence over other uses for choline. Despite such shunting of choline, eventually choline deficiency decreases the absolute contribution of the CDP-pathway (the pathway is ultimately limited by choline availability).

Once CDP-choline is formed it is rapidly combined

with diacylglycerol, forming phosphatidylcholine in a reaction catalyzed by CDP-choline: 1,2,diacylglycerol choline phosphotransferase (EC 2.7.8.2). This enzyme is located on the cytoplasmic surface of the endoplasmic reticulum.[91]

De novo biosynthesis of choline

Three enzymatic pathways catalyze phosphatidylcholine biosynthesis, yet only one generates new choline molecules. The cytidine diphosphocholine (CDP-choline) and base exchange pathways do not cause a net synthesis of choline, but only redistribute preexisting molecules.[96,102] The only source of choline other than diet is from the *de novo* biosynthesis of phosphatidylcholine catalyzed by phosphatidylethanolamine-N-methyltransferase (PeMT; EC 2.1.1.17). This enzyme synthesizes phosphatidylcholine via sequential methylation of phosphatidylethanolamine using S-adenosylmethionine as a methyl donor.[103-106] Most PeMT activity is found in the liver,[107] but significant activity is present in brain[104,108] and mammary gland[21] and detectable activity is found in other tissues.

PeMT from liver microsomes of adult rats has been purified to apparent homogeneity.[106] A single integral membrane protein with a molecular mass of 18.3 kDa catalyzes the three methylations required to convert phosphatidylethanolamine to phosphatidyl-monomethylethanolamine (PMME), phosphatidyl-dimethylethanolamine (PDME) and finally phosphatidylcholine (using S-adenosylmethionine as the methyl donor). Both intermediates are bound to the enzyme and do not diffuse away.[119] The phospholipid precursors and products appear to compete for a single catalytic site.[119] The first methylation (phosphatidylethanolamine → PMME) is rate limiting.[119] Thus, under physiologic conditions, PMME and PDME never accumulate.[120] Though most data support the hypothesis that there is a single catalytic site for all three methylations catalyzed by PeMT, it is difficult to reconcile this model with the observation of Higgins that intermediates translocate across the membrane bilayer during formation of phosphatidylcholine by PeMT.[121]

There are no accurate estimates of the activity of phosphatidylethanolamine-N-methyltransferase *in vivo*. Investigators have attempted to assess activity by measuring excretion of labile methyl groups in humans eating diets devoid of choline.[122,123] These studies have assumed that choline can only be derived from the diet or from phosphatidylethanolamine-N-methyltransferase activity, but such assumptions are not valid, as choline can also, at least temporarily, be withdrawn from storage pools such as the phosphatidylcholine in membranes. Best estimates, based upon *in vitro* data, are that 15 to 40% of the phosphatidylcholine present in liver is synthesized via the phosphatidylethanolamine-N-methyltransferase pathway, with the remainder coming from the CDP pathway.[107,124] PeMT activity is minimal in the liver of the fetus and newborn rat and increases to a maximum at 6 to 10 days postnatal, thereafter declining slightly.[125-127]

The methylation pathway may be especially important in brain, where it provides choline for acetylcholine synthesis.[128] In brain, PeMT is primarily localized within nerve endings.[104,108] The phosphatidylcholine formed by this pathway in brain constitutes a metabolic pool that turns over rapidly, liberating free choline,[128] some of which may be available as a precursor of a neurotransmitter, acetylcholine. The activity of PeMT changes in a complex fashion during the postnatal development of rat brain.[103] Synthesis of phosphatidylcholine was highest in brains of neonatal animals (2 days of age) because of the presence of relatively large amounts of a novel form of PeMT that catalyzed the first (and probably rate-limiting) methylation. This form of PeMT has a low affinity for S-adenosylmethionine [requiring 90 μM S-adenosylmethionine to reach half-maximal velocity] and could not be detected in brains of rats older than 5 days of age. This novel PeMT was very similar to the form of PeMT present in the mammary epithelial cell.[21] Later in the animals' lives, brain PMME was synthesized by a PeMT that had a high affinity for S-adenosylmethionine (apparent K_m 1.6 μM) and whose activity reached its maximum by 12 to 20 days of age. This activity (V_{max}), however, was lower than that of the neonatal form of PeMT that catalyzed the conversion of phosphatidylethanolamine to PMME. The activity of PeMT that catalyzed the conversion to PDME to phosphatidylcholine (not the rate-limiting step) was highest in the 12 to 20-day-old brain and had a tendency to decrease thereafter. Its affinity for S-adenosylmethionine was fairly constant (apparent K_m 100 μM). In the brains of newborn rats S-adenosylmethionine concentrations are 40 to 50 nmol/g of tissue and S-adenosylhomocysteine levels are 1 nmol/g.[126,129] These levels probably are sufficient to enable the neonatal form of PeMT to maintain high rates of PMME synthesis, and it is in this concentration range that this enzyme is sensitive to changes in S-adenosylmethionine levels. Once the PeMT is of the adult type (exhibiting high affinity for S-adenosylmethionine), it probably is saturated with S-adenosylmethionine and the rate of phosphatidylcholine formation would be expected to be slower and less affected by substrate (See discussion below on the S-adenosylmethionine/S-adenosylhomocysteine ratio and regulation of PeMT). Hoffman *et al.*,[126] studied developmental changes in PeMT activity of rat brain microsomes (we studied whole brain) and found a fairly constant specific activity in all ages examined. Chida and Arakawa[130] observed that phosphatidylcholine synthesis via the methylation pathway was highest in young rats *in vivo*, a finding confirmed by our observations *in vitro*. Developmental changes in the activity or structure of purified PeMT have never been characterized.

The regulation of PeMT activity has not been completely characterized. In adult liver, PeMT seems to be regulated by the availability of phosphatidylethanol-

amine, the S-adenosylmethionine/S-adenosylhomocysteine concentration ratio, and by the composition of the boundary lipids which surround this transmembrane protein. Manipulations which depleted phosphatidylethanolamine levels in membranes tended to diminish formation of phosphatidylcholine via the PeMT enzyme—manipulations which increased phosphatidylethanolamine levels in membranes tended to enhance formation of phosphatidylcholine via the PeMT enzyme.[131] In these studies, enzyme mass was constitutive and activity was determined by the changed availability of phosphatidylethanolamine. Under *in vitro* conditions, the inclusion of phosphatidylcholine in the phospholipid vesicle presented to PeMT enhanced activity, and Ridgway and Vance have suggested that the enzyme may have a secondary phosphatidylcholine binding site which modulates PeMT activity.[119] The phosphatidylethanolamine content of rat liver mitochondria is relatively constant during development; phosphatidylcholine concentrations are 1.6-fold higher in the fetal liver.[132] PeMT displays selectivity for molecular species of phosphatidylethanolamine *in vivo* (two or more double bonds are preferred in the substrate phosphatidylethanolamine), while *in vitro* the enzyme does not display specificity for molecular species of phosphatidylethanolamine, PMME or PDME.[133] The availability of S-adenosylmethionine relative to S-adenosylhomocysteine also determines PeMT activity.[119,134] S-adenosylhomocysteine, a product of the reactions, inhibits the methyltransferase.[119,134] In liver, S-adenosylmethionine concentrations are 70 nmol/g from birth through 30 days of age.[134] S-adenosylhomocysteine is 5 nmol/g in neonatal rat liver and is 14 nmol/g in adults.[126,129] The S-adenosylmethionine/S-adenosylhomocysteine ratio in rat liver drops from 12:1 at birth to 5:1 at 30 days of age.[134] At an S-adenosylmethionine/S-adenosylhomocysteine ratio of 12:1 fifteen percent of total PeMT activity (phosphatidylethanolamine methylation) would be inhibited, while at a ratio of 5:1, thirty percent of activity would be inhibited.[134] Thus, if S-adenosylmethionine were the sole regulator of hepatic PeMT, activity in liver should have been highest during the perinatal period—it was not. As discussed earlier, in brain the availability of S-adenosylmethionine enhances the activity of perinatal PeMT (the enzyme has comparatively low affinity for S-adenosylmethionine). Choline deficiency is associated with increased hepatic PeMT activity measured *in vitro*,[135,136] but this is only seen when exogenous S-adenosylmethionine is added to the incubation mixture.[84] The availability of S-adenosylmethionine in the liver of choline deficient animals limits the activity of this pathway.[137,138]

In addition to precursor and product regulation of PeMT, several other factors influence activity of this enzyme in liver. PeMT is inhibited by increased concentration of fatty acids;[139] unsaturated fatty acids were the most effective inhibitors. Hashizume and colleagues[140] have isolated two different protein inhibitors of PeMT in rat liver cytosol. Both appear to inhibit the methylation of phosphatidylethanolamine → PMME but not the subsequent methylations. These studies were performed using crude enzyme preparations. A peptide isolated from liver, methinin, inhibits several methyltransferases and might be the endogenous inhibitor of PeMT.[141,142]

The regulation of PeMT by hormones has been the focus of a number of reports, but there is no clear story that has emerged.[120] In brain and red blood cells, stimulation of noradrenergic receptors acts to increase the rate of phosphatidylethanolamine methylation.[116] Glucagon inhibits activity *in vivo* and stimulates *in vitro*.[120] Insulin also has variable effects.[120] We did not observe differences in PeMT activity in brain due to sex differences between postnatal days 9 to 61.[103] However, PeMT activity in some tissues is influenced by sex hormones. In rat pituitary, estradiol activates PeMT 6-fold.[143] PeMT activity in livers of adult female rats was 2-fold greater than in male rats.[107,144] It has been suggested that PeMT is phosphorylated by a cAMP dependent serine kinase and by protein kinase C, and that activity is regulated by such phosphorylation.[145-147] However, the purified PeMT used in these studies contained a 50 kDa protein which coeluted with PeMT but was not associated with enzyme activity.[148,149] This 50 kDa protein was the site of phosphorylation. PeMT (18 kDa protein) can be phosphorylated *in vitro* by a cAMP dependent serine kinase.[149] However, *in vitro* PeMT was not phosphorylated in hepatocytes treated with a cAMP analog.[149]

Choline and methyl-group metabolism

The demand for choline as a methyl donor is probably the major factor which determines how rapidly a diet deficient in choline will induce pathology. The pathways of choline and 1-carbon metabolism intersect at the formation of methionine from homocysteine (see *Figure 3*).[122,150,151] Methionine is regenerated from homocysteine in a reaction catalyzed by betaine: homocysteine methyltransferase, in which betaine, a metabolite of choline, serves as the methyl donor.[150] Betaine concentrations in livers of choline deficient rats are markedly diminished,[150-152] as are total folate concentrations.[153] The only alternative mechanism for regeneration of methionine is via a reaction catalyzed by 5-methyltetrahydrofolate:homocysteine methyltransferase (EC 2.1.1.13) which uses a methyl group generated *de novo* from the 1-carbon pool.[150,154] Methionine is converted to S-adenosylmethionine in a reaction catalyzed by methionine adenosyl transferase. S-adenosylmethionine is the active methylating agent for many enzymatic methylations.

A disturbance in folate or methionine metabolism results in changes in choline metabolism and visa versa. During choline deficiency hepatic choline concentration decreases rapidly (see *Figure 4*). At the same time, hepatic S-adenosylmethionine concentra-

tions are decreased (see *Figure 5*).[155-158] It has been suggested that the availability of methionine limits S-adenosylmethionine synthesis during choline deficiency because the 5-methyltetrahydrofolate homocysteine methyltransferase reaction alone cannot fulfill the total requirement for methionine and the betaine dependent remethylation of homocysteine is limited by the availability of betaine.[150] Choline deficiency is also associated with inhibition of hepatic glycine-N-methyltransferase activity (EC 2.1.1.20), which is believed to be important for the removal of excess S-adenosylmethionine from liver.[159] Folate metabolism is also altered in choline deficiency.[153] Methotrexate which is widely used in the treatment of cancer, psoriasis, and rheumatoid arthritis, limits the availability of methyl groups by competitively inhibiting dihydrofolate reductase, a key enzyme in intracellular folate metabolism. When 1-carbon metabolism is poisoned, the only alternative to choline as a source of methyl groups for regeneration of methionine is lost. Hepatic S-adenosylmethionine and betaine concentrations are diminished after treatment with methotrexate.[160-163] Choline supplementation reverses the fatty liver caused by MTX administration.[163-166] Methotrexate treatment increases the susceptibility of tissues to chemical carcinogens,[167] perhaps by creating a relative state of choline deficiency (see discussion below).

Biochemical and physiologic consequences of choline deficiency

Chronic ingestion of a diet deficient in choline has major consequences that include hepatic, renal, pancreatic, memory, and growth disorders. In the rat,[168] hamster,[169] guinea pig,[170] pig[171,172] dog,[173-175] monkey,[11] trout,[176] quail[177] and chicken,[178] choline deficiency results in liver dysfunction. Hepatocyte turnover is greatly increased during choline deficiency.[179,180] During choline deficiency, extremely large amounts of lipid (mainly triglycerides) can accumulate in liver, eventually filling the entire hepatocyte.[168,181-184] Fatty infiltration of the liver starts in the central area of the lobule and spreads peripherally.[181] This process is different from that occuring in kwashiorkor or essential amino acid deficiency, where fatty infiltration usually begins in the portal area of the lobule. The accumulation of triacylglycerol within hepatocytes begins within hours after rats are started on a choline deficient diet, peaks within the first 6 months (at > 2000 mg/liver; in control rats was 28 mg/liver) and

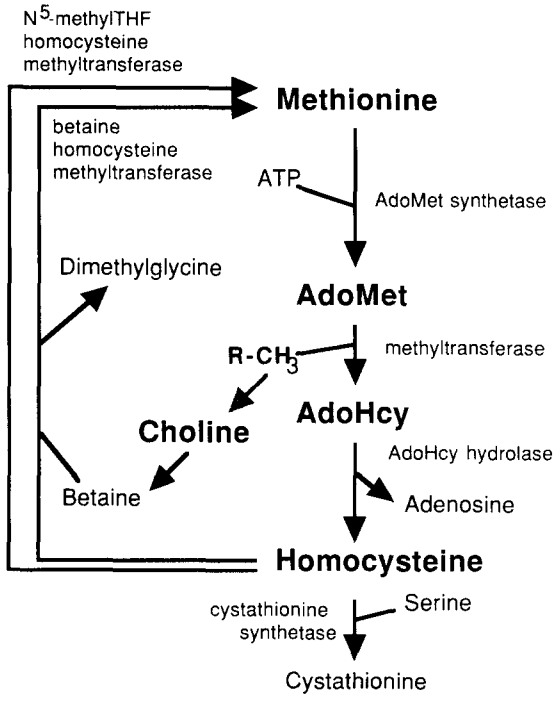

Figure 3 Interrelationships between choline and 1-carbon metabolism. AdoMet = S-adenosylmethionine, AdoHcy = S-adenosylhomocysteine. R-CH_3 = methylated acceptor. Taken from Zeisel *et al.*[158] with permission.

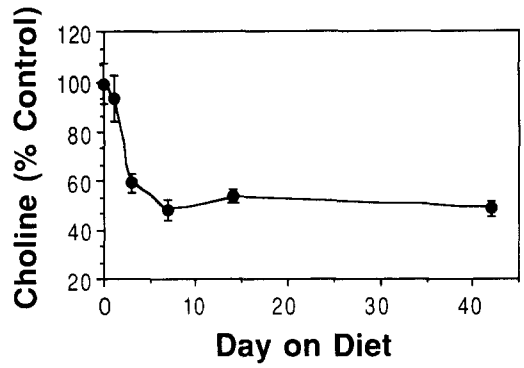

Figure 4 Changes in hepatic choline concentration during choline deficiency in the rat. Rats were fed a control or choline deficient diet for 6 weeks. Choline was determined using a gas chromatography mass spectrometry assay. Control choline concentration in liver was 105 nmol/g on day 0. Data are expressed as means % control ± SEM (n = 5/point). From Zeisel *et al.*[158] with permission.

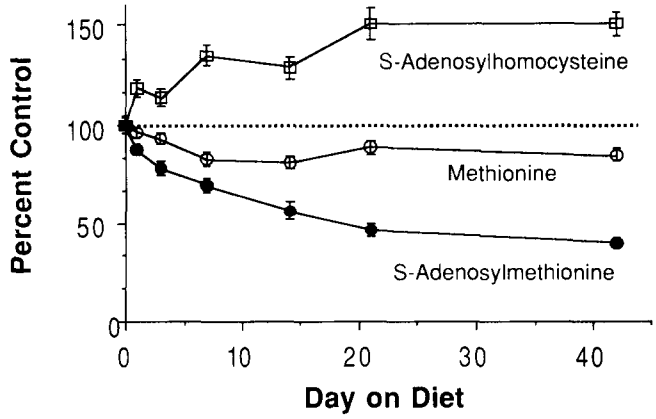

Figure 5 Hepatic concentrations of S-adenosylmethionine, S-adenosylhomocysteine, and methionine during choline deficiency. Rats were pair fed a control or choline deficient diet for 6 weeks. Liver was collected at timed intervals, and S-adenosylmethionine, S-adenosylhomocysteine and methionine were assayed using high pressure liquid chromatography and a UV absorbance detector. Data are expressed as mean percent control (n = 4–5/point). Taken from Zeisel *et al.*[158] with permission.

then diminishes as liver becomes fibrotic.[185] Triacylglycerol accumulation occurs because triglyceride must be packaged as very low density lipoprotein (VLDL) to be exported from liver. Phosphatidylcholine is an essential component of VLDL; other phospholipids cannot substitute.[182,183] Electronmicroscopic studies of hepatocytes from rats fed a choline deficient diet have demonstrated ultrastructural abnormalities of the endoplasmic reticulum and Golgi system associated with delayed VLDL transport.[186] Hepatocytes, isolated from choline-deficient rats were unable to export VLDL until choline or methionine was made available.[182] The methylation of phosphatidylethanolamine can be blocked with 3-deazadenosine without disturbing hepatic lipoprotein secretion.[187]

The defect in hepatic VLDL excretion may be the most apparent problem, but there are abnormalities of secretion in other organs as well in choline deficient animals. When animals are made choline deficient and treated with ethionine, they develop pancreatitis caused by inability to secrete zymogen granules normally.[188,189] This treatment does not affect membrane fusion-fission, but seems to interfere specifically with exocytosis.[190]

Renal function is also compromised, with abnormal concentrating ability, free water reabsorption, sodium excretion, glomerular filtration rate, renal plasma flow, and gross renal hemorrhage.[138-142,191-194] Infertility, growth impairment, bony abnormalities, decreased hematopoiesis, and hypertension have also been reported to be associated with diets low in choline content.[195-198]

Maintaining adult rats on a choline deficient diet lowered brain choline, but did not lower brain acetylcholine levels in some studies.[67,199] However, Nagler reported lower levels of choline and actelylcholine in brain, kidney and intestine of choline deficient rats.[200] Striatal and hippocampal slices from adult rats fed a choline deficient diet for 30 to 40 days had diminished acetylcholine content and synthesis, and formed less free choline during incubations (from hydrolysis of membrane phosphatidylcholine).[69] The absence of choline in the medium superfusing electrically stimulated rat brain slices diminished the release of acetylcholine from these slices, as compared to spontaneous and evoked release in the presence of physiologic (20 μM) concentrations of choline.[201]

Choline supplementation increases the number of dendritic spines in the cerebral cortex of old mice.[202,203] In these same animals, memory, as assessed by learning performance was improved by choline supplementation.[204] A modest degree of choline deficiency (3 mg/day versus 12 mg/day in controls; fed these diets between the ages of 8.5 to 18 months) had no effect on dendritic spine density.[202,203] Unfortunately, the total methyl content of the choline deficient diet used was not enumerated, and no biochemical assessment of choline pool sizes were made. This makes it difficult to be certain that a state of choline deficiency actually existed.

Choline deficiency may alter the hypothalamic-pituitary-adrenal response to stressors. Plasma and adrenal corticosterone were the same in unstressed control and choline deficient rats. However, after auditory or hypercapnic stress, the deficient rats had impaired cortisol response.[205]

The bladder is normally under the influence of parasympathetic (cholinergic) stimulation. Choline deficient mice exhibited a 46% increase over controls in contractile respones of isolated bladders, while mice on a choline enriched diet showed a 15% decrease in contractile response.[206] These data suggest that muscarinic receptors are up-regulated (i.e., increased in number) during choline deficiency.

Choline deficiency and carnitine

Carnitine is a cofactor for long-chain acetylCoA carnitine transferase; human deficiency syndromes have been identified.[207] Rats fed a choline-deficient diet had reduced levels of carnitine in liver, heart, and skeletal muscle.[208,209] This finding has been attributed to a methyl-group deficiency, i.e., carnitine is derived from trimethyllysine. However, a single injection of choline (but not of methionine, betaine, or sarcosine) was able to raise the concentration of hepatic carnitine in these animals to control values within 1.5 hours.[209] This suggests that choline was capable of facilitating carnitine release from some storage pool, as *de novo* synthesis of carnitine would have taken much more time. Paradoxically, plasma carnitine was higher in choline-deficient rats,[209] probably because transport into tissues was inhibited. Perhaps a choline molecule must exit the cell in order to flip the carnitine carrier from the inside to the outside of the plasma membrane.

Choline deficiency and hepatocarcinogenesis

Choline deficient animals (fed diets just adequate in methionine and folate content; i.e., lipotrope limited) are much more likely to develop hepatocarcinomas.[179,180,185,210-218] Deficiency alone is sufficient to trigger carcinogenesis, there is no need for exposure to any known carcinogen.[180,185,219] Chandar and Lombardi[185] observed that 26% of rats fed a choline deficient diet for 16 months (versus 0% of controls) developed hepatocellular carcinoma. These investigators also made the intriguing observation that if, after 12 months of being fed a choline devoid diet, rats were fed a choline sufficient diet for 4 months the incidence of hepatocellular carcinoma increased to 73%. In this latter group, foci of enzyme-altered hepatocytes which synthesize γ-glutamyl transpeptidase (GGT) were detected at a 10-fold higher rate than in controls.[185] These observations are consistent with the hypothesis that, during a crucial period, choline deficiency can either initiate carcinogenesis, or promote endogenously initiated cells, or make hepatocytes susceptible to initiation. The enhancement of carcinogenesis observed when choline was restored to the diet after a year of deprivation may have occurred because choline

deficiency inhibited growth or survival of initiated cells by increasing the rate of death of all hepatocytes.[220] Perhaps when choline was restored initiated cells were able to grow and multiply more rapidly.

There are several mechanisms which have been suggested for the cancer-promoting effect of a choline devoid diet. In the choline deficient liver there is a progressive increase in cell proliferation, related to regeneration after parenchymal cell death.[185,219,220] Cell proliferation, with associated increased rate of DNA synthesis, could be the cause of greater sensitivity to chemical carcinogens.[179] Other stimuli for increased DNA synthesis can be associated with carcinogenesis: hepatectomy and necrogenesis chemicals are examples. However, Shinozuka and Lombardi[218] found that the overall rate of liver cell proliferation could be dissociated from the rate at which preneoplastic lesions formed during choline deficiency, suggesting that cell proliferation is not the sole condition acting as a promoter of liver cancer. Methylation of DNA is important for the regulation of expression of genetic information. It has been suggested that the undermethylation of DNA (decreased 5-methylcytosine content in nuclear DNA), observed during choline deficiency (despite adequate dietary methionine), is responsible for carcinogenesis.[216] Another proposed mechanism is based upon the observation that, when rats are fed a choline deficient diet, increased lipid peroxidation occurs within liver (presence of diene conjugates in lipids isolated from purified hepatic nuclei).[221] Lipid peroxides in the nucleus could be a source of free radicals which could modify DNA, and case carcinogenesis. Though each of these factors probably contributes to carcinogenesis in choline deficient animals, none of the above hypotheses is entirely satisfactory.

1,2-sn-diacylglycerol (1,2-DAG) is an important intermediate for the biosynthesis of triacylglycerol and membrane phospholipids. Choline-containing phospholipids are one of the important sources of 1,2-DAG release during transmembrane signalling.[222,223] 1,2-DAG is also a second messenger, formed when plasma membrane receptors for certain hormones, neurotransmitters or growth factors are coupled to phospholipase C.[222] The 1,2-DAG molecule remains within the membrane after hydrolysis of phospholipids, and can activate a regulatory enzyme, protein kinase C (PKC).[224] During activation, which requires the presence of calcium and phosphatidylserine, PKC is translocated from the cytosol to the plasma membrane.[225] 1,2-DAG markedly increases the affinity of PKC for calcium, thereby activating the enzyme without a net increase in intracellular calcium concentration.[224] 1,2-DAGs containing unsaturated fatty acids are the most potent in this respect, while 1,3- or 2,3-*sn*-diacylglycerols neither activate or inhibit the enzyme.[224] This means that the 2,3-*sn*-diacylglycerol liberated from triglyceride by the action of lipoprotein lipase, and heparin-released hepatic lipase will not activate PKC.[224] The appearance of 1,2-DAG in membranes is usually transient, and therefore PKC is activated only for a short time after a receptor has been stimulated. Previously most interest was focussed upon 1,2-DAG present in plasma membranes as an activator of PKC, but Azhar, *et al.*, [226] and Buckley *et al.*[227] have recently reported that protein kinase C activity is associated with hepatic microsomal and nuclear membranes as well as with plasma membranes.

Several lines of evidence indicate that cancers might develop secondary to abnormalities in PKC-mediated signal transduction. Some of the most potent mitogens and tumor promoters, the phorbol esters, are analogs of 1,2-DAG which have higher affinity than 1,2-DAG for the same site on PKC; they cause PKC translocation to membranes and long lasting activation.[224] Prolonged activation of PKC by these compounds leads to down regulation of the enzyme (i.e., proteolysis to a form which is not bound to the membrane. It is believed that the tumor promoting effects of phorbol esters may be explained by their interactions with PKC. Many mitogens activate PKC, and this activation can be very impressive. Buckley *et al.*[227] found that prolactin, a mitogen for liver, stimulated PKC activity several hundred-fold in rat liver nuclear membrane; probably by a phospholipid-1,2-DAG mediated pathway. Gene expression abnormalities that are often associated with tumors, can also be associated with alterations in 1,2-DAG and PKC mediated pathways. Fibroblasts normally respond to excess 1,2-DAG by activating diacylglycerol kinase activity (the enzyme translocates from cytosol to membranes); in erbB-transformed fibroblasts this does not occur.[228] 1,2-DAG is elevated *in vivo* in *ras*-transformed liver of neonatal transgenic mice bearing a hybrid gene construct consisting of mouse albumin enhancer/promoter fused to the coding sequence of an activated human Ha-*ras* oncogene.[229] NIH 3T3 cells transformed with Ha-*ras* or Ki-*ras*, v-*src*, and *v-fms* oncogenes have elevated 1,2-DAG levels as well as tonic activation and partial down regulation of PKC.[230,231] Activated PKC, in turn, may participate in mechanisms leading to the induction of expression of the c-*myc* oncogene.[232,233] Fibroblasts, transfected with a gene for a mutant PKC which is constantly in the active conformation, become transformed and form tumors in mice.[234] This is the strongest evidence to date that activation of PKC is a key event in carcinogenesis.

We have observed that 1,2-DAG accumulates in choline deficient liver (*Figure 6*).[184] 1,2-DAG content of plasma membrane was significantly increased as well (*Figure 7*). We did not observe an increase in the 1,2-DAG content of mitochondria or microsomes. Thus, the increase in 1,2-DAG appears to be specific, occurring at a site where 1,2-DAG is known to be able to activate PKC. In human control livers we measure similar concentrations of 1,2-DAG (unpublished data).

It has been reported that the sum of 1,2-DAG and 2,3-*sn*-DAG increased from 300 to 1800 nmol/g in rat liver after one week of choline deficiency.[235] Unfortunately, at the time of these investigations, no convenient technique existed to measure 1,2-DAG

specifically. Recent studies indicate that the physiological state of the cell determines the ratio of 1,2-DAG to 2,3*sn*-DAG. For example in the parotid, 2,3-*sn*-DAG constituted approximately 8% of DAG at rest, and stimulation of β-adrenergic receptors specifically increased the formation of 2,3-*sn*-DAG, such that it constituted over 30% of the total DAG.[236] This 2,3-*sn*-DAG was ineffective in stimulating PKC activity.[236] Thus, it is important that we observed specific increases in 1,2-DAG levels in choline deficient liver because only this stereoisomer can activate PKC. Choline deficiency was associated with a remarkable increase in hepatic 1,2-DAG concentrations, reaching values higher than those occurring after stimulation of a receptor linked to phospholipase C activation (e.g., vasopressin receptor[237,238] and of the order of magnitude needed to activate PKC *in vitro*. The concentrations of 1,2-DAG achieved in choline deficient liver were several fold higher than the concentrations of exogenous 1,2-DAG used to activate PKC in platelets[239,240] or to modify responses to α1-adrenergic receptors in the liver.[241] The activation, as well as down regulation, of PKC has been observed in cells transformed with Ha-*ras* or Ki-*ras*, v-*src*, and *v-fms* oncogenes which have elevated 1,2-DAG levels.[230,231] It is expected that accumulation of 1,2-DAG in cells from choline deficient animals may result in one of two phenomenona, a) constitutive translocation of PKC to the membrane such that membrane-associated PKC activity is higher in these cells than that in the controls and that therefore there would be less enzyme activity that could be translated to the membrane by the action of phorbol ester, or b) an initial translocation of PKC to the membrane that will occur immediately with the onset of increased intracellular 1,2-DAG concentrations, followed by a decrease in PKC activity in the cells [due to higher PKC protein turnover]. In preliminary studies using animals fed a choline deficient diet for 6 weeks we did not observe a change in the amount of membrane associated PKC activity.[242] Other investigators, studying shorter periods of choline deficiency in rats (one to four weeks) also did not find that PKC translocated to membranes.[243] It is possible that PKC will be activated (translocated) after longer periods of choline deficiency, or that only one of the several isozymes of PKC in liver[244,245] are activated. Metabolites generated from some choline-containing phospholipids (sphingosine and lysosphingolipids from sphingomyelin, and lysophosphatidylcholine from phosphatidylcholine) act as negative effectors modulating PKC activation.[222] Perhaps net activation of PKC depends upon a balance between production of 1,2-DAG and these negative effectors.

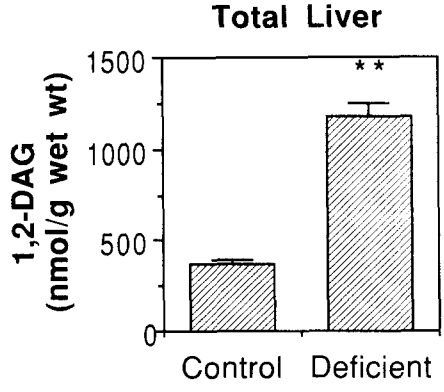

Figure 6 Total 1,2-diacylglycerol in rat liver. Rats were pair-fed control or choline-devoid diets for six weeks. Hepatic 1,2-*sn*-diacylglycerol was measured by a radioenzymatic assay. Results are expressed as means ± SEM. Statistical significance of differences between groups was determined by t-test. From Blusztajn and Zeisel[184] with permission. ** = $p < 0.01$ by t-test.

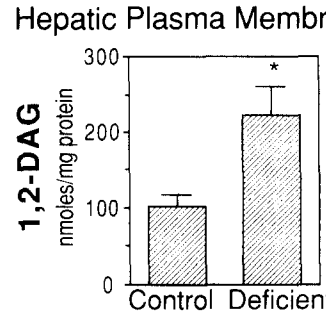

Figure 7 1,2-diacylglycerol in plasma membranes of rat liver. Rats were pair-fed a choline deficient or choline-containing (control) diet for six weeks. Membranes were prepared from liver using the method of Aronson and Touster.[256] 1,2-*sn*-diacylglycerol was measured by a radioenzymatic assay. Results are expressed as means ± SE (n = 3/group). * = $p < 0.05$ by t-test.

Pathophysiologic events which could result in choline deficiency

Choline and phosphatidylcholine are so ubiquitous in the food supply that a deficiency syndrome in humans has not yet been proven (see discussion below). The rat requires cystine for hair formation. This requirement may increase the demand for methionine and the methyl groups of choline relative to the human. There are certain clinical situations which act to increase demands for choline, and therefore might be more likely to result in organ dysfunction secondary to choline deficiency.

Hepatic complications associated with total parenteral nutrition (TPN), which include fatty infiltration of the liver and hepatocellular damage, have been reported by many clinical groups.[246] Frequently, TPN must be terminated because of the severity of the associated liver disease. It is possible that some of the liver disease associated with TPN is related to choline deficiency. When rats were fed intravenously with choline-free TPN solutions (4.25% FreAmine II in 25% glucose), they developed fatty infiltration of the liver, and had elevated serum levels of conjugated bilirubin and transaminases.[247] In these animals, oral or intravenous supplements of choline were effective in reversing hepatic lipid accumulation. This finding suggests that these rats were choline-deficient and that the methyl groups supplied by methionine within the TPN

solution were not available in adequate amounts or were not utilized to spare choline requirements. Other investigators, however, have observed that intravenously-administered choline did not prevent fatty liver in rats treated with TPN.[248]

Amino acid-glucose solutions used in TPN of humans contain no choline.[9,249] The lipid emulsions used to deliver extra calories and essential fatty acids during total patenteral nutrition contain choline in the form of phosphatidylcholine (20% emulsion contains 13.2 μmol/ml.).[9] Burt *et al.*[250] reported that plasma choline concentrations were decreased in TPN patients at the same time that liver dysfunction was present. Malnourished humans, at the time they were referred for TPN therapy, had significantly lower plasma choline concentrations than did well-fed control subjects.[9,249] Plasma choline concentrations in these patients declined further when they were treated with an amino acid-glucose solution lacking choline during the first week of therapy.[9] However, when patients were treated with lipid emulsion as well as an amino acid-glucose solution, their plasma choline concentrations rose slightly. Neither group received sufficient choline to restore plasma choline concentrations to normal. We calculated that humans treated with TPN required 1,000 to 1,700 μmol of choline-containing phospholipid per day during the first week of TPN therapy to maintain plasma choline levels.[9] Enteral food supplements, which contained choline, contributed to the rising plasma choline observed after the first week of TPN therapy. Malnourished humans with cirrhosis who were fed enterally also had diminished plasma choline content.[10]

Conditions that enhance hepatic triglyceride synthesis (such as carbohydrate loading) increase the requirement for the choline-containing lipoprotein envelope surrounding these compounds in plasma.[251] Thus, treatment of malnourished patients with high-calorie TPN solutions, at a time when choline stores are depleted, might cause hepatic dysfunction. The definitive experiment, in which supplemental choline is administered and found to decrease the incidence of hepatic dysfunction during TPN, has not yet been performed. Until such data are available, it is impossible to state that humans require choline during TPN. The information available to date only suggests that this may be so.

Bypass surgery involving large segments of the bowel (i.e., to produce weight loss in very obese humans) is associated with fatty liver. In obese rats which have had 90% of their small intestine bypassed, fatty liver develops. Choline supplementation prevents this, and choline deficient diets in such patients exacerbate the accumulation of fat in the liver.[252]

Pregnancy is associated with increased requirements for tissue (fetus) biosynthesis. As discussed earlier, a placental transport system withdraws choline from mother into fetus. The choline concentration of the liver fell from a mean of 130 nmol/g in adult non-pregnant rats to 38 nmol/g in late pregnancy.[253] Pregnant women, especially those in their third trimester, are particularly susceptible to development of fatty liver, and it has been suggested that this may be related to an increased choline requirement.[254] The neonate requires especially large amounts of choline to sustain rapid tissue growth, yet the rate of *de novo* biosynthesis of choline is minimal in the newborn (see earlier discussion of the methyltransferase). For this reason choline deficiency associate fatty liver is easiest to induce in young, growing mammals.[127]

Experimental choline deficiency in humans

We have characterized the effects of making normal humans choline deficient.[255] For a week at the beginning and end of our study, healthy human subjects ate a diet that contained choline, while for 3 weeks in the middle of the study the subjects may, or may not, have eaten a diet containing choline. The choline content of the deficient diet was 13 mg/70 kg body weight/day, the choline-containing diet contained 713 mg/70 kg body weight/day. Both diets delivered 40 Kcal/kg body weight (10% protein, 35% fat, 55% carbohydrate) in the form of liquid shakes. The diet met the recommended daily allowance for all amino acids, vitamins and minerals; of special interest—folate (300 μg/70 kg body weight/day) and vitamin B_{12} (9 μg/70 kg body weight/day). The protein source was α-soy protein (STA-PRO 3200, Central Soya) which contained adequate amounts of methionine (921 mg/70 kg body weight/day). Six subjects were fed the choline-

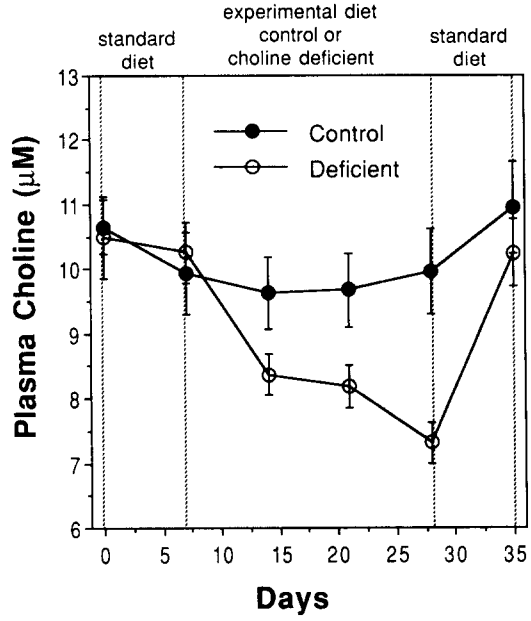

Figure 8 Plasma choline concentrations in humans during ingestion of choline deficient or control diets. Healthy human volunteers were fed a semisynthetic diet containing choline for the first and last weeks of the study (days 1 to 7 and days 29 to 35). For three weeks (days 8 to 28), eight of the subjects ingested the same semisynthetic diet devoid of choline (deficient) and six subjects ingested the diet containing choline (control). Plasma choline was determined using a mass spectrometric method.[18] Data are expressed as mean ± SEM. Taken from Zeisel *et al.*[255] with permission.

containing (control) diet during the experimental period, 8 subjects were fed the choline deficient (deficient) diet during the experimental period.

Plasma concentrations of choline dropped significantly in the deficient group (decreased in all subjects) between day 7 and day 28 (period on defiient diet); there were no changes observed in the control group (*Figure 8*). As soon as the deficient subjects were returned to a choline-containing diet their plasma choline concentrations returned to normal. The drop in plasma choline concentrations associated with ingesting a choline deficient diet observed in these humans is similar to that which was observed in rats.[158] Malnourished humans present with plasma choline concentrations that are similar to those observed in our choline deficient subjects on day 28.[9,10,249] Humans deprived of choline appear not to be able to make up this dietary deficit by the *de novo* biosynthesis pathway (see earlier discussion)—therefore, it is likely that choline is an essential nutrient for humans.

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