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# CHOLINE AND HUMAN NUTRITION

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## CONTENTS



## INTRODUCTION

Choline is a dietary component that is crucial for normal function of all cells. We believe that humans require dietary choline in order to sustain normal life. Choline or its metabolites (see Figure 1) are important for the structural



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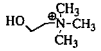
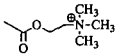
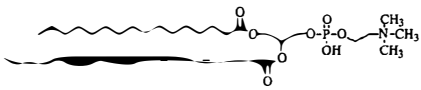
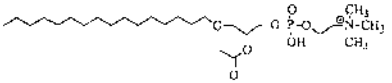
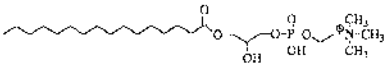
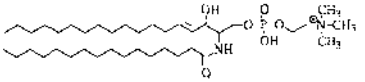
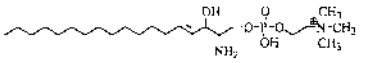
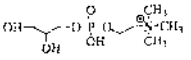
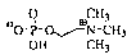
| Compound | Chemical structure | Biologic function |
|---|---|---|
| *Choline* |  | |
| Betaine |  | Methyl group donor; renal osmolyte |
| Acetylcholine |  | Neurotransmitter |
| Phosphatidylcholine |  | Necessary building block of biomembranes; VLDL component necessary for hepatic VLDL secretion |
| Platelet-activating factor |  | Hormone |
| Lysophosphatidylcholine |  | Putative second messenger modulating PKC activity |
| Sphingomyelin |  | Necessary building block of biomembranes |
| Lysosphingomyelin |  | Putative second messenger mediating growth-factor actions, mitogen |
| Glycerophosphocholine |  | Renal osmolyte |
| Phosphocholine |  | Intracellular storage pool of choline |

***Figure 1*** Choline-containing compounds of physiologic importance.

integrity of cell membranes, methyl-metabolism, cholinergic neurotransmission, and transmembrane signaling as well as for lipid-cholesterol transport and metabolism. This quaternary amine is present in tissues predominantly as one of the choline-phospholipids (phosphatidylcholine and sphingomyelin). Although they represent a smaller proportion of the total choline pool, impor-

tant metabolites include platelet-activating factor (PAF), acetylcholine, choline plasmalogens, lysophosphatidylcholine, phosphocholine, glycerophosphocholine, and betaine.

Several comprehensive reviews explore the metabolism and functions of choline (181, 183, 184). Here we do not attempt to be comprehensive but rather to present exciting new observations that greatly enhance our understanding of choline and cellular function. We discuss new information about how choline, methionine, and methyl-folate metabolism are interrelated. This information is especially pertinent because of the recent recommendations about folate supplementation during pregnancy. We present new perspectives on the regulation of phosphatidylcholine synthesis and on the signal transduction functions of phosphatidylcholine, PAF, and sphingomyelin. These major signaling functions for choline phospholipids were unknown a decade ago when we wrote our first review on choline for the *Annual Review of Nutrition.* Choline deprivation is the only single-nutrient deficiency that causes spontaneous carcinogenesis in animals; we review recent studies on the mechanisms involved. We discuss how membrane phospholipids act as sources of choline for acetylcholine biosynthesis in neurons. Exposure to choline during critical periods in brain development significantly affects brain structure and function. This observation highlights the importance of perinatal nutrition for normal brain function in the elderly. Finally, we present a review of several clinical studies that suggest that humans require dietary choline.



## UPDATE OF CHOLINE METABOLISM

### *Choline and Methyl-Group Metabolism*

The metabolic pathways of choline, methionine, and methyl-folate are closely interrelated, and the use of choline molecules as methyl donors is probably the major factor that determines how rapidly a diet deficient in choline will induce pathologic changes (121). The pathways of choline and 1-carbon metabolism intersect at the formation of methionine from homocysteine. The three methyl groups of choline can be made available from one-carbon metabolism upon conversion to betaine. This process requires two enzymes (191): The mitochondrial choline dehydrogenase converts choline to betaine aldehyde, which can then be oxidized in the mitochondria or cytoplasm to betaine. Liver and kidney are the major sites for choline oxidation; in kidney betaine serves as an osmolyte (63). Betaine:homocysteine methyltransferase catalyzes the methylation of homocysteine, using betaine as the methyl donor (see Figure 2) (60, 120, 175). In an alternative pathway, 5-methyltetrahydrofolate:homocysteine methyltransferase regenerates methionine using a methyl group derived de novo from the 1-carbon pool (59, 60). Methionine adenosyltransferase

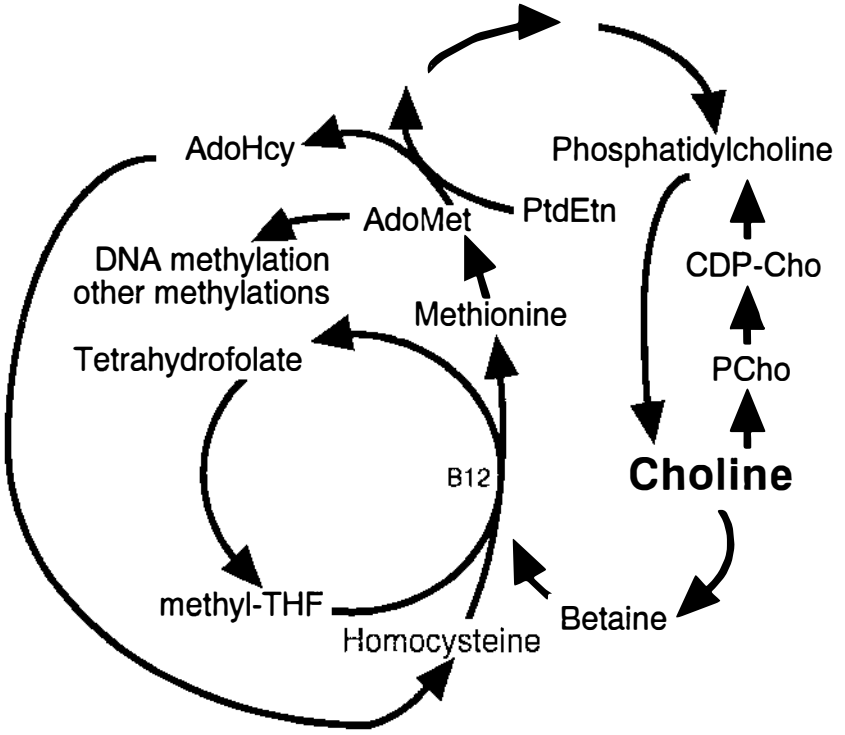



*Figure 2* Choline, folate, and methionine metabolism are closely interrelated. CDP-Cho, cytidine diphosphocholine; PCho, phosphocholine; PtdEtn, phosphatidylethanolamine; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; B12, vitamin B12.

converts methionine to S-adenosylmethionine, the active methylating agent for many enzymatic methylations, including the methylation of phosphatidylethanolamine to form phosphatidylcholine (144).

Perturbing metabolism of one of the methyl donors reveals intermingling of these metabolic pathways. Hepatic choline and choline metabolite concentrations decreased during choline deficiency in the rat (190). Phosphocholine in liver was the best marker for choline status because it was most sensitive to modest dietary choline deficiency, decreasing to 10–20% of control values after rats were fed a deficient diet for 2 weeks (136). Total hepatic folate content decreased 31% after rats were 2 weeks on a choline-deficient diet, and an elongation of the glutamate chains of the folates was observed, which suggests prolonged retention time within liver (152). Rats fed diets deficient in both methionine and choline for five weeks had hepatic folate concentrations half those of controls (83). During choline deficiency, hepatic S-adenosylmethionine concentrations also decreased as much as 50% (7, 135, 155, 190).

Hepatic glycine-*N*-methyltransferase activity decreases as well (39). This enzyme may be important for the removal of excess S-adenosylmethionine from liver. Tetrahydrofolate deficiency, induced by treatment with methotrexate (8, 9, 62, 136, 160) or by dietary folate deficiency (SH Zeisel, unpublished data), results in diminished hepatic total choline, with the greatest decrease occurring in hepatic phosphocholine concentrations.

Redundant parallel pathways protect the capacity to donate methyl groups. Clearly, if investigators had first established that choline was essential, they would believe that methionine is not an essential amino acid because it can be replaced by homocysteine and choline. The amounts of choline, methyl-folate, or methionine required in the diet depend on the availability of the other two. Does this observation mean that other methyl donors can completely substitute for choline? Many investigators make this assumption, but the question has never been sufficiently investigated. As we discuss below, some important functions of choline require the whole molecule, not just the methyl groups. Can de novo synthesis of choline (see discussion of phosphatidylethanolamine *N*-methyltransferase) proceed at rates adequate to meet these demands? In rats this is not the case (183), but rats destroy choline (by oxidation) more rapidly than humans (156) and need more cysteine (derived from methionine) for hair synthesis. These factors may increase the dietary choline requirement of rats compared with humans. Recent studies of choline requirements in humans (discussed below) suggest that humans do require some dietary choline.

## *Phosphatidylcholine Biosynthesis*

Elegant regulatory mechanisms control phosphatidylcholine biosynthesis and hydrolysis. Many of these mechanisms have been elucidated during the last decade. Synthesis occurs by two pathways. In the first, choline is phosphorylated then converted to cytidine diphosphocholine (CDP-choline). This high-energy intermediate, in combination with diacylglycerol, forms phosphatidylcholine and cytidine monophosphate (91). In the alternative pathway, phosphatidylethanolamine is sequentially methylated to form phosphatidylcholine by using S-adenosylmethionine as the methyl donor (167).

Choline kinase, the first enzyme in the CDP-choline pathway, has been purified, and its properties have been reviewed elsewhere (86, 137). It is a cytosolic enzyme that also catalyzes the phosphorylation of ethanolamine. Three isoforms of choline kinase are present in liver (86).

The next step in the CDP-choline pathway, catalyzed by CTP:phosphocholine cytidylyltransferase, is rate limiting (132) and is the regulated step in phosphatidylcholine biosynthesis (166). A deficient activity of this enzyme in the lungs of prematurely born human infants causes them to develop respiratory distress syndrome (55). Cytidylyltransferase is present in the cytosol (166)

[and probably within the nucleus (169)] as an inactive dimer of two 42-kDa subunits and is found in the endoplasmic reticulum, Golgi, and nuclear envelope as an active membrane-bound form (166, 170). The presence of significant cytidylyltransferase activity in the nuclear membranes is remarkable because heretofore we thought that phosphatidylcholine synthesis occurred exclusively in the endoplasmic reticulum and golgi apparatus.

Diacylglycerol content, membrane phosphatidylcholine, and the state of phosphorylation of cytidylyltransferase regulate the reversible translocation of the enzyme between cytosol and membranes (166). When cytidylyltransferase is phosphorylated by a cAMP-dependent protein kinase, it translocates from membranes into the cytosol and becomes inactivated. This process reverses when protein phosphatase dephosphorylates the enzyme (169). The phosphatidylcholine content of the membrane also regulates the binding of the enzyme to membranes. Cytidylyltransferases bind to membranes more avidly when their phosphatidylcholine content decreases, whereas the reverse occurs when membrane phosphatidylcholine content increases (87, 170). This finding may explain why cytidylyltransferase activity increases in choline-deficient hepatocytes (177). Diacylglycerol also regulates cytidylyltransferase. Treatments that increase intracellular diacylglycerol [exposure to oleate (78)] or that mimic the actions of diacylglycerol [exposure to phorbol ester (165)] activate cytidylyltransferase. Fatty acids and diacylglycerol may act indirectly on cytidylyltransferase by modulating protein kinase C (PKC) and protein phosphatase activities (169).

The third enzyme in the CDP-choline pathway (CDP-choline:1,2-diacylglycerol choline-phosphotransferase) is present in the membranes of the endoplasmic reticulum. Cornell recently reviewed its properties (40). Because it is not the rate-limiting enzyme in the pathway, CDP-choline does not accumulate in significant concentrations within cells.

The alternative pathway for phosphatidylcholine biosynthesis (through the methylation of phosphatidylethanolamine by phosphatidylethanolamine *N*-methyltransferase) is most active in liver but has been identified in many other tissues, including brain (22, 41) and mammary gland (176); detectable activity exists in most other tissues (44, 61, 77, 80, 101, 122, 126, 129, 146, 149). This is the major (and perhaps only) pathway for de novo synthesis of the choline moiety in adult mammals. However, plants (119) and possibly embryonic neurons [from chicken or rat; (1, 2)] can catalyze the methylation phosphoethanolamine to form phosphocholine. Vance & Ridgway recently reviewed the properties of phosphatidylethanolamine-*N*-methyltransferase (167). No accurate estimates of the activity of phosphatidylethanolamine-*N*-methyltransferase in vivo are available. The best figures, based on in vitro data, estimate that 15–40% of the phosphatidylcholine in liver derives from the phosphatidylethanolamine-*N*-methyltransferase pathway, with the remainder

coming from the CDP-choline pathway (16, 159). Phosphatidylethanolamine-*N*-methyltransferase is membrane bound and has at least two isoforms (42, 143, 145). In adult liver, the availability of phosphatidylethanolamine, the S-adenosylmethionine/S-adenosylhomocysteine concentration ratio, and the composition of the boundary lipids that surround phosphatidylethanolamine-*N*-methyltransferase regulate its activity (145). S-adenosylhomocysteine, a product of the reactions, inhibits the methyltransferase (82, 144). The availability of S-adenosylmethionine in the liver of choline-deficient animals limits the activity of this pathway (68, 131). We discuss the regulation of the hydrolysis of phosphatidylcholine below in the section entitled "Signal Transduction".



## *Platelet-Activating Factor: a Choline-Containing Phospholipid with Hormonal Functions*

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a choline phospholipid characterized by a fatty alcohol (usually hexadecanol) at the *sn*-1 position of the glycerol backbone and by an acetate residue at the *sn*-2 position. It has diverse biological functions exerted through specific receptors located on many different cell types. When ligand binding activates cells, PAF forms in a two-step pathway that involves the initial conversion of alkyl choline phospholipids to the corresponding lyso-intermediate (lyso-PAF) by phospholipase $A_2$ ($PLA_2$), followed by the addition of an acetate group to the *sn*-2 position (138). Receptor-mediated synthesis of PAF requires calcium and is mediated by a guanosine triphosphate (GTP)-binding protein (G protein). Synthesis of PAF by this pathway can be stimulated by exposure to complement-coated zymosan particles, calcium ionophores, or phorbol esters in human polymorphonuclear leukocytes and by thrombin, bradykinin, and ATP in endothelial cells. De novo synthesis of PAF also occurs via a pathway that uses cytidine-diphosphocholine as a precursor (138).

The effects of PAF include activation of phosphoinositide-specific phospholipase C and release of fatty acids from phospholipids, possibly by activation of phospholipase $A_2$ (138). These actions in turn generate the active metabolites diacylglycerol and inositol-1,4,5-trisphosphate (causing the subsequent mobilization of calcium and activation of PKC) and arachidonic acid (with subsequent metabolism to form eicosanoids). In addition to activating platelet aggregation, PAF lowers blood pressure; increases vascular permeability; activates monocytes, macrophages, and polymorphonuclear neutrophils; and stimulates hepatic glycogenolysis. PAF mediates many processes of inflammation and allergy and may mediate parturition as well. Many excellent reviews describe biosynthesis, turnover, and actions of PAF (35, 100, 138, 157).

## *Sphingomyelin Metabolism*

Recently, investigators began to realize that the choline-phospholipid sphingomyelin is an important precursor for the generation of intracellular signals. Sphingomyelin metabolism has been reviewed extensively (115). Ceramide, the basic structural unit of the sphingolipids, is formed by adding a fatty acid to the amino group of sphingosine. Sphingolipid head groups can be divided into several classes; addition of a phosphocholine group to the terminal hydroxyl group of ceramide forms sphingomyelin, which resembles phosphatidylcholine in that it contains two long carbon chains and a phosphocholine group. Most of this phosphocholine group comes from phosphatidylcholine via a transfer catalyzed by phosphatidylcholine:ceramide choline phosphotransferase (115). Alternative minor pathways for sphingomyelin synthesis include combination of the phosphocholine from CDP-choline with ceramide and acylation of sphingosylphosphocholine (115).

Extracellular signals regulate sphingomyelin synthesis. For example, phorbol esters increase sphingomyelin synthesis in several cell lines (49, 93, 94, 96). Corticosteroids (142), diacylglycerol (95), vitamin $D_3$ (128), tumor necrosis factor $\alpha$ (TNF$\alpha$) (51, 92), and $\gamma$-interferon ($\gamma$IFN) (92) all can invoke increased sphingomyelinase activity. Activation of sphingomyelinase may constitute one of the signal transduction pathways for triggering differentiation (51, 92). It causes elevations in levels of cellular ceramide, a compound with potent biological activities, including the triggering of programmed cell death (apoptosis) (127). Ceramide can be phosphorylated to form ceramide 1-phosphate (50) by a calcium-dependent kinase (97), or it can be hydrolyzed to form sphingosine (115). The formation of sphingosine from ceramide may be critical for regulating PKC signal transduction (discussed in more detail in the section entitled "Signal Transduction").

## *Acetylcholine Biosynthesis*

Choline and its relationship to acetylcholine synthesis have been thoroughly reviewed elsewhere (20). Choline and acetyl-CoA form acetylcholine in a reaction catalyzed by choline acetyltransferase. Cholinergic neurons require choline not only for membrane synthesis but also for acetylcholine synthesis. The choline-phospholipids in these cells constitute a large precursor pool of choline for acetylcholine synthesis. This precursor pool may be especially important in neurons with increased demands for choline to sustain acetylcholine release (e.g. when particular cholinergic neurons fire frequently or when the supply of choline from the extracellular fluid is inadequate). A decrease in the amount of membrane phosphatidylcholine was observed in repeatedly depolarized preparations containing cholinergic nerve terminals such as superior cervical ganglia (130) or striatal slices (164), but no reduction occurred in preparations devoid of cholinergic innervation [cerebellar slices (164)].

Human cholinergic neuroblastoma cells transferred choline from phosphatidylcholine to acetylcholine (18, 102). These cells liberated choline into the cytoplasm from phosphatidylcholine in a one-step process catalyzed by a phospholipase D (102). Evidence consistent with hypotheses that abnormal membrane phospholipid turnover may impart selective vulnerability to cholinergic neurons has been forthcoming from studies on Alzheimer's disease—a disorder characterized by the extensive degeneration of nerve terminals of cholinergic neurons whose cell bodies reside in the basal forebrain and that project to the cortex and hippocampus (24, 133). Abnormalities of metabolism of membrane phospholipids appear to be a feature of this disease (125). These include reduced levels in brain (at autopsy) of phosphatidylcholine, phosphatidylethanolamine, choline, and ethanolamine, concomitant with elevated levels of glycerophosphocholine and glycerophosphoethanolamine.

## *Functions Of Choline And Choline Phospholipids*

The numerous functions of choline as a methyl donor and of choline phospholipids as structural elements of cells have been reviewed extensively elsewhere (181). We focus on new observations concerning physiologic roles for these compounds in mammals.

## *Hepatic Secretion of Very Low-Density Lipoprotein*

In the human (187), rat (106), hamster (70), guinea pig (161), pig (17, 54), dog (13, 14, 79), and monkey (81), choline deficiency results in liver dysfunction owing to massive accumulation of triacylglycerol within the hepatocyte (21, 106, 107, 178, 179). In rats, fatty liver begins within hours to days after initiation of a choline-deficient diet (43).

The triacylglycerol produced by liver is delivered to other tissues, mainly in the form of very low-density lipoprotein (VLDL). Phosphatidylcholine is a required component of the VLDL particle (178, 179), and in choline deficiency, the diminished capacity of liver cells to synthesize new phosphatidylcholine molecules results in intracellular accumulation of triglycerides whose synthesis continues unchecked. In choline-deficient rat hepatocytes, even compounds as close in structure to choline as dimethylethanolamine will not suffice to synthesize the phospholipid needed for VLDL formation (179). Methionine can substitute for choline, but only as long as phosphatidylethanolamine-*N*-methyltransferase activity (see above discussion of phosphatidylcholine biosynthesis) continues (178). Secretion of high-density lipoprotein (HDL) from hepatocytes does not require synthesis of new phosphatidylcholine molecules (180). Choline-deficient humans have diminished plasma LDL cholesterol (187). This observation is consistent with the hypothesis that in humans, as in other species, choline is required for VLDL secretion.

## *Signal Transduction*

Our understanding of choline phospholipid–mediated signal transduction has increased considerably during the last decade. Stimulation of membrane-associated receptors activates neighboring phospholipases, resulting in the formation of breakdown products that are signaling molecules either by themselves (i.e. they stimulate or inhibit the activity of target macromolecules) or after conversion to signaling molecules by specific enzymes (Figure 3). Much of signaling research focused on minor membrane phospholipid components, particularly phosphatidylinositol derivatives. However, metabolism of choline phospholipids, especially phosphatidylcholine and sphingomyelin, forms biologically active molecules that can amplify external signals or terminate the signaling process by generating inhibitory second messengers (185).

Signal transduction via receptor-mediated hydrolysis of phosphatidylinositol-bis-phosphate has been extensively reviewed elsewhere (11, 162). For example, activation of heptahelical receptors leads to altered conformation of the receptor so that it can activate a G protein. These G proteins are a highly conserved family of membrane-associated heterotrimeric proteins. Activation of the G protein results in subsequent activation of phospholipase C activity within the plasma membrane (Figure 3). Phospholipase Cs are a family of phosphodiesterases that hydrolyze the glycerophosphate bond of intact phospholipids to generate 1,2-*sn*-diacylglycerol and an aqueous-soluble head group. Numerous phosphatidylinositol-bis-phosphate–specific phospholipase Cs exist, and specific receptors are thought to couple to specific phospholipase C isotypes (114). In a similar manner, specific receptors appear to be linked to activation of specific phosphatidylcholine-phospholipase Cs (53) (see discussion below).

The action of phospholipase C triggers the next event in the signal cascade: activation of PKC (serine/threonine kinase). Products generated by phosphatidylinositol-bis-phosphate-phospholipase Cs include inositol-1,4,5-trisphosphate (Ins-1,4,5-$P_3$) and diacylglycerol (Figure 3). Ins-1,4,5-$P_3$ is a water-soluble product that acts to release calcium from stores in the endoplasmic reticulum. Some PKC isotypes are $Ca^{2+}$ dependent (PKC$\alpha$, $\beta_2$, and $\gamma$). PKC$\delta$, $\varepsilon$, $\zeta$, $\theta$, and $\eta$ lack a calcium-binding domain (124). By increasing membrane occupancy of PKC, calcium places the enzyme in close proximity to phosphatidylserine, a cofactor for PKC activation. Diacylglycerol, the other product of phospholipase C, remains in the plasma membrane and is both a messenger molecule and an intermediate in the metabolism of lipids. Normally PKC is folded so that an endogenous pseudosubstrate region on the protein is bound to the catalytic site, thereby inhibiting activity. The combination of diacylglycerol and $Ca^{2+}$ causes a conformational change in PKC, resulting in flexing at a hinge region so as to withdraw the pseudosubstrate and unblock

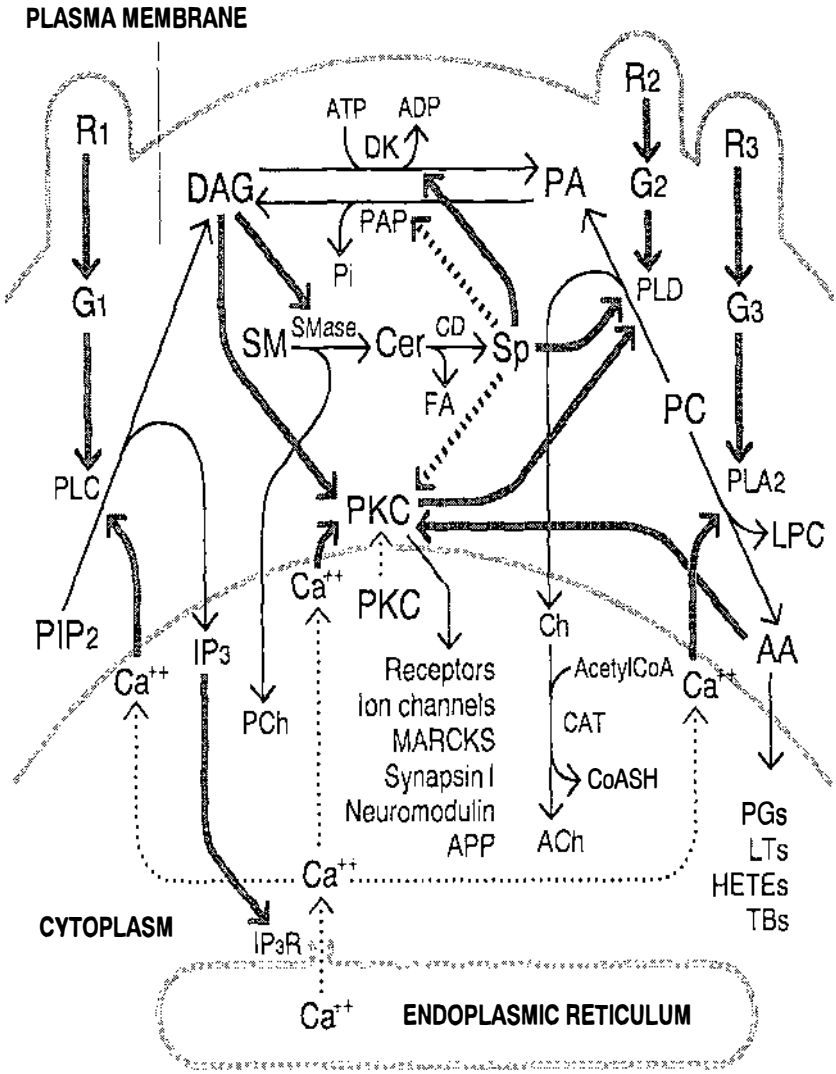



*Figure 3* Phospholipids and transmembrane signaling. Phospholipid breakdown products as signaling molecules are described in the text. The diagram shows only the key features of the system. Solid black lines represent enzymatic reactions, thick solid lines represent activation, and thick dashed lines represent inhibition (e.g. DAG activates PKC, whereas Sp inhibits it). Dotted lines represent translocation. PKC substrates are indicated by a thin gray arrow. Abbreviations for compounds are: AA, arachidonic acid; ACh, acetylcholine; Cer, ceramide; Ch, choline; DAG, diacylglycerol; FA, fatty acid; HETEs, hydroxy eicosatetraenoic acids; $IP_3$, inositol-1,4,5-trisphosphate; LPC, lysophosphatidylcholine; LTs, leukotrienes; PA, phosphatidic acid; PC, phosphatidylcholine; PCh, phosphocholine; PGs, prostaglandins; $PIP_2$, phosphatidylinositol-4,5,-bisphosphate; SM, sphingomyelin; Sp, sphingosine; TBs, thromboxanes. Abbreviations for macromolecules are: APP, amyloid precursor protein; CAT, choline acetyltransferase; CD, ceramidase; G, G proteins; PAP, PA phosphohydrolase; PKC, protein kinase C; $PLA_2$, phospholipase $A_2$; PLC, phospholipase C; PLD, phospholipase D; DK, DAG kinase; R, receptors; SMase, sphingomyelinase. Adapted from Ref. 19.

the PKC catalytic site. The appearance of diacylglycerol in membranes is usually transient, and PKC is therefore activated only for a short time after a receptor has been stimulated.

Generation of diacylglycerol from membrane phosphatidylcholine amplifies the signal (Figure 3). Phospholipase C and (indirectly) phospholipase D generate this diacylglycerol (38, 53, 103, 141). Changes in intracellular calcium may regulate phospholipase D activity (3, 103). Evidence also indicates that receptors with intrinsic tyrosine kinase activity [e.g. epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) receptors] stimulate phosphatidylcholine hydrolysis (53). Sustained activation of PKC is essential for triggering cell differentiation and proliferation (124). Other products of phosphatidylcholine hydrolysis, such as phosphatidic acid, lysophosphatidylcholine, and free fatty acids, are also second messengers (15, 53). Phosphatidic acid can act as a mitogen (168). Lysophosphatidylcholine stimulates PKC activity (124) but is a membrane-lytic detergent with potential toxic effects. Lysophosphatidylcholine generation is important in chemotaxis, relaxation of smooth muscle, and activation of T lymphocytes (124). Thus, lysophosphatidylcholine may be a choline-containing putative second messenger generated by phospholipase $A_2$. Recent data indicate that phosphatidylcholine may synergize with diacylglycerol and calcium to activate PKC in lymphocytes (5) and that modulation of PKC isozymes by phosphatidylcholine may be isoform specific (151).

The characterization of events that occur downstream from PKC is just beginning. Serine-threonine kinases and tyrosine kinases catalyze phosphorylation of target proteins distal to PKC. Phosphorylation alters the biochemical properties of these substrates, resulting in a range of cellular responses. These phosphorylation cascades enhance amplification of the original signal. PKC signals impinge on several known intracellular control circuits (158). Targets for phosphorylation by PKC include receptors for insulin, EGF, and many proteins involved in control of gene expression (123, 171).

Although sphingolipids are ubiquitous components of mammalian cells, they have only recently been proven necessary for cellular survival and growth (69). Whereas phosphatidylcholine hydrolysis generates a series of messengers that sustain the PKC phosphorylation cascade, hydrolysis of sphingomyelin generates messengers that terminate the cascade (see above discussion of sphingomyelin hydrolysis; Figure 3). Sphingomyelin, sphingomyelinase, and ceramidase (which produces sphingosine and fatty acid from ceramide) are present in the outer leaflet of the plasma membrane. Ceramide is a potent inhibitor of cell growth as well as a promoter of cell differentiation. Its metabolite, sphingosine, is a potent inhibitor of PKC that acts by blocking diacylglycerol-mediated activation (67, 71, 72, 74–76, 116, 117). Furthermore, sphingosine's inhibition of phosphatidic acid phosphohydrolase (an enzyme that generates

the PKC activator, diacylglycerol) diminishes activation of PKC (65, 104). Direct activation of diacylglycerol kinase by sphingosine also removes diacylglycerol (150). These actions of sphingosine tend to increase the cellular levels of phosphatidic acid. Sphingosine concentrations in cells are in the micromolar range, perhaps high enough to inhibit PKC (115). Lysosphingomyelin, also formed during hydrolysis of sphingomyelin (Figure 3), is a known PKC inhibitor (73). In addition, sphingosylphosphocholine (lysosphingomyelin) was recently described as a potent mitogen (45, 46). Synthesis of sphingomyelin from ceramide and phosphatidylcholine generates diacylglycerol (Figure 3), but whether this diacylglycerol is delivered to a subcellular location where it is available for PKC activation remains unknown.

Sphingosine also inhibits protein phosphorylation catalyzed by insulin receptor kinase (4), *src* kinase (85), and [through its inhibitory action on calmodulin (88)], calmodulin-sensitive kinase while activating the protein phosphorylation catalyzed by casein kinase II (110). Finally, sphingosine activates a class of novel protein kinases (140) and thus may play an important role in regulating the degree of protein phosphorylation catalyzed by a variety of kinases.

## *Carcinogenesis*

Choline is the only single nutrient for which dietary deficiency causes development of hepatocarcinomas without any known carcinogen (121). Interestingly, choline-deficient rats not only have a higher incidence of spontaneous hepatocarcinoma, but they are markedly sensitized to the effects of administered carcinogens (121). Choline deficiency is therefore considered to stimulate both cancer-initiating and cancer-promoting activities.

Several mechanisms have been suggested for the cancer-promoting effect of a diet devoid of choline. In the choline-deficient liver, a progressive increase in cell proliferation occurs that is related to regeneration of liver after parenchymal cell death (32, 33, 121). Cell proliferation (with an associated increased rate of DNA synthesis) could be responsible for the greater sensitivity to chemical carcinogens (64). Stimuli for increased DNA synthesis, e.g. hepatectomy and necrogenic chemicals, increase carcinogenesis. However, the overall rate of liver cell proliferation could be dissociated from the rate at which preneoplastic lesions form during choline deficiency (154). This possibility suggests that cell proliferation is not the sole condition acting as a promoter of liver cancer. Methylation of DNA is important for the normal expression of genetic information. Undermethylation of DNA, observed during choline deficiency (despite adequate dietary methionine), may be responsible for carcinogenesis (48, 105). Another proposed mechanism derives from the observation that when rats eat a choline-deficient diet, increased lipid peroxidation occurs within liver (148). Lipid peroxides in the nucleus may be a source of

free radicals that could modify DNA and cause carcinogenesis. Recently, we proposed that choline deficiency perturbs PKC signal transduction, thereby promoting carcinogenesis.

As discussed above, choline deficiency causes massive fatty liver (see section entitled "Hepatic Secretion of Very Low-Density Lipoprotein"). We have observed that 1,2-*sn*-diacylglycerol accumulates in this fatty liver (21, 43). In plasma membrane from livers of choline-deficient rats, diacylglycerol reaches values higher than those occurring after stimulation of a receptor linked to phospholipase C activation (e.g. vasopressin receptor). This process results in stable activation of PKC and/or an increase in the total PKC pool in the cell (43), with changes in several PKC isotypes (at 6 weeks of choline deficiency, amounts of PKCα and δ increased 2-fold and 10-fold, respectively).

The accumulation of diacylglycerol and subsequent activation of PKC within liver during choline deficiency may be the critical abnormality that eventually contributes to the development of hepatic cancer in these animals (43). Abnormalities in PKC-mediated signal transduction may trigger carcinogenesis (171). Many mitogens activate PKC (124); the phorbol esters (diacylglycerol analogs) are potent tumor promoters (123). Okadaic acid (an inhibitor of protein phosphatases whose net effect is to increase protein phosphorylation of PKC targets) is also a potent tumor promoter (84). The expression of oncogenes can perturb PKC-mediated signal transduction in a manner similar to that described above for choline deficiency. In *erbB*-transformed fibroblasts, diacylglycerol accumulates because diacylglycerol kinase activity does not remove it (90). Transformation by *ras* or *src* oncogenes translocates PKC to the plasma membrane (47). Diacylglycerol concentrations are elevated in vivo in *ras*-transformed liver of neonatal transgenic mice (173). NIH 3T3 cells transformed with Ha-*ras*, Ki-*ras*, v-*src*, or v-*fms* oncogenes have increased diacylglycerol levels as well as tonic activation and partial downregulation of PKC (174). Degradation of phosphatidylcholine produces these elevated diacylglycerol levels (47, 139). Transfection of fibroblasts so that they overexpress PKC (α, $\beta_1$, or ε) activity causes them to become transformed and tumorigenic (29, 99, 113, 134, 158). Thus, many observations suggest that choline deficiency causes cancer by increasing diacylglycerol concentration with subsequent sustained activation of PKC.

## *Brain Development and Function*

The central role of choline as a precursor of acetylcholine and phosphatidylcholine has led Nature to develop several mechanisms to ensure that a developing animal receives adequate amounts of choline. Perhaps the most striking example of such a mechanism is the egg of birds. The yolk sac of the egg contains primarily precursors of membranes, i.e. phospholipids and cholesterol. The most abundant phospholipid in the yolk is phosphatidylcholine.

In mammals, the placenta transports choline to the fetus (172); amniotic fluid choline concentration is 10-fold greater than that in maternal blood (SH Zeisel, unpublished observations). At birth all mammals studied, including the human, had plasma choline concentrations much higher than those in adults (188). The capacity of brain to extract choline from blood is greatest during the neonatal period (25). A novel phosphatidylethanolamine-*N*-methyltransferase (which synthesizes choline de novo) in neonatal rat brain is extremely active (23). Human and rat milk contain especially large amounts of choline (147, 186). The evolution of these multiple mechanisms suggests that choline supply might be crucial during this period.

The time during which rat brain develops includes two sensitive periods during which treatment with choline results in long-lasting facilitation of spatial memory. The first period occurs during embryonic days 12–17 and the second during postnatal days 16–30 (108, 111, 112). Choline supplementation during these critical periods elicits a constant percentage improvement in choice performance at all stages of training for a visuospatial task (12-arm radial maze), which suggests a memory rather than a learning rate effect. This effect is apparent months after the brief exposure to extra choline. Other findings also indicate memory enhancement: (a) Compared with controls, perinatal choline-treated rats retain information about more maze locations in working memory; (b) these rats are more likely to use prospective than retrospective coding of information, which requires larger memory capacity; and (c) they exhibit little or no proactive interference, which implies greater accuracy as well as increased memory capacity. These effects of perinatal choline treatment on memory appear to be permanent, since both working and reference memory performance continue to show facilitation relative to controls, even at 26 months of age (W Meck, personal communication). Recent studies (163) confirmed these results and revealed that the improved visuospatial memory in rats treated perinatally with choline can be demonstrated using another experimental paradigm (water maze).

The two sensitive periods correlate with neurogenesis of cholinergic cells (prenatal) and with synaptogenesis (postnatal). Neurogenesis of cholinergic cells of the basal forebrain [i.e. the groups of cells involved in memory processes (52, 58, 109)] occurs between embryonic days 12 and 17 in the rat. The establishment of neuronal projections and synapses of the brain cholinergic system occurs during the postnatal period of development. These processes reach the fastest rate approximately two to three weeks after birth and are largely complete by postnatal day 30 (6).

The observation that perinatal supplementation with choline results in improved memory performance of adult rats suggests that choline availability is critical for brain development. The choline content of rat chow has been established based on choline requirements of nonpregnant adult rats. However,

we do not know the minimal requirement for the pregnant or lactating female or for the fetus. Perhaps changes in eating behavior during pregnancy allow rats in the wild to adjust their choline intake. These considerations are also important when considering choline requirements for humans.

## DIETARY REQUIREMENT FOR CHOLINE

### *Dietary Sources of Choline*

Many foods consumed by humans contain significant amounts of choline and esters of choline (Table 1). No information is available about the phosphocholine or glycerophosphocholine content of foods, but these metabolites are likely present in significant amounts. Some choline is added during food processing [especially when preparing infant formula (56)]. Average choline dietary intake (as free choline and the choline in phosphatidylcholine and other choline



**Table 1** Choline content of some common foods[a]

| Food | Concentration (μmol/kg) | | |
|---|---|---|---|
| | Choline | Phosphatidylcholine | Sphingomyelin |
| Apple | 27 | 280 | 15 |
| Banana | 240 | 37 | 20 |
| Beef liver | 5831 | 43,500 | 1850 |
| Beef steak | 75 | 6030 | 506 |
| Butter | 42 | 1760 | 460 |
| Cauliflower | 1306 | 2770 | 183 |
| Corn oil | 3 | 12 | 5 |
| Coffee | 90 | 34 | 23 |
| Cucumber | 218 | 76 | 27 |
| Egg | 42 | 52,000 | 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 |

[a] Choline, phosphatidylcholine, and sphingomyelin were measured using a gas chromatography/mass spectrometry assay in foods prepared in the form that they would normally be consumed. Modified from Ref. 182.

esters) in the adult human is more than 7–10 mmol/day (57, 181). When Zeisel et al switched humans from a diet of normal foods to a defined diet containing 5 mmol/day we observed that plasma choline and phosphatidylcholine concentrations decreased in most subjects (187). This result suggests that average adult dietary intake of choline exceeds this level. A kilogram of beef liver contains 50 mmol of choline equivalents; thus, one can easily consume a diet of normal foods (eggs, liver, etc) that delivers much more choline per day than the calculated average intake (189).

The human infant consumes a choline-rich diet. Breast milk contains ~ 1.5 mmol/liter choline and choline esters; we have only recently discovered that the major forms of choline in mature human and rat milk are phosphocholine and glycerophosphocholine (147, 186). Thus, an infant consuming 500 ml breast milk ingests 750 μmol choline. Choline is routinely added to commercially available infant formulas (approximately 1 mmol free choline per liter). Formulas made from bovine milk proteins contain slightly less total choline than human breast milk, whereas formulas made from soybean proteins contain approximately the same amount of choline as breast milk (147, 186).

## *Choline Deficiency in Animals*

Liver dysfunction and fatty liver associated with choline deficiency are discussed above. Choline deficiency compromises renal function, resulting in abnormal concentrating ability, free water reabsorption, sodium excretion, glomerular filtration rate, renal plasma flow, and gross renal hemorrhage (10, 12, 66, 118). Diets low in choline content also cause infertility, growth impairment, bony abnormalities, decreased hematopoiesis, and hypertension (30, 34, 89, 98).

## *Choline Deficiency in Humans*

Humans require choline to sustain normal life. Is a dietary source of choline required? As discussed above, normal diets deliver sufficient choline. When healthy humans were fed a choline-deficient diet for three weeks they developed biochemical changes consistent with choline deficiency (187). These included diminished plasma choline (Figure 4) and phosphatidylcholine concentrations as well as diminished erythrocyte membrane phosphatidylcholine concentrations. Serum alanine transaminase (ALT) activity, a measure of hepatocyte damage, increased significantly during choline deficiency (Figure 5). This experiment established a choline requirement in the diet of normal humans.

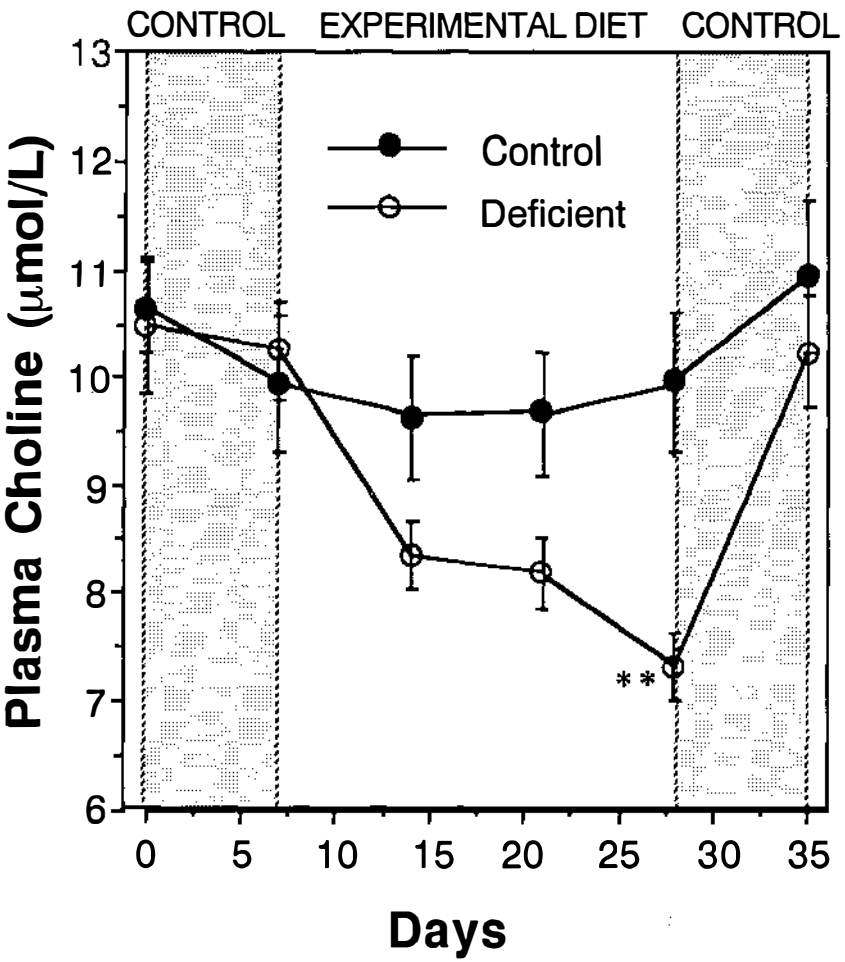



*Figure 4* Plasma choline in humans ingesting a control or choline-deficient diet. Healthy male subjects were hospitalized for five weeks. During the first and last weeks of the study all subjects consumed the same choline-containing standard diet. During the middle three weeks of the study subjects were divided into two groups: one that continued on the choline-containing diet, and one that consumed the same diet without choline. During the fifth week of the study all subjects consumed the choline-containing diet. Plasma choline was measured by a mass spectrometric method. Data are expressed as mean choline concentration (μmol/L) ± standard error of the mean. ** = $p < 0.01$ different from day 7 value. From Ref. 187.

This choline requirement may be more apparent in specific human populations. The demand for choline in normal adults is likely smaller than that of infants, since large amounts of choline are needed in order to make phospholipids in growing organs (183). The changes observed in choline-deficient

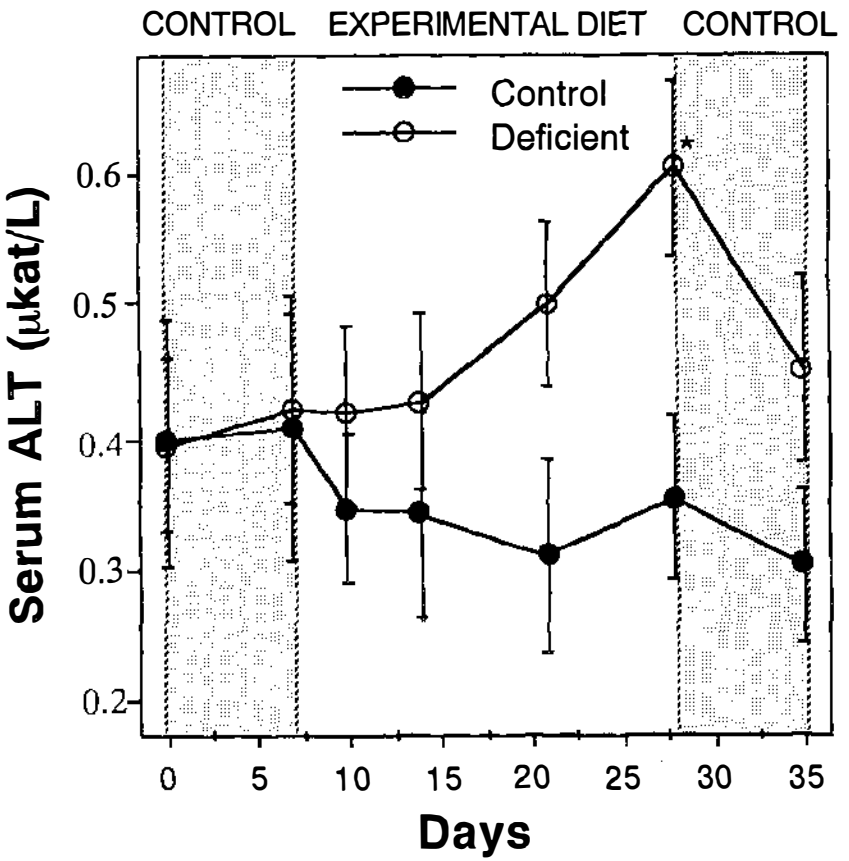



*Figure 5* Serum alanine aminotransferase activity in humans ingesting a control or choline-deficient diet. Subjects were treated as described in the legend to Figure 4. Serum alanine aminotransferase was determined using an automated spectrophotometric assay. Data are expressed as mean activity (μkat/liter) ± standard error of the mean. * = $p < 0.05$ different from day 7 value. From Ref. 187.

adult humans might have been greater had we studied growing children. Malnourished humans, in whom stores of choline, methionine, and folate have been depleted (37, 153), also appear to need more dietary choline than did our healthy adult subjects. The liver is the primary site for endogenous synthesis of choline. Alcoholics with liver cirrhosis have diminished plasma choline concentration and fatty liver, which resolves when patients are supplemented with choline (37).

Amino acid-glucose solutions used in total parenteral nutrition of humans

contain no choline (36, 153). The lipid emulsions used to deliver extra calories and essential fatty acids during parenteral nutrition contain choline in the form of phosphatidylcholine (20% emulsion contains 13.2 mmol/L). Humans treated with parenteral nutrition required 1–1.7 mmol of choline-containing phospholipid daily during the first week of parenteral nutrition therapy to maintain plasma choline levels (153). Burt et al (28) reported decreased plasma choline concentrations in parenteral nutrition patients at the same time that they detected liver dysfunction. Conditions that enhance hepatic triglyceride synthesis (such as carbohydrate loading) increase the requirement for choline needed for export of triglyceride from liver (31). Thus, treatment of malnourished patients with high-calorie parenteral nutrition solutions at a time of depleted choline stores might enhance the likelihood of hepatic dysfunction. A recent clinical trial supports the requirement for supplemental choline during total parenteral nutrition (26, 27). Patients were treated with total parenteral nutrition (average duration 7 years), including 4 kcal/kg per day as 20% Intralipid® (delivering ~ 50 μmoles/kg per day choline as phosphatidylcholine). These patients had low plasma choline concentrations (average 6.3 μM). In a double-blind protocol, investigators administered either 20 g lecithin (30% phosphatidylcholine delivering ~ 300 μmoles choline moiety/kg per day) or placebo (soybean oil) orally twice daily for six weeks. At the end of this experimental period, plasma choline had risen more than 50% in the lecithin group, whereas in the placebo group it had decreased 25%. Fatty liver was defined using computed tomography. In the treated group, liver density increased (and fat decreased) ~ 30% ($p < 0.05$); in the placebo group, liver density increased by only 8% (nonsignificant). These findings strongly suggest that choline is an essential nutrient during long-term total parenteral nutrition.



## SUMMARY

Choline is crucial for sustaining life. It modulates the basic signaling processes within cells, is a structural element in membranes, and is vital during critical periods in brain development. Choline metabolism is closely interrelated with the metabolism of methionine and folate. We believe that the normal human diet provides sufficient choline to sustain healthy organ function. However, vulnerable populations may become choline deficient, including the growing infant, the pregnant or lactating woman, the cirrhotic, and the patient fed intravenously. Further studies of choline requirements in these groups are required.

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