

Use of Phosphatide Precursors to Promote Synaptogenesis

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Abstract

New brain synapses form when a postsynaptic structure, the dendritic spine, interacts with a presynaptic terminal. Brain synapses and dendritic spines, membrane-rich structures, are depleted in Alzheimer's disease, as are some circulating compounds needed for synthesizing phosphatides, the major constituents of synaptic membranes. Animals given three of these compounds, all nutrients—uridine, the omega-3 polyunsaturated fatty acid docosahexaenoic acid, and choline—develop increased levels of brain phosphatides and of proteins that are concentrated within synaptic membranes (e.g., PSD-95, synapsin-1), improved cognition, and enhanced neurotransmitter release. The nutrients work by increasing the substrate-saturation of low-affinity enzymes that synthesize the phosphatides. Moreover, uridine and its nucleotide metabolites activate brain P2Y receptors, which control neuronal differentiation and synaptic protein synthesis. A preparation containing these compounds is being tested for treating Alzheimer's disease.

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INTRODUCTION

If animals are treated for several weeks with a mixture of three food constituents normally present in the plasma—uridine, docosahexaenoic acid (DHA), and choline—the amounts of the phosphatides that are formed from these compounds increase significantly (202), both in whole brain and per brain cell. Moreover, the brains also exhibit parallel changes in proteins

known to be associated with pre- and postsynaptic membranes (202) and show such signs of enhanced synaptic activity as increased numbers of dendritic spines (153) and increased release of certain neurotransmitters (189, 190). The animals also exhibit improvements in cognitive behaviors (79, 80, 174). This mixture is currently undergoing testing in humans with neurodegenerative diseases (159) that cause the loss of synapses (e.g., Alzheimer's disease) (175).

This review considers the biochemical mechanisms through which consumption of these food constituents increases the quantity of synaptic membrane and perhaps the number of brain synapses. These mechanisms appear to involve an unusual kinetic property—poor affinities for their substrates—of some brain enzymes that transform the three constituents to biologically active products. They also involve the activation of neuronal P2Y receptors that influence neuronal differentiation and protein synthesis by uridine and its phosphorylated products. Unfortunately, no data are available on the effects of this or of nontoxic biochemical treatments in general on the actual numbers of synapses in brain regions. Hence, estimates of such numbers must be extrapolated from surrogate measurements, such as numbers of dendritic spines, or concentrations of synaptic proteins, or behaviors known to involve particular neurons. Of these surrogates, the number of dendritic spines is generally believed to provide the best correlation with the actual number of synapses (cf. 5, 7, 8, 13, 52, 53, 72, 98, 127, 179).

Precursor Control

The brain is unusual among organs in the extent to which the rates of some of its most characteristic biochemical reactions are controlled not by the amount or activity of a key enzyme, but rather by the extent to which that enzyme is saturated with its substrate, which usually is both a nutrient and precursor for a physiologically active reaction product (200). This usually occurs because the enzyme protein has a relatively poor affinity for its substrate, relative to available

substrate concentrations. Thus, for example, the rates at which brain neurons synthesize and release the neurotransmitters serotonin (33, 158), acetylcholine (185), histamine (160), and dopamine (54) can all be increased by administering their precursors tryptophan, choline, histidine, and tyrosine, respectively. Similar relationships, described below, apparently influence the ability of brain neurons to synthesize the phosphatide molecules that are the main constituent of their synaptic membranes (202). Thus, giving animals the three normally circulating compounds—uridine, an omega-3 fatty acid (e.g., DHA), and choline—that are needed for phosphatide synthesis can substantially increase the levels of phosphatidylcholine (PC) and the other major membrane phosphatides per brain cell. The mechanisms by which these dietary constituents affect phosphatide synthesis and, ultimately, the amount of synaptic membrane are described in detail below. They allow nutritional state to affect brain function and perhaps also provide a strategy for treating brain diseases in which synapses are deficient.

Synapses and Synaptogenesis

Most of the communications that take place between pairs of brain neurons occur at synapses, highly specialized structures that consist of a presynaptic terminal originating on an axon, the synaptic cleft, and the postsynaptic membrane, usually on a dendrite or cell body (**Figure 1**). Presynaptic terminals synthesize and store the neuron's neurotransmitter, generally in cytologically identifiable bodies, the synaptic vesicles, and eventually releasing it into the synaptic cleft when the neuron's action potential arrives at the terminals. The synaptic cleft is a fluid-filled space between the two neurons through which the neurotransmitter either diffuses to the postsynaptic membrane or is inactivated by enzymatic degradation or by reuptake into its neuron of origin. The postsynaptic membrane contains receptors to which the neurotransmitter can attach and additional protein molecules that transduce the functional consequences of

the receptor's activation. Pre- and postsynaptic membranes contain similar lipids—principally phospholipids and cholesterol; however, the membranes differ from each other and from membranes elsewhere in the brain by virtue of the particular fatty acids in their phosphatides and the particular proteins each contains, as described below.

The postsynaptic membranes on which glutamate—the most widely used excitatory neurotransmitter in the brain—acts often contain characteristic postsynaptic densities, each housing a large number of different proteins, which initiate the further transduction of biological signals generated by the transmitter-receptor complex. This transduction is accomplished by the opening or closing of protein channels in the membranes, which allow specific ions that affect the cell's voltage to pass into or out of the cell, or by activating membrane-bound enzymes that synthesize intracellular second messengers.

The formation of a new synapse among the well-studied hippocampal neurons that use glutamate as their neurotransmitter can be initiated by the coming together of a presynaptic terminal and a postsynaptic dendritic spine, a process that is facilitated by the latter's motility (127). A number of treatments apparently can increase dendritic spines, for example, giving the hormone ghrelin to mice caused parallel increases in the density of hippocampal dendritic spines and in memory performance and long-term potentiation; in contrast, targeted disruption of the gene for ghrelin in other mice decreased dendritic spines and memory performance (52)—indirectly affirming the importance of dendritic spines in hippocampal synaptic transmission. Dendritic spines are also known to be particularly vulnerable in Alzheimer's disease (97), a disorder that decreases the number of synapses and impairs memory.

Although most brain synapses are formed during pre- or early postnatal development, each survives for only days to months and thus must be renewed periodically throughout the individual's life span (101). This continuing

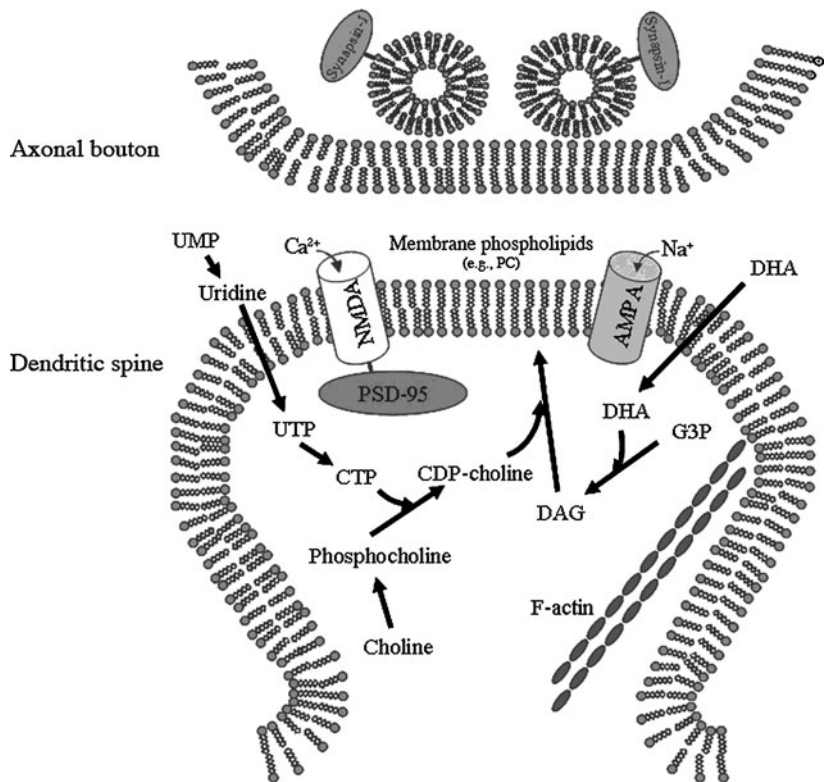


Figure 1

A model for spine formation by supplementation with DHA and UMP. The diagram shows phosphatidylcholine (PC) synthesis as an example. In neurons, DHA is acylated into the sn-2 position of glycerol-3-phosphate (G3P) to form DAG, together with a saturated fatty acid at the sn-1 position. UMP, a precursor of uridine, is metabolized to CTP, the major rate-limiting precursor in membrane phosphatide synthesis. CTP then reacts with phosphocholine to form CDP-choline. PC is synthesized by combining DAG and CDP-choline to form the phosphatide in dendritic spines. DHA and uridine may also activate other neuronal mechanisms (e.g., axonal growth and exocytosis) by receptor-mediated cell-signaling pathways to induce spine formation. Adapted from Reference 153. Abbreviations: CDP, cytidine diphosphate; CTP, cytidine-5'-triphosphate; DAG, diacylglycerol; DHA, docosahexaenoic acid; UMP, uridine monophosphate.

necessity is probably of major importance in underlying the brain's plasticity and the individual's ability to learn since it allows specific, perhaps newly formed, synapses to be associated with newly learned material (cf. 13, 65). Early in development, most synaptogenesis occurs in the absence of neuronal depolarization and neurotransmitter release (112, 187). In adulthood, however, the rate at which new synapses form and the ways that new synaptic connections become configured are largely governed by neuronal activity. This allows very active

synapses to facilitate the formation of additional synapses (13). Synaptogenesis can also be enhanced by the activation of neuronal genes, for example, those for transcription factors like CREB (cAMP response element-binding protein), which enhances synapse formation (2, 93, 108), and for myocyte enhancer factor 2, which limits the potentially excessive formation of new synapses (13, 59).

Among new neurons forming in adult mouse hippocampus that are making their initial synaptic contacts, it can be readily

demonstrated (179) that a new synapse starts to come into existence when a dendritic spine from one neuron comes into contact with a presynaptic bouton of another. Hence, the rate of synaptogenesis can depend in part on the numbers of dendritic spines that happen to be available, and treatments such as the nutrient mixture described in this review—uridine, an omega-3 fatty acid, and choline—that increase the numbers of dendritic spines (**Figure 2**) can also thereby promote synaptogenesis. All three of the compounds are needed to maximize synthesis of the phospholipids in synaptic membranes. Uridine acts via its phosphorylated products cytidine-5'-triphosphate (CTP, an intermediate in the Kennedy cycle) and uridine-5'-triphosphate (UTP, which activates P2Y receptors that stimulate the synthesis of specific proteins); the polyunsaturated omega-3 fatty acids DHA or eicosapentaenoic acid (EPA) are incorporated into diacylglycerols, which then become actual components of the phosphatides; and choline is needed to form PC, the most abundant brain phosphatide. The omega-3 fatty acids are derived solely from dietary sources; uridine and choline are obtained partly from endogenous synthesis in the liver and elsewhere, and partly from the diet.

BIOSYNTHESIS OF MEMBRANE PHOSPHATIDES AND PROTEINS; EFFECTS OF URIDINE, CHOLINE, AND OMEGA-3 FATTY ACIDS

All cells utilize DHA and other fatty acids, uridine, and choline to form the phosphatide subunits [e.g., PC (**Figure 3**)] that, when aggregated, constitute the major components of their membranes. PC, the principal such subunit in brain, is synthesized from these precursors by the CDP-choline cycle or Kennedy cycle (94) (**Figure 3**). PC also provides the phosphocholine moiety needed to synthesize sphingomyelin (SM), the other major choline-containing brain phospholipid. The phosphatide phosphatidylethanolamine (PE) is also synthesized via the Kennedy Cycle, utilizing ethanolamine instead of choline, while

phosphatidylserine (PS), the third major structural phosphatide, is produced by exchanging a serine molecule for the choline in PC or the ethanolamine in PE (155).

The CDP-choline cycle involves three sequential enzymatic reactions (**Figure 3**). In the first, catalyzed by choline kinase (CK), a monophosphate is transferred from ATP to the hydroxyl oxygen of the choline, yielding phosphocholine. The second, catalyzed by CTP:phosphocholine cytidyltransferase (CT), transfers cytidine-5'-monophosphate (CMP) from CTP to the phosphorus of phosphocholine, yielding cytidine-5'-diphosphocholine (also known as CDP-choline or as citicoline). As discussed below, much of the CTP that the human brain uses for this reaction derives from circulating uridine (201). The third and last reaction, catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT), bonds the phosphocholine of CDP-choline to the hydroxyl group on the 3-carbon of diacylglycerol (DAG), yielding the PC. All three PC precursors must be obtained by brain entirely or in large part from the circulation, and because the PC-synthesizing enzymes that act on all three have low affinities for these substrates, blood levels of all three can affect the overall rate of PC synthesis (32, 202).

Thus, choline administration increases brain phosphocholine levels in rats (117) and humans (12) because CK's K_m for choline (2.6 mM) (167) is much higher than usual brain choline levels (30–60 μ M) (95, 150, 169). Typically the second, CT-catalyzed reaction most influences the overall rate of PC synthesis, either because not all of the CT enzyme is fully activated by being attached to a cellular membrane (186) or because local CTP concentrations are insufficient to saturate the CT (150). Thus, when brain CTP levels are increased by giving animals uridine (32), CTP's circulating precursor in human blood (201), PC synthesis is accelerated (32). The activity of CPT and the extent to which this enzyme is saturated with DAG can also control the overall rate of PC synthesis, as has been demonstrated in, for

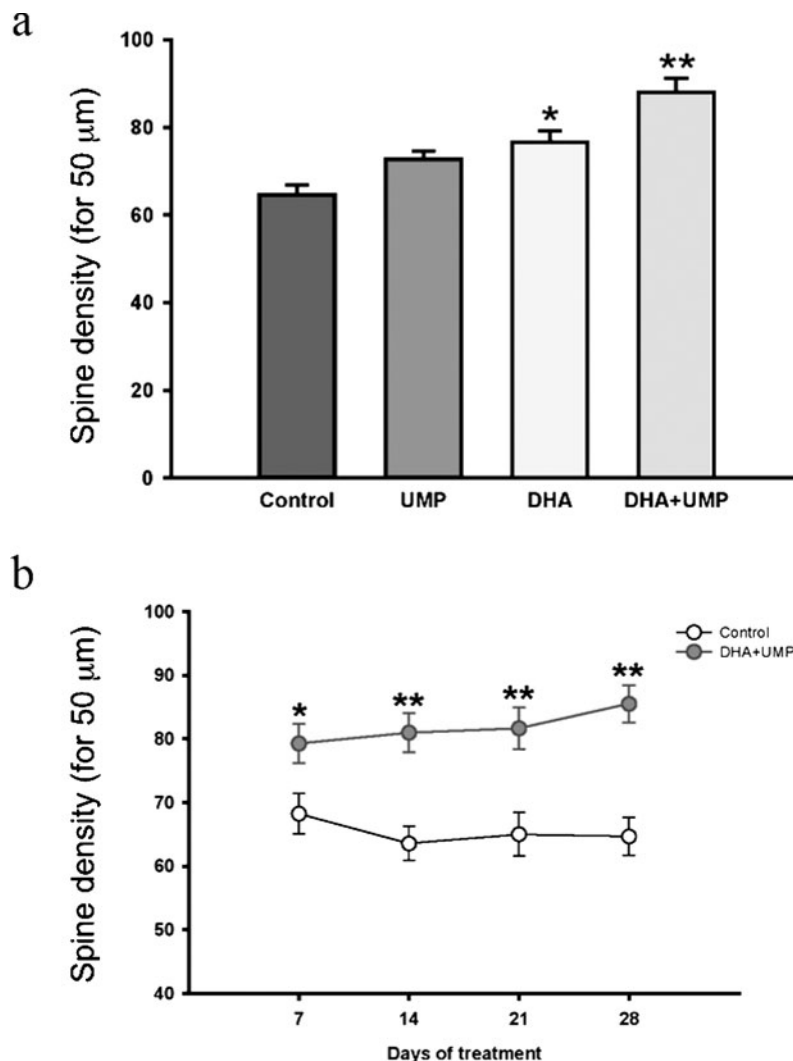


Figure 2

DHA-induced dendritic spine formation in adult gerbil hippocampus is enhanced by cosupplementation with uridine monophosphate (UMP). Animals received UMP (0.5%), docosahexaenoic acid (DHA) (300 mg/kg), or both daily for four weeks; control gerbils received neither. (a) Animals supplemented with DHA exhibited a significant increase in spine density (by 19%, $*p = 0.004$ versus control); those receiving both DHA and UMP exhibited a greater increase (by 36%, $**p < 0.001$ versus control or by 17%, $p = 0.008$ versus DHA supplemented). $n = 20$. ~ 25 neurons from four animals per group. One-way ANOVA followed by Tukey's test. (b) The effect of DHA-plus-UMP on spine density was apparent by one week after the start of the treatment. The treated groups received both UMP (0.5%) and DHA (300 mg/kg) daily for one, two, three, or four weeks; the control groups were given only a regular diet. $n = 12$. ~ 20 neurons from two animals per group. Two-way ANOVA followed by Tukey's test. $*p = 0.02$; $**p < 0.001$. Figure and data obtained from Reference 153.

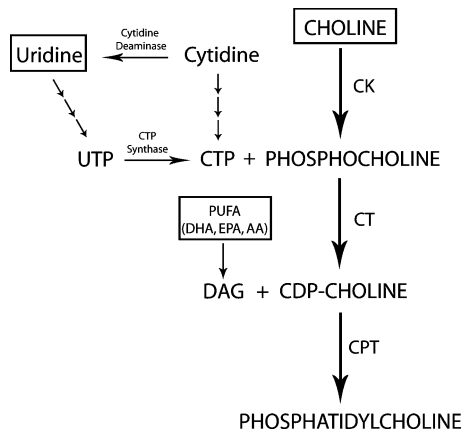


Figure 3

Phosphatidylcholine (PC) biosynthesis via the Kennedy cycle. In rats, plasma cytidine is the major circulating pyrimidine; in gerbils and humans, the primary circulating pyrimidine is uridine. Only small amounts of circulating cytidine are converted to brain CTP since the blood-brain barrier (BBB) high-affinity transporter for pyrimidines (CNT2) has a very low affinity for cytidine. Uridine, in contrast, readily enters the brain via CNT2, yielding UTP, which can then be converted to CTP by CTP synthase. CTP then reacts with phosphocholine to form endogenous CDP-choline, which combines with DAG species containing polyunsaturated fatty acids such as DHA, EPA, or AA to form PC. Boxes indicate the compounds that are obtained from the circulation. Abbreviations: AA, arachidonic acid; CDP, cytidine diphosphate; CTP, cytidine-5'-triphosphate; DAG, diacylglycerol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; UTP, uridine triphosphate.

example, permeabilized Hela cells exposed to glycerol-3-phosphate and acyl-CoA (107), or in PC12 cells extending neurites following exposure to the nerve growth factor (NGF) (6). In PC-12 cells, NGF increased DAG levels fivefold, CPT activity by 70%, and the incorporation of choline into PC by twofold. DAG species containing DHA or other PUFA on the middle carbon apparently are preferentially utilized for phosphatide synthesis instead of for triglyceride synthesis (115).

If rodents are given a standard diet supplemented with choline and uridine (as its monophosphate, UMP) and, by gavage, DHA, brain PC synthesis rapidly increases (32, 202), and absolute levels of PC per cell (i.e., DNA) or per mg protein rise substantially (e.g., by 40%–50% after several weeks of daily treatment) (202) (Table 1).

This treatment also increases the levels of each of the other principal membrane phosphatides (Table 1) as well as those of particular proteins known to be localized within presynaptic and postsynaptic membranes (for example, synapsin-1, PSD-95, and syntaxin-3) (50) (Table 2), but not of β -tubulin, a ubiquitously distributed protein (34, 202).

These changes in synaptic proteins are probably mediated by an additional mechanism—the activation of P2Y receptors by uridine or uridine-containing nucleotides (139), as discussed below. Administration of DHA,

Table 1 Effects of uridine monophosphate (UMP)-containing diet and/or docosahexaenoic acid (DHA) on brain phospholipid levels

Treatment	Total PL	PC	PE	SM	PS	PI
Control diet + vehicle	351 ± 8	152 ± 6	65 ± 4	45 ± 2	33 ± 3	21 ± 2
UMP diet + vehicle	367 ± 22	171 ± 8 ^a	84 ± 8 ^a	52 ± 5	35 ± 3	31 ± 2 ^b
Control diet + DHA	392 ± 20	185 ± 12 ^a	78 ± 5 ^a	56 ± 3 ^a	39 ± 3	32 ± 2 ^b
UMP diet + DHA	442 ± 24 ^c	220 ± 12 ^c	113 ± 6 ^c	73 ± 4 ^c	46 ± 6 ^c	36 ± 3 ^c

Groups of eight gerbils were given either a control or a UMP-containing (0.5%) diet, and received orally (by gavage) DHA (300 mg/kg in a vehicle of 5% gum Arabic solution) or just its vehicle for 28 days. On the 29th day their brains were harvested and assayed for phospholipids. Data are given as means ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey test.

^aP < 0.05, ^bP < 0.01, ^cP < 0.001 when compared with the values for control diet + vehicle group.

Reprinted from Reference 202.

Table 2 Effects of uridine monophosphate (UMP)-containing diet and/or docosahexaenoic acid (DHA) on synaptic protein levels

Treatment	Synapsin-1	PSD-95	Syntaxin-3	β -tubulin
Control diet + vehicle	100 \pm 8	100 \pm 11	100 \pm 6	100 \pm 1
UMP diet + vehicle	116 \pm 8	116 \pm 8	116 \pm 6	100 \pm 1
Control diet + DHA	137 \pm 7 ^b	125 \pm 11 ^a	120 \pm 10 ^b	93 \pm 2
UMP diet + DHA	151 \pm 4 ^c	142 \pm 5 ^c	131 \pm 8 ^c	102 \pm 1

Groups of eight gerbils were given either a control or a UMP-containing (0.5%) diet, and received orally (by gavage) DHA (300 mg/kg in a vehicle of 5% gum Arabic solution) or just its vehicle for 28 days. On the 29th day their brains were harvested and assayed for synaptic proteins using western blots. In rodents receiving the control diet + vehicle (i.e., the control group), arbitrary values obtained from protein band intensities were normalized to 100 in order to compare data obtained from treatment groups as percents of those of the control group. Statistical analysis was performed using one-way ANOVA followed by Tukey test.

^aP < 0.05, ^bP < 0.01, ^cP < 0.001 when compared with the values for control diet + vehicle group.

Adapted from Reference 34.

UMP, and choline to adult gerbils also promotes the formation of hippocampal dendritic spines (153); improves hippocampus-dependent cognitive behaviors in rats [e.g., those reared in a socially deprived environment (79, 174)] and gerbils (80); and can amplify neurotransmitter release (189, 190). Providing supplemental UMP or DHA without the other compound can also increase brain phosphatide levels; however, the increase is less than when all three precursors (including choline, which is present in all of the test diets) are given.

Sources of Plasma and Brain Uridine, Choline, and Omega-3 Fatty Acids

Uridine

Sources of plasma uridine. Few data are available as to whether foods other than milk contain significant quantities of free uridine or uridine-containing nucleotides. What is known is that pyrimidines, as well as purines, are constituents of nucleic acids, i.e., ribonucleic acid (RNA), which contains uridine and cytidine, and deoxyribonucleic acid (DNA), which contains cytidine. Since RNA and DNA are components of all cells, any food consumed by humans that contains cells (e.g., meats, poultry, fish, vegetables, fruits) is, at least theoretically, a good source of nucleic acids and perhaps also of plasma pyrimidines. Evidence

from in vitro studies suggests that, following enzymatic breakdown of dietary nucleic acids, pyrimidine compounds are taken up into the blood from the intestine. However, it has yet to be compellingly demonstrated that eating any food elevates plasma uridine levels in adult humans. The nucleic acids in foods or in breast milk have been shown in in vitro studies to be degraded to yield purine and pyrimidine nucleotides, nucleosides, and free bases (196, 197). In vitro, RNA is digested by ribonucleases to yield uridine nucleotides including uridine-3'-monophosphate (3'-UMP), uridine-5'-monophosphate (5'-UMP), and traces of 2',3'-cyclic UMP. These can be further hydrolyzed to the nucleoside uridine by phosphatases on the external side of the intestinal brush-border membrane of the mucosa (25).

Uridine is present as such in breast milk, but also as constituents of RNA, nucleotides (5'-UMP), and nucleotide adducts (UDP-glucose, UDP-galactose) (103, 177). The total available uridine contents of pooled milk samples from 100 European women determined by a method that simulated in vivo digestion (103) (i.e., by enzymatically degrading nucleic acids, nucleotides, and nucleotide adducts) were 32, 48, and 47 μ M, respectively, for mothers of 2- to 10-day-old, 1-month-old, and 3-month-old babies; available cytidine contents in the same samples were 86, 102, and 96 μ M, respectively, for

2- to 10-day-old, 1-month-old, and 3-month-old babies (103). Hence, uridine and cytidine are provided to the small intestines of infants up to three months of age in average concentrations of 40 μM and 95 μM . Synthetic infant formulas are also routinely fortified with uridine and cytidine monophosphates.

Uridine in the intestinal lumen can be transported across the mucosal epithelium as such (57, 129) and in the form of uracil, the free base, which is formed by cleaving uridine's phosphate bond. In the rat's small intestine, the cytidine derived from RNA or DNA is partly deaminated to uridine (which may also partly be converted to uracil) in the mucosa (196). In humans, this deamination in epithelial cells of intestinal mucosa and other tissues, particularly liver, is probably much greater than in rats, since exogenously administered cytidine is almost undetectable as such in human plasma (201).

The transport of pyrimidine nucleosides and bases across the small intestine is mediated by the sodium-dependent concentrative nucleoside transporters CNT1 and CNT2 (126). The kinetic properties of intestinal uridine (or uracil) uptake have not yet been determined, nor the extents to which dietary RNA (and DNA, by yielding cytidine) is hydrolyzed to uridine and uracil, nor that to which luminal uridine and uracil are absorbed into the circulation.

Following intestinal absorption, uridine and uracil are transferred via the portal vein to the liver. In rats, the liver has been suggested as the major organ modulating plasma uridine concentrations. In vitro and in vivo tracer studies on rats showed that more than 90% of the uridine entering the liver via the portal vein is metabolized in a single pass (64). Moreover, the concentration of uridine in plasma leaving the liver via the hepatic vein ($1.32 \pm 0.45 \mu\text{M}$) is slightly higher than the concentration in portal ($1.03 \pm 0.3 \mu\text{M}$) or arterial ($1.06 \pm 0.2 \mu\text{M}$) blood, which suggests that the uridine in the rat's hepatic venous blood may be derived from de novo hepatic synthesis. The sources of plasma uridine in humans are not yet known. There may be multiple sites of uridine uptake

and degradation besides the liver. For example, erythrocytes have been reported to take up orotic acid and convert it to UDP-glucose, and peripheral tissues and brain can release free uridine and glucose made from the orotic acid (67).

Sources, transport, and phosphorylation of brain pyrimidines. Uridine and cytidine are transported across cellular membranes in all tissues, including the brain, via two families of transport proteins, i.e., the Na^+ -independent, low-affinity, equilibrative transporters (ENT1 and ENT2; SLC29 family) and the Na^+ -dependent, high-affinity, concentrative (CNT1, CNT2, and CNT3; SLC28 family) nucleoside transporters (reviewed in 28).

The two ENT proteins, which transport uridine and cytidine with similar affinities, have been cloned from rat and mouse blood-brain barrier (BBB). Since their K_m values for pyrimidines are in the high micromolar range (100–800 μM) (136), they probably mediate BBB pyrimidine uptake only when plasma levels of uridine and cytidine have been elevated experimentally. In contrast, CNT2, which transports both uridine and purines like adenosine, probably mediates uridine transport across the BBB under physiologic conditions. K_m values for the binding of uridine and adenosine to CNT2 (which has been cloned from rat BBB) (105) are in the low micromolar range (9–40 μM in kidney, intestine, spleen, liver, macrophage, and monocytes), whereas plasma uridine levels are subsaturating, i.e., 0.9–3.9 μM in rats, 3.1–4.9 μM in humans (180), and around 6.5 μM in gerbils. Cytidine, although not a preferred substrate for CNT2 (70), is transported by this protein but with a much lower affinity than that for uridine (102, 122). Pyrimidines also may be taken up into brain via the choroid plexus (CP) epithelium and the ENT1, ENT2, and CNT3 transporters, all of which are found in CP epithelial cells of rats and rabbits. However, because the surface area of BBB is so much greater than that of the CP epithelium (i.e., in humans 21.6 m^2 versus 0.021 m^2) (134) it is clear that the BBB is the major locus at which

circulating uridine enters the brain. Once in brain's extracellular fluid, pyrimidines can also be taken up into brain cells by the two nucleoside transport families (143).

Uridine and cytidine are converted to their respective nucleotides following successive phosphorylations by various kinases. Uridine-cytidine kinase (UCK) (ATP:uridine 5'-phosphotransferase, EC 2.7.1.48) catalyzes the phosphorylations of uridine and cytidine to form uridine-5'-monophosphate (UMP) and cytidine-5'-monophosphate (CMP), respectively (133, 163). UMP and CMP are then converted to uridine-5'-diphosphate (UDP) and cytidine-5'-diphosphate (CDP), respectively, by UMP-CMP kinase (UMP-CMPK) (ATP:UMP phosphotransferase, EC 2.7.4.14) (84, 152, 170). These, in turn, are phosphorylated to UTP and CTP by nucleoside diphosphate kinases (NDPK) (nucleoside triphosphate:nucleoside diphosphate phosphotransferase, EC 2.7.4.6) (15, 135).

Interconversions of uridine and cytidine, and of their respective nucleotides, also occur in mammalian cells. Cytidine and CMP can be deaminated to uridine and UMP (192), while UTP is aminated to CTP by CTP synthase [UTP:ammonia ligase (ADP-forming), E.C. 6.3.4.2] (83, 106). CTP synthase activity has been demonstrated in rat brain (66).

All of the enzymes described above apparently are unsaturated with their respective nucleoside or nucleotide substrates in brain and other tissues. For example, the K_m values for uridine and cytidine of UCK prepared from various tissues varied between 33–270 μM (71, 133, 163), and the K_m for uridine of recombinant enzyme cloned from mouse brain was 40 μM (148, 149). Brain uridine and cytidine levels are about 22–46 pmol/mg wet weight (32, 116) and 6–43 pmol/mg wet weight (32, 138), respectively. Hence, the syntheses of UTP and CTP, and the subsequent syntheses of brain PC and PE via the Kennedy pathway, depend on the availability of their pyrimidine substrates. Indeed, an increase in the supply of uridine or cytidine to neuronal cells, *in vitro* (139, 144, 156, 184) or *in vivo* (32), enhanced the phos-

phorylation of uridine and cytidine, elevating the levels of UTP, CTP, and CDP-choline.

The time courses of the increases in brain levels of particular uridine-containing compounds following uridine administration were examined in gerbils given a single dose of UMP (1 mmol/kg) (32) by gavage. Plasma samples and brains were collected for assay between 5 min and 8 h thereafter. Thirty minutes after gavage, plasma uridine levels were increased from 6.6 ± 0.58 to 32.7 ± 1.85 μM ($P < 0.001$) and brain uridine from 22.6 ± 2.9 to 89.1 ± 8.82 pmol/mg tissue ($P < 0.001$). UMP also significantly increased plasma and brain cytidine levels. However, both basally and following UMP administration, these levels were much lower than those of uridine, rising from 1.2 μM to 1.9 μM in plasma and from 5 pmol/mg tissue to 12 pmol/mg tissue in brain 30 to 60 minutes after gavage. In human subjects receiving oral cytidine as CDP-choline, plasma cytidine levels do not rise detectably at all (201). Brain UTP, CTP, and CDP-choline were all elevated in gerbils 15 min after UMP (from 254 ± 31.9 to 417 ± 50.2 ($P < 0.05$); 56.8 ± 1.8 to 71.7 ± 1.8 ($P < 0.001$); and 11.3 ± 0.5 to 16.4 ± 1 , ($P < 0.001$ pmol/mg tissue, respectively), returning to basal levels after 20 and 50 min. The smallest UMP dose that significantly increased brain CDP-choline was 0.5 mmol/kg. These results show that oral UMP, a uridine source, enhances the synthesis of CDP-choline, the immediate precursor of PC, in gerbil brain, but that the increases in nucleotides or CDP-choline are short-lived and disappear long before increases in brain phosphatides become detectable. How, then, does repeated daily intake of supplemental uridine (as UMP in the test diet) ultimately raise brain PC? Probably, in part, via uridine's other mechanism of action, its activation of P2Y receptors, which then elicit longer-term downstream effects.

Choline. Choline, a normal constituent of plasma (and brain), is present as the free base (40, 77, 157), as a constituent of phospholipids (including PC, SM, lyso-PC,

choline-containing plasmalogens, and the platelet-activating factor), and as PC's water-soluble metabolites (principally phosphocholine and glycerophosphocholine) (128). Free choline is also found in other biologic fluids (199). In blood, choline is concentrated within erythrocytes through the action of an uptake molecule that is unsaturated [$K_m = 5\text{--}10\ \mu\text{M}$ (145)] at normal plasma choline concentrations.

Sources of plasma choline. Plasma choline derives from three sources: dietary choline, consumed as the free base or as a constituent of phospholipids; endogenous synthesis, principally in liver; and liberation from its reservoir within the membrane phosphatides of all mammalian cells. Choline is present within many foods (99; also see <http://www.nal.usda.gov/fnic/foodcomp/Data/Choline/Choline.html>) and also in breast milk and infant formulas (81), principally as the free molecule or as phosphatides, and its plasma levels can rapidly increase several fold after ingestion of choline-rich foods (77). For example, consumption by humans of a five-egg omelet (containing about 1.3 g of choline) increased these levels from $9.8\ \mu\text{M}$ to $36.6\ \mu\text{M}$ within four hours. Prolonged fasting reduced human plasma choline levels from $9.5\ \mu\text{M}$ to $7.8\ \mu\text{M}$ after seven days. Similarly, removal of all choline-containing foods from the diet for 17–19 days gradually lowered plasma choline, from $10.6\ \mu\text{M}$ to $8.4\ \mu\text{M}$ in humans (205) and from $12.1\ \mu\text{M}$ to $6.3\ \mu\text{M}$ in rats, indicating that plasma choline can be partially but not fully sustained by release from endogenous stores.

Dietary PC is deacylated within the gut to form lyso-PC. About half of this product is further degraded to free choline within the gut or liver. The remainder is reacylated to regenerate PC (82), which is then absorbed into the lymphatic circulation (60). Much of the dietary choline that reaches the liver via the portal circulation is destroyed by oxidation to betaine, ultimately providing methyl groups that can be used to regenerate S-adenosylmethionine (SAM) from homocysteine. The rest of the

choline in portal venous blood passes into the systemic circulation.

In 1998, the Food and Nutrition Board of the U.S. Institute of Medicine established a dietary reference intake for choline (87, 205). Since the Food and Nutrition Board did not believe that existing scientific evidence allowed calculation of a Recommended Daily Allowance for choline, it instead set an Adequate (daily) Intake level, and an Upper (daily) Limit that should not be exceeded. The main criteria for determining the Adequate Intake and Upper Limit were, respectively, the amount of choline needed to prevent liver damage and the choline intake associated with choline's most sensitive adverse effect, i.e., hypotension (87). It should be noted that subsequent studies have shown that the enzymes, described below, that synthesize and metabolize choline can be affected by common genetic polymorphisms that cause important person-to-person variations in dietary choline needs. For further details about dietary reference intakes and the choline contents of various foods, the reader is referred to the official Web sites of the Institute of Medicine (<http://www.nap.edu/catalog/6015.html#toc>) and the United States Department of Agriculture (<http://www.nal.usda.gov/fnic/foodcomp/Data/Choline/Choline.html>).

Endogenous choline is synthesized, principally in liver but also to a small extent within brain (19), by the sequential addition of three methyl groups to the amine nitrogen of PE; this forms PC, which can then be hydrolyzed to liberate the choline. The methylation reactions are catalyzed by phosphatidylethanolamine-N-methyltransferase (PEMT1; EC 2.1.1.17), which converts PE to its monomethyl derivative, and phosphatidyl-N-methylethanolamine-N-methyltransferase (PEMT2; EC 2.1.1.71), which adds the second and third methyl groups (a single enzyme may catalyze all three methylations in liver). Both enzymes utilize SAM as the methyl donor; their K_m values for SAM are $2\text{--}4 \times 10^{-6}\ \text{M}$ and $20\text{--}110 \times 10^{-6}\ \text{M}$, respectively (48), while brain SAM concentrations are $10\text{--}17\ \mu\text{g/g}$ wet weight

(50–85 μM assuming about 50% of the brain mass is aqueous). Hence, PEMT1 is probably fully saturated with SAM whereas PEMT2 is not. PEMT activity has been identified in membranous fractions from homogenates of rat and bovine brain (121); highest specific activities were present in synaptosomes (19), suggesting that nerve terminals are able to synthesize choline. In the course of these transmethylation, the phosphatide intermediates “flip” from the membrane’s cytoplasmic side, where most of the less polar PE and phosphatidylserine (PS) are found, to the more polar external leaf. PE itself can be formed in liver, kidney, or brain from free ethanolamine, via the CDP-ethanolamine cycle (Kennedy cycle) or from the decarboxylation of PS (155). PS is produced, in nerve terminals (78) and elsewhere, by the process of base exchange, in which a serine molecule substitutes for the ethanolamine in PE or the choline in PC.

The biosynthesis of PC, and thus of endogenous choline, by the methylation of hepatic PE is diminished among animals given inadequate amounts of vitamins required for methyl group production, i.e., B6, B12, and folate. This relationship provides a basis for administering supplemental quantities of these vitamins to subjects receiving uridine, DHA, and choline to promote membrane phosphatide formation.

Free choline is liberated from newly synthesized PC, and from PC molecules formed from pre-existing choline, by the phospholipase enzymes. Phospholipase D directly cleaves the choline/phosphate bond to generate choline and phosphatidic acid. Phospholipase A2 acts on the bond connecting a fatty acid to the hydroxyl group on PC’s number-2 carbon to yield that fatty acid [often arachidonic acid (AA) or DHA] and lyso-PC. This lyso-PC can then be further metabolized to choline, either directly through the action of a phosphodiesterase, or first to glycerophosphocholine by phospholipase A1 and then to choline by a phosphatase. Phospholipase C (PLC) acts on the bond connecting the phosphate and the hydroxyl group on PC’s number-3 carbon to yield DAG and phosphocholine; the phosphocholine can then

be metabolized to free choline through the action of a phosphatase.

It is estimated that, on average, about 15% of the free choline that enters the human bloodstream derives from endogenous synthesis, and the rest comes principally from dietary sources (204). Acute or chronic liver disease or deficiencies in methionine, folic acid, or vitamin B12 intake can thus lower plasma choline levels by impairing hepatic PC synthesis. Running a marathon (43) or undergoing hemodialysis (85) or surgery (86) also decreases circulating choline levels.

Cellular membranes contain most of the choline in the body, principally in the form of the phosphatide PC, but also as PC’s products sphingomyelin and lyso-PC, or as less-abundant choline-containing phospholipids such as the platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine). Membranes also contain the phosphatides PS, PE, and phosphatidylinositol (PI), as well as specific proteins, cholesterol, and various minor lipids. The quantities of choline present in brain as PC (2–2.5 mmoles/g) or as SM (0.25 mmoles/g) are orders of magnitude greater than those of free choline (30–60 μM). The proportion of any membrane’s phospholipids represented by PC can vary depending on the species and age of the animal, the particular brain region or cell type being studied, and the membrane’s function within the cell (e.g., nuclear membrane, plasma membrane). In the gray matter of human brain, PC constitutes 42% of total phospholipids and sphingomyelin 10%; in white matter, these proportions are 33% and 15%, respectively.

Moreover, PC is itself highly heterogeneous, actually representing a family of compounds with differing fatty acid compositions and, consequently, differing chemical and physical properties. The fatty acid in the C-1 position of PC tends most often to be saturated (e.g., stearic or palmitic acid), whereas that in position C-2 is more likely to be monounsaturated (oleic acid) or polyunsaturated [e.g., the omega-3 fatty acids DHA (22:6) and EPA (20:5) or the omega-6 fatty acid AA (20:4)]. Newly

synthesized phosphatide molecules contain relatively larger quantities of polyunsaturated fatty acids (PUFAs) than do the phosphatide molecules present at steady state (173). This reflects faster turnover of PUFA-containing phosphatides or their rapid deacylation followed by reacylation with more-saturated fatty acid species, or both. Membranes of retinal and brain cells are especially rich in PUFAs, particularly DHA [which comprises about 20% of the total fatty acids in retinal phospholipids (62) and about 7% of those in brain phospholipids, respectively]. As described below, administration of supplemental DHA accelerates PC synthesis and increases brain levels of PC and other phosphatides.

Dietary choline or choline secreted into the gut can be broken down by intestinal bacteria to form trimethylamine and related amine products (124). This process is responsible for the “fishy odor” sometimes detected in people taking large doses of choline supplements.

Sources of brain choline. Because choline is, by virtue of its quaternary nitrogen atom, highly polar, it had generally been assumed that plasma choline was unavailable to the brain. And since brain cells were also thought to be incapable of synthesizing choline *de novo*, the ability of cholinergic neurons to maintain the intracellular choline concentrations needed for acetylcholine (ACh) synthesis was usually attributed either to an extraordinarily effective reuptake mechanism for reutilizing choline formed from the hydrolysis of ACh or to the uptake into brain of circulating PC or lyso-PC. And since the poor affinity of choline acetyltransferase (ChAT), the enzyme that catalyzes choline's conversion to ACh, for choline made it likely that intracellular choline concentrations would control brain ACh synthesis, it was broadly conjectured that choline's high-affinity uptake from the synaptic cleft controlled the rate of brain ACh synthesis.

It is no longer held that brain choline levels are sustained solely by circulating phosphatides or by the high-affinity uptake of free choline from synapses, or that variations in high-affinity

uptake are responsible for observed variations in brain choline levels. Choline molecules (but not those of PC or lyso-PC) do readily cross the BBB (47), and brain cells do indeed synthesize choline *de novo* (18). Physiological variations do occur in choline levels within brain neurons; however, these result principally from changes in plasma choline concentrations after eating choline-rich foods, or from choline's metabolism (185).

Mammalian brains contain choline as the free base, as such water-soluble phosphorylated metabolites as phosphocholine and glycerophosphocholine (128), and as constituents of membrane phospholipids including PC, sphingomyelin, and lyso-PC. Free choline levels in brains of humans and rats reportedly vary between 36–44 μM and 30–60 μM , whereas PC and sphingomyelin levels are orders of magnitude higher (approximately 2000–2500 μM and 250 μM , respectively) (113). These high levels reflect the ubiquity of phospholipids and the numerous essential roles they mediate when they form membranes. Membrane phospholipids also serve as reservoirs for choline and for such “second messenger” molecules as DAG, AA, inositol trisphosphate, and phosphatidic acid.

Free choline molecules in brain derive from four known sources: uptake from the plasma, liberation from the PC in brain membranes, high-affinity uptake from the synaptic cleft after ACh released from a cholinergic terminal has been hydrolyzed, and, probably to a minor extent, the breakdown of newly synthesized PC formed from the methylation of PE.

The brain can obtain circulating choline via two routes. Small amounts pass from the blood to the cerebrospinal fluid through the action of a specific transport protein, organic cation transporter 2, which is present in cells that line the choroid plexus (172). However, orders of magnitude more choline pass bidirectionally between the blood and the brain's extracellular fluid by facilitated diffusion. This process is catalyzed by a different transport protein, which is localized within endothelial cells that line the brain's capillaries (47, 132). Its action is

independent of sodium and can be blocked by hemicholinium-3.

Studies using an *in situ* brain perfusion technique, or a cell line of immortalized endothelial microvessels from rat brain (RBE4), have demonstrated the existence of a transport protein with a relatively low K_m for choline (39–42 μM or 20 μM) that could mediate choline's bidirectional flux across the BBB (61). Other investigators using other experimental systems had proposed substantially higher K_m values for endothelial choline transport, *i.e.*, 220–450 μM (47, 119, 132). The differences among the affinities noted in these studies might reflect the different methods used for their measurement. But in any case, the protein would still be unsaturated at physiological plasma choline concentrations and its net activity still affected by variations in these concentrations. It might constitute a kind of pore through which choline can pass in either direction, based on the gradient between its blood and brain levels (96). Hence, when consumption of a choline-rich meal elevates plasma choline levels (*e.g.*, to 50 μM in the rat), choline tends to enter the brain, but when plasma choline levels are low, its flux is in the opposite direction. It has been estimated that the plasma choline concentration in rats required in order for the net choline flux to be from blood to brain is about 15 μM ; below this concentration, net choline flux is presumably from brain to blood (96).

Once circulating choline has entered the brain's extracellular fluid, it can be taken up into all cells by a low-affinity transport protein ($K_m = 30\text{--}100 \mu\text{M}$) or into cholinergic nerve terminals by a high-affinity uptake protein ($K_m = 0.1\text{--}10 \mu\text{M}$). The high-affinity process—unlike the passage of choline across the BBB—is energy and sodium dependent.

The choline in membrane PC can be liberated through the actions of the phospholipase enzymes, described above. The activation of each phospholipase is tightly regulated and, in general, initiated by the interaction of a neurotransmitter or other biologic signal with a receptor coupled to a G-protein. For example, both the PLC enzymes (which act on PC to

yield DAG and phosphocholine, or on PI) and phospholipase D (which acts on PC to yield phosphatidic acid and choline) are activated when ACh attaches to M1 or M3 muscarinic receptors.

The release of choline from PC can also be enhanced, and its reincorporation into PC diminished, by sustained neuronal depolarization (56). This process has been termed “autocannibalism” when some of the choline is diverted for the synthesis of ACh (17, 185). Autocannibalism may underlie the particular vulnerability of cholinergic neurons in certain diseases by decreasing the quantities of phosphatide molecules and thus of neuronal membranes. It can be blocked by providing the brain with supplemental choline.

Acetylcholine released into synapses is very rapidly hydrolyzed to free choline and acetate; this process terminates the neurotransmitter's physiologic actions. The enzymes that catalyze ACh hydrolysis, the acetylcholinesterases (EC 3.1.1.7, AChE), are particularly abundant within the cholinergic synapse; they are synthesized in the cholinergic neuron and secreted into the synapse, along with ACh, when the neuron is depolarized. Most of the free choline liberated by the intrasynaptic hydrolysis of ACh is taken back up into its nerve terminal of origin by a high-affinity choline transporter and either reacylated to form ACh or phosphorylated for ultimate conversion to membrane PC.

Sources of plasma and brain DHA and EPA.

The omega-3 PUFAs DHA (22:6n-3) and EPA (20:5n-3) and the omega-6 PUFA AA (22:4n-6) are long-chain derivatives of α -linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6), respectively. ALA and LA are essential dietary constituents for vertebrates because these animals cannot synthesize them or their polyunsaturated products *de novo*. Although DHA, EPA, and AA can be produced in the human body through the elongation and desaturation of ALA and LA, respectively, the conversion of ALA to EPA or DHA is slow, since about 75% of available ALA is shunted to β -oxidation (23). Furthermore, the commercial oils that provide

dietary ALA and LA, such as safflower, sunflower, and corn oils, also contain very high proportions of LA and thus give rise to disproportionately large amounts of AA in the bodies of people who consume them, i.e., most people on Western diets. This increase, in turn, decreases the conversion of ALA to EPA and DHA in the body because these PUFAs compete for the same delta-6 desaturase enzyme as the one that converts LA to AA. Thus, in order to compensate for the effect of AA and to provide the body with sufficient amounts of omega-3 PUFAs, additional EPA and DHA must be obtained from the diet, particularly from high-fat fish or foods fortified with deodorized omega-3-rich oils. No authoritative body has defined a requirement for DHA (162); intakes of 3 g per day, or even more, have been used to lower plasma triglyceride levels in diabetes mellitus (73).

The processes by which circulating PUFAs are taken up into the brain and brain cells are thought to include both simple diffusion (also termed “flip-flop”) (90) and protein-mediated transport (3). One such transport protein (BFATP) (38) has been cloned (161). DHA, EPA, and AA are then transported from the brain’s extracellular fluid into cells and can be activated to their corresponding CoA species (e.g., docosahexaenoyl-CoA, eicosapentaenoyl-CoA, arachidonoyl-CoA) and acylated to the sn-2 position of DAG (147) to form PUFA-rich DAG species (14, 176) for incorporation into phosphatides. DHA is acylated by a specific acyl-CoA synthetase, *Acs16* (114), which exhibits a low affinity for this substrate ($K_m = 26 \mu\text{M}$) (142) relative to usual brain DHA levels (1.3–1.5 μM) (44). Hence, treatments that raise blood DHA levels rapidly increase its uptake into and retention by brain cells.

EPA can be acylated to DAG by the acyl-CoA synthetase (125) or it can be converted to DHA by brain astrocytes (120), allowing its effects on brain phosphatides and synaptic proteins, described below, to be mediated by DHA itself. Exogenously administered AA, like DHA, is preferentially incorporated into brain phosphatides (51, 154) as well as into other lipids, e.g., the plasmalogens (58, 123). AA shares some

neurochemical effects with DHA, e.g., the ability to activate syntaxin-3 (50), and also has other important functions, e.g., as the precursor of prostaglandins. However, unlike DHA, AA administered orally to laboratory rodents apparently does not promote synaptic membrane synthesis (34) or dendritic spine (153) formation.

DHA and AA are major components of brain membrane phospholipids (130). Although AA is widespread throughout the brain and particularly abundant in PI and PC, DHA is concentrated within synaptic regions of gray matter (22) and is especially abundant in PE and PS (171). In contrast, EPA is found only in trace amounts in brain phosphatides, mostly in PI. No significant differences have been described between the proportions of ingested omega-3 and of omega-6 PUFAs that actually enter the systemic circulation (16, 36); the rates at which radioactively labeled DHA and AA are incorporated into brain phospholipids following systemic injection also are similar (51, 141).

Properties of Enzymes that Mediate Brain Phosphatide Synthesis

The ability of each of the three circulating phosphatide precursors to affect the rate of phosphatide synthesis derives from the low affinities of these enzymes for their substrates, as described below.

Choline Kinase (CK). The synthesis of PC is initiated by the phosphorylation of choline, in which CK (EC 2.7.1.32) catalyzes the transfer of a monophosphate group from ATP to the hydroxyl oxygen of the choline. In some neurons, choline is also used to synthesize the neurotransmitter ACh, the enzyme ChAT transferring an acetyl group from acetyl-CoA to the hydroxyl oxygen of the choline. Like CK, ChAT has a very low affinity for its choline substrate (76, 182). The K_m values of CK and ChAT in brain (which, of course, describe the choline concentrations at which the enzymes operate at only half-maximal velocity) are

reportedly 2.6 mM (167) and 540 μ M (151), respectively, whereas brain choline levels are only about 30–60 μ M (95, 150, 169). Hence, the syntheses of both phosphocholine and ACh are highly responsive to treatments that raise or lower brain choline levels.

CTP:phosphocholine cytidyltransferase.

CTP:phosphocholine cytidyltransferase (CT; EC 2.7.7.15) catalyzes the condensation of CTP and phosphocholine to form CDP-choline (**Figure 3**). CT is present in both the soluble and particulate fractions of the cell (195, 198); the cytosolic form is reportedly inactive, and the membrane-bound form is active (181, 186). Increases in the association of CT with membranes reportedly correlate with increases in CT activity and in the net synthesis of PC in vitro (137, 164, 165). Some other lipids (e.g., PS) (46) and DAG (164) also stimulate the translocation of CT from the cytosol to membranes in vitro, thereby activating the enzyme. However, translocation is clearly not the sole mechanism for CT activation, inasmuch as increases in the activity of membrane-bound CT often do not correlate with decreases in that of the cytosolic enzyme (194) as would be expected if translocation were the only means whereby CT became activated. The phosphorylation state of CT may also be important (193), as well as the enzyme's substrate saturation with CTP and perhaps with phosphocholine, as described below.

The K_m values of CT for CTP and phosphocholine in brains of laboratory rodents and humans are reportedly 1–1.3 mM and 0.30–0.31 mM (111, 150), respectively, whereas brain levels of these compounds are only 70–110 μ M (32) and 0.32–0.69 mM (95, 117, 128), respectively. Hence, brain CT also is highly unsaturated with CTP and only about half-saturated with phosphocholine in vivo, suggesting that its degree of substrate saturation, particularly with CTP, exerts important limiting roles in PC synthesis. Thus, treatments that increase cellular CTP (e.g., administration of a uridine or cytidine source) have been shown to enhance CDP-choline and PC synthesis in

poliovirus-infected HeLa cells (39), undifferentiated PC12 cells (109, 144), slices of rat corpus striatum (156, 184), and gerbil brain in vivo (32).

CDP-choline:1,2-diacylglycerol choline-phosphotransferase.

CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT; EC 2.7.8.2) catalyzes the final reaction in the Kennedy cycle; it transfers the phosphocholine moiety from CDP-choline to DAG (198), thus yielding PC (**Figure 3**). CPT is an integral membrane protein that is now cloned in humans (75) and is present primarily in the endoplasmic reticulum (41). CPT may be a reversible enzyme, synthesizing CDP-choline from PC and CMP in microsomal preparations from liver (91, 92) and brain (68, 69, 146).

The choline phosphotransferase reaction is also unsaturated with the enzyme's substrates: Its K_m values for CDP-choline and DAG in rat liver are 200 μ M and 150 μ M (45), respectively, whereas the concentrations of these compounds in liver are approximately 40 μ M and 300 μ M (183). (A DAG concentration of at least 1000 μ M thus would probably be needed to saturate the enzyme.) Brain CDP-choline and DAG levels are even lower, i.e., about 10–30 μ M (32) and 75 μ M, respectively. Levels of cellular DAG have been shown to limit PC synthesis in permeabilized HeLa cells (107), cultured rat hepatocytes (89), and PC12 cells (described above) (6).

P2Y Receptors as Mediators of Uridine Effects

How does exogenous uridine—a precursor for the cytidine compounds utilized in the syntheses of PC and other cellular lipids—increase levels of cellular proteins, specifically of certain pre- and postsynaptic neuronal proteins? Most likely by a second, entirely different mechanism in which uridine's phosphorylated products act as ligands for P2Y receptors, which then can activate protein synthesis and normal neuronal differentiation.

Extracellular nucleotides can serve as ligands for a variety of ionotropic P2X and metabotropic P2Y receptors. While P2X receptors recognize adenine nucleotides, P2Y receptors can recognize both adenine and uridine nucleotides. Members of the P2Y family, G protein-coupled receptors, are widely distributed throughout the body, including in the brain (26). To date, eight P2Y receptors of human origin (P2Y1, 2, 4, 5, 11, 12, 13, and 14) have been cloned and characterized (1). [The missing numbers represent either nonhuman orthologs or receptors having some sequence homology to P2Y receptors but for which there is currently no functional evidence of responsiveness to nucleotides (26).]

P2Y receptors that recognize adenine but not uridine nucleotides, and comprising the P2Y1, P2Y11, P2Y12, and P2Y13 subtypes, exist principally outside the brain (26). P2Y2 receptors, in contrast, are abundant in brain and are activated by UTP or ATP (110); P2Y4 receptors are activated by UTP (42), and P2Y6 receptors are activated by UDP. Their activation, through coupling to PLC, increases intracellular concentrations of DAG, inositol trisphosphate, and calcium (4, 9).

That uridine nucleotides affect neurite outgrowth as well as neuronal differentiation and function by stimulating P2Y receptors (cf. 29, 104) has been demonstrated mainly by using in vitro assay systems (10, 37, 139). UTP increases neurite outgrowth by NGF-stimulated PC-12 cells (139) and the expression of some neurofilament proteins, and these effects are blocked by P2Y receptor antagonists or by apyrase, a drug that degrades extracellular nucleotides (49, 139). The uridine nucleotides also cause receptor-mediated neuronal proliferation and differentiation [e.g., of dopaminergic neurons during development (118)] and can mediate signaling events such as release of the neurotransmitters noradrenaline and glutamate (20, 21, 188), long-term potentiation (140), and neuroprotection [perhaps by

stimulating astrogliosis (191)]. The nucleotides also reportedly antagonize apoptosis (11) and, in vivo, diminish β -amyloid deposits (27). Such P2Y-receptor-mediated actions could argue for the possible utility of P2Y agonists in treating Alzheimer's disease, especially since P2Y2 receptors are known to be deficient in parietal cortex of brains of patients with Alzheimer's disease (100).

EFFECTS OF URIDINE ON NEURITE OUTGROWTH IN CULTURED PC-12 CELLS AND OF URIDINE AND DHA, IN VIVO, ON DENDRITIC SPINE FORMATION

The formation of a new brain synapse generally follows the interaction of a highly differentiated outgrowth, a dendritic spine, from what will become the postsynaptic neuron, with a terminal bouton of a presynaptic neuron. The number of dendritic spines at steady state in a brain region depends on genetic factors and on the frequency with which the neuron is depolarized or stimulated by synaptic transmission. It is also increased in hippocampus of animals treated with the uridine-DHA-choline mixture (**Figure 2**) or, less so, with DHA alone. Moreover, uridine (139) or DHA (49) can increase the number of neurites projecting from PC-12 cells. Arachidonic acid, an omega-6 PUFA, fails to increase dendritic spines in vivo (153) but does stimulate neurite outgrowth (50).

Uridine and Neurite Formation by PC-12 Cells

PC-12 cells that had been differentiated by nerve growth factor were exposed to various concentrations of uridine, and the number of neurites that the cells produced was measured (139). After four but not two days, uridine significantly and dose-dependently increased the number of neurites per cell. This increase was accompanied by increases in neurite branching and in the levels of the neurite proteins neurofilament M and neurofilament 70. Uridine

treatment also increased intracellular levels of CTP and UTP, which suggests that it enhanced neurite output both by stimulating PC synthesis and by activating P2Y2 receptors (9). The increase in neurite output was mimicked by exposing the cells to UTP and could be blocked by various drugs known to antagonize P2Y receptors [e.g., suramin, Reactive Blue 2, pyridoxal-phosphate-6-azophenyl-2',4' disulfonic acid (PPADS)]. Treatment of the cells with uridine or UTP also enhanced their accumulation of inositol phosphates, and this effect was also blocked by PPADS. Moreover, degradation of nucleotides by apyrase blocked the stimulatory effect of uridine on neuritogenesis. Like uridine, choline (203) or DHA (50) also enhance neurite outgrowth *in vitro* (50).

Uridine is not unique in regulating cell differentiation and metabolism via two separate mechanisms; i.e., as a receptor agonist and a bulk precursor of CTP needed for phosphatide synthesis. Diacylglycerol also acts in two ways, both as a potent "second messenger" that activates protein kinase C and as a bulk precursor in phosphatide synthesis, the intracellular levels of which modulate the substrate-saturation of CPT (6). The density of P2Y2 receptors is selectively reduced in brains of patients with Alzheimer's disease (100). This could reflect a loss of postsynaptic structures that contain this protein (e.g., postsynaptic densities) or perhaps the action of a toxin that inhibits neurite outgrowth and ultimately suppresses synapse formation in Alzheimer brains.

Stimulation of Hippocampal Dendritic Spine Formation *in vivo* by Uridine-DHA-Choline Treatment or DHA Alone

As noted above, mature dendritic spines—the small membranous protrusions extending from postsynaptic dendrites of neurons—form and then represent excitatory glutamatergic synapses. Their numbers in particular brain regions are highly correlated with numbers of synapses (5, 7, 8, 13, 52, 53, 72, 98), and

it has been proposed (179) that "more than 90% of excitatory synapses occur on dendritic spines." This suggests that processes that damage the spines [e.g., beta-amyloid, amyloid plaques (88, 97, 168)] or that increase spine number [treatment with uridine, DHA, and choline, discussed below (153)] will cause parallel changes in synapse number. The formation of dendritic spines in the hippocampus is induced physiologically by synaptic inputs that induce long-term potentiation in CA1 pyramidal neurons, probably mediated by enhanced calcium influx into the postsynaptic neuron (55, 178).

The effects of administering the phosphatide precursors DHA (300 mg/kg) and uridine (as UMP, 0.5%) on dendritic spine number (in CA1 pyramidal hippocampal neurons) were examined in adult gerbils treated daily for 1–4 weeks; animals received one or both compounds (153). DHA alone caused dose-related increases in spine density, accompanied by parallel increases in membrane phosphatides and in specific pre- and postsynaptic proteins; its effect was doubled if animals also received uridine (UMP). In contrast, administration of the omega-6 PUFA arachidonic acid, with or without uridine, had no effect on spine density or on phosphatide or synaptic protein levels. DHA administration has been described as promoting cognition (63, 74), yet its effects on neurotransmission have been obscure. Perhaps its effect on cognition is mediated in part by the increases it produces in numbers of dendritic spines or synapses.

Similar studies were performed on pregnant rats and their offspring (30). The dams consumed UMP, DHA, or both compounds for 10 days prior to parturition and 21 days while nursing. By day 21, brains of weanlings exhibited significant increases in membrane phosphatides, various pre- and postsynaptic proteins (synapsin-1, mGluR1, and PSD 95), and in hippocampal dendritic spine density. Perhaps administering the phosphatide precursors to lactating mothers or to infants could be useful in treating developmental disorders characterized by deficient synapses.

EFFECTS OF URIDINE OR UMP ON NEUROTRANSMITTER RELEASE AND OF UMP PLUS DHA ON BEHAVIOR

Consumption by rats of a diet containing uridine (as UMP) and choline can increase dopamine (DA) and ACh levels in, and—as assessed using *in vivo* microdialysis—their release from, corpus striatum neurons. Dietary supplementation of aged male Fischer 344 rats with 2.5% w/w UMP for six weeks, *ad libitum*, increased the release of striatal DA evoked by potassium-induced depolarization, from $283 \pm 9\%$ in control rats to $341 \pm 21\%$ in those receiving the UMP ($P < 0.05$) (190). Giving both uridine and DHA amplifies uridine's effect on DA levels (31).

In general, each animal's DA release correlated with its striatal DA content, measured postmortem. The levels of neurofilament-70 and neurofilament-M proteins, two markers of neurite outgrowth, were also increased after UMP treatment, to $182 \pm 25\%$ of control levels for the neurofilament-70 ($P < 0.05$) and to $221 \pm 34\%$ ($P < 0.01$) for the neurofilament-M (190).

In a similar microdialysis study, ACh release, basally as well as after administration of atropine (a muscarinic antagonist that blocks inhibitory presynaptic cholinergic receptors), was found to be enhanced following UMP consumption. Among aged animals consuming a 2.5% uridine diet for one or six weeks, baseline ACh levels in striatal microdialysates rose from 73 to 148 fmol/min after one week of treatment ($P < 0.05$) to 197 fmol/min after six weeks ($P < 0.05$) (189). A lower dose of dietary UMP (0.5%, one week) also amplified the increase in ACh release caused by giving atropine (10 μM , via the artificial CSF); atropine alone increased ACh concentrations from 81 to 386 fmol/min in control rats and from 127 to 680 fmol/min in those consuming UMP ($P < 0.05$). Young rats eating the UMP-containing diet exhibited similar responses. These data suggest that giving a uridine source may enhance some cholinergic

functions, perhaps by increasing the amount of synaptic membrane or the quantities of ACh stored in synaptic vesicles. Apparently no data are available on effects of UMP plus DHA on neurotransmitter release.

A few behavioral studies provide indirect evidence that treatment with UMP alone, or with UMP plus DHA, can affect brain neurotransmission (79, 80, 174).

The effects on cognitive performance of DHA, alone or with dietary UMP, in environmentally impoverished or enriched rats were assessed using the Morris water maze (80). Animals received DHA (300 mg/kg) by gavage, UMP (0.5%) in the diet, or both compounds, and hippocampal and striatal forms of memory were measured in rats exposed to either environment for one month starting at weaning and consuming a choline-containing diet. Giving either DHA or UMP improved performance in the hidden version of the Morris water maze (all $P < 0.05$), a hippocampal-dependent task; coadministration of both phosphatide precursors further enhanced performance among environmentally impoverished rats ($P < 0.001$). Neither giving UMP or DHA alone, nor giving both compounds, affected performance by rats raised in the enriched environment or the performance by either group on the visible version of the Morris water maze, a striatal-dependent task. [Simply rearing animals in the enriched environment also elevated brain levels of individual phosphatides and total phospholipids ($P < 0.01$).] Chronic dietary administration of UMP (0.1%) alone for three months also ameliorated this impairment among the impoverished rats (174).

In normal adult gerbils, DHA plus choline improved performance on the four-arm radial maze, T-maze, and Y-maze tests; coadministering UMP enhanced these increases (79). These findings demonstrate that a treatment that increases synaptic membrane can enhance cognitive functions in normal animals as well as in those reared in a restricted environment.

CLINICAL APPLICATIONS

Brains of patients with Alzheimer's disease are deficient in choline (128) and in DHA (166) and exhibit selective decreases in numbers of P2Y₂ receptors (100) and dendritic spines (97) and synapses (175). Because the loss of dendritic spines or synapses precedes neuronal degeneration and is associated with cognitive deficits in both patients and animal models of Alzheimer's disease, it can be hypothesized that impaired synaptic signaling is an initial process in developing the pathologic findings and behavioral characteristics of Alzheimer's disease. The loss of spines may result from toxic effects of beta-amyloid, particularly that in senile plaques (88, 97, 168).

Because administering the uridine-DHA-choline mixture improves cognition, increases dendritic spine number, and raises brain levels of the phosphatides and synaptic proteins that comprise synaptic membrane in normal or experience-deprived rats and gerbils (35), it seemed reasonable to explore whether this treatment might also improve cognition in impaired patients with Alzheimer's disease. [In support of this hypothesis, treatment of transgenic APP^{swe}/PS1^{oE9} mice for three to four months with a DHA-enriched diet has been shown to decrease hippocampal A-beta levels and microglial activation (131), while treatment of these mice with a multi-nutrient-enriched diet that included both UMP and DHA also decreased A-beta plaque burden and neuritic plaque burden (24).] Similar arguments can be adduced for determining whether nutrient mixtures shown to enhance synaptogenesis should be tested in other neurodegenerative diseases, or in neurovascular diseases, or in cognitive disorders following brain trauma.

A randomized, controlled, double-blind, parallel group, multi-center, multi-country clinical trial, involving 212 drug-naïve subjects with mild Alzheimer's disease and directed by Prof. Philip Scheltens (159) was thus

performed to examine the effects of a mixture (SouvenaidTM) including DHA, UMP, choline, and other nutrients (e.g., vitamins B₆, B₁₂, and folic acid) on a delayed verbal memory task (derived from the Wechsler Memory Scale-Revised) and the item-modified ADAS-cog at 12 weeks (159). The trial was preregistered with the Dutch Trial Registry (No. ISRCTN 722254645).

In the group receiving the mixture, a significant benefit was found on the verbal memory task in patients with mild and very mild Alzheimer's disease. The unadjusted analyses showed no significant effect on the modified ADAS-cog test. However, the baseline modified ADAS-cog score was a predictor for the intervention effect, i.e., patients with a higher baseline score showed a greater effect after treatment with the mixture. Intervention with the mixture was well tolerated (compliance was 94%) and safe. This proof-of-concept study was interpreted as demonstrating that giving a drink that contains DHA, uridine, choline, and other nutrients for 12 weeks can improve memory in mild and very mild Alzheimer's disease, and that further studies would be justified. A second study, on 500 patients in the United States, was initiated in March 2009.

SUMMARY

Brain phosphatide synthesis requires three circulating compounds: DHA, uridine, and choline. Oral administration of these phosphatide precursors to experimental animals increases the levels of phosphatides and synaptic proteins in the brain and per brain cell, as well as the numbers of dendritic spines on hippocampal neurons. AA fails to reproduce these effects of DHA. If similar increases occur in human brain, giving these compounds to patients with diseases that cause the loss of brain synapses—such as Alzheimer's disease—could be beneficial.

DISCLOSURE STATEMENT

Richard Wurtman is a professor at MIT, which owns patents and has filed patent applications relating to the uses of compounds, described in this review, to increase synapse formation and to treat diseases associated with the loss of synapses. These patents have been licensed to the Danone Company, and mixtures including the compounds are undergoing testing for treating Alzheimer's disease. RW is also a paid consultant to Danone on scientific matters and will receive a share of whatever royalties MIT receives. The MIT research has all been supported by the NIH; however, Danone has on occasion provided support for a postdoctoral fellow or technician. None of the other authors of this review is included in the patents, nor has any received direct support—salary or research—from Danone.

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