Chimeras of Two Isoprenoid Synthases Catalyze All Four Coupling Reactions in Isoprenoid Biosynthesis

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Chimeras of Two Isoprenoid Synthases Catalyze All Four Coupling Reactions in Isoprenoid Biosynthesis

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The carbon skeletons of over 55,000 naturally occurring isoprenoid compounds are constructed from four fundamental coupling reactions: chain elongation, cyclopropanation, branching, and cyclobutanation. Enzymes that catalyze chain elongation and cyclopropanation are well studied, whereas those that catalyze branching and cyclobutanation are unknown. We have catalyzed the four reactions with chimeric proteins generated by replacing segments of a chain-elongation enzyme with corresponding sequences from a cyclopropanation enzyme. Stereochemical and mechanistic considerations suggest that the four coupling enzymes could have evolved from a common ancestor through relatively small changes in the catalytic site.

Isoprenoid metabolism is the most chemically diverse of nature's biosynthetic pathways. Over 55,000 naturally occurring isoprenoid molecules have been discovered, with a large number of new structures reported each year. Isoprenoid compounds play crucial metabolic and structural roles in cells, and the pathway is found in all forms of life. Among the better-known classes of isoprenoids are sterols (hormones and membrane components), carotenoids (visual pigments and photoprotective agents), prenylated proteins (membrane structure and cell signaling), dolichols (glycoprotein synthesis and bacterial cell wall synthesis), monoterpenes (insect sex pheromones and fragrances), and sesquiterpenes (plant defensive agents). The carbon skeletons of isoprenoid compounds are constructed from 3-methyl-1-butyl units, which are joined in one of nine different patterns (Fig. 1) (*1*–*3*). In four of these structures, C1′ of one unit (red) is joined to a single carbon of the other unit (black) (1′-1, 1′-2, 1′-3, and 1′-4). In one case, C1′ is inserted between atoms in the other unit (2-1′-3), and in three cases, C1′ is joined to the other unit in a cyclic structure (c1′-1-2, c1′-2-3, and c1′-2-3-2′). The example that does not involve C1′ is the 4′-4 pattern seen in membrane lipids from Archaea (*3*).

Except for the 4′-4 attachment, the isoprenoid skeletons in Fig. 1 are formed early in the isoprenoid pathway, before the modification reactions that are required to synthesize specific natural products. Four of the basic isoprenoid structures (1′-2, 1′-4, c1′-2-3, and c1′-2-3-2′) are synthesized by joining simpler units, whereas the other four (1′-1, 1′-3, 2-1′-3, and c1′-1-2) are produced by rearrangement of 1′-2-3 structures (*4*, *5*). The basic building reaction in the pathway is chain elongation, where the growing isoprenoid chain of an isoprenoid allylic diphosphate is added to the double bond in isopentenyl diphosphate (IPP) (Fig. 2). This reaction occurs in all organisms. The 1′-4 linkage is by far the most common that is seen in isoprenoid compounds, and compounds with this attachment between isoprene units are called head-to-tail or regular terpenes. Other attachment patterns are designated as non–head-to-tail or irregular. The cyclopropanation reaction is the first pathway-specific step in the biosynthesis of sterols (*6*) and carotenoids (*7*) to give metabolites widely distributed in Eukarya, Archaea, and some Bacteria. Branching is only found in a limited number of plants (*1*, *8*). The only documented compounds from cyclobutanation are mealybug mating pheromones (*2*, *9*).

The enzymes that catalyze chain elongation can be divided into two convergently evolved families based on the stereochemistry, *E* or *Z*, of the double bond in the newly added isoprene unit (*10*). Members of the *E* family typically synthesize shorter-chain isoprenoid diphosphates found early in the pathway, whereas those in the *Z* family synthesize longer-chain diphosphates. Farnesyl diphosphate synthase (FPPase) is the prototypal representative of the *E* family. FPPase catalyzes two reactions: the sequential condensation of dimethylallyl diphosphate (DMAPP, C_5) and geranyl diphosphate (GPP, C_{10}) with IPP to give farnesyl diphosphate (FPP, C_{15}). Structural studies and phylogenetic comparisons indicate that the α-helical "isoprenoid fold" originally discovered in avian FPPase is found in all of the *E*-family chain-elongation enzymes (*11*) and in more distant relatives that catalyze the cyclopropanation reactions in sterol biosynthesis (*12*) and terpenoid cyclization reactions (*13*). An ancestral FPPase or a closely related relative is a likely candidate for the protein from which these other enzymes evolved.

We recently isolated the genes for chrysanthemyl diphosphate synthase (CPPase), an enzyme with cyclopropanation activity, from *Chrysanthemum cinerariaefolium* (chrysanthemum) (*14*) and *Artemisia tridentata* ssp. *spiciformis* (snowfield sagebrush) (*15*). The encoded sagebrush FPPase and CPPase proteins have similar sequences (75% identity and 96% similarity), suggesting that they share a recent common ancestor. Because FPPases are universally distributed, whereas CPPases are confined to members of a closely related family of higher plants, it is apparent that the cyclopropanation activity evolved from the chain-elongation activity of a parental FPPase. In contrast, the cyclopropanation enzymes squalene (*13*) and phytoene (*16*) synthase have no discernable sequence similarity with chain-elongation enzymes, suggesting an independent divergence of chain-elongation and cyclopropanation activities at a much earlier time, probably at the very beginning of cellular life.

On closer examination, we discovered that CPPase also synthesized lesser amounts of the 1′-2 branched product lavandulyl diphosphate (LPP) and the chain-elongation product GPP. In preliminary experiments with a series of chimeric proteins constructed by replacing amino acids of sagebrush FPPase with corresponding sequences from sagebrush CPPase, we found a transition in activities from chain elongation to branching and finally to cyclopropanation (*17*). Studies with a full set of chimeric proteins demonstrate that they catalyze all four of the fundamental isoprenoid-coupling reactions and that the absolute stereochemistries of the chiral products are identical to their naturally evolved counterparts.

Synthesis and expression of chimeric enzymes. By means of procedures reported previously, a common set of specific restriction

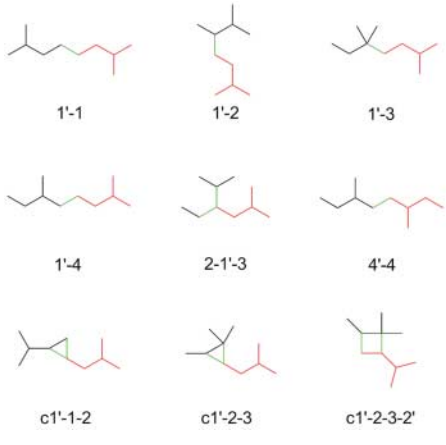

Fig. 1. Patterns found in nature for connecting isoprene units. Colors indicate isoprene units (red and black) and bonds joining the isoprene units (green).

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sites at identical locations was introduced into the open reading frames (ORFs) for FPPase and CPPase (fig. S1) (*18*). The kinetic constants for the modified (M) proteins, FPPase-M and CPPase-M, were similar to those of the wild-type (WT) enzymes (table S1). Eleven chimeras were constructed from FPPase-M and CPPase-M by fusing fragments of the two proteins at the common restriction sites. In six of the constructs, amino acids in FPPase-M, beginning at the N terminus, were replaced with increasingly longer segments from CPPase-M. A complementary set of six constructs was prepared in a similar manner by replacing amino acids in CPPase-M, again beginning at the N terminus, with the corresponding segments from FPPase-M. The chimeras were named by designating the site of the junction and the origin of the amino acids, either FPPase-M or CPPase-M, on either side of the junction. For example, the "C69F" chimera (where C indicates CPPase and F indicates FPPase) has a junction site constructed at the common BseR I restriction sites in the ORFs for FPPase-M and CPPase-M where the first 69 amino acids (FPPase-M numbering) are from CPPase-M and the remaining amino acids are from FPPase-M. The chimeric proteins were obtained for 11 of the 12 constructs (only F243C failed to give protein) and were purified (>95%) by Ni^{2+}-affinity chromatography. Removing the His_6 tag in FPPase-M and CPPase-M did not alter their activity, and the histidine tags were not removed from the parental enzymes or chimeric enzymes used in this study.

A screen for chain-elongation and "irregular" products. FPPase-M, CPPase-M, and 11 chimeric proteins were screened for synthesis of chain-elongation and irregular products (Fig. 3). The screen for chain elongation involved incubation of the proteins with [^{14}C]IPP and either DMAPP or GPP. Products and substrates were separated by thin-layer chromatography (TLC) and analyzed by phosphor autoradiography (fig. S2). The screen for irregular monoterpene synthesis involved incubation with [^{14}C]DMAPP. Incubation of FPPase-M with [^{14}C]IPP and DMAPP gave products that comigrated with C_{10} (GPP) and C_{15} (FPP) isoprenoid diphosphates. No products were seen when the enzyme was incubated with [^{14}C]DMAPP. Incubation of CPPase-M with [^{14}C]DMAPP gave products that comigrated with chrysanthemyl diphosphate (CPP). However, the enzyme also gave products characteristic of a C_{10} diphosphate when incubated with [^{14}C]IPP and DMAPP in the chain-elongation assay. TLC traces for the series of chimeras from C69F to C243F reflect a gradual transition from the C_{10} and C_{15} chain-elongation products typically synthesized by FPPase to the irregular products synthesized by CPPase. Between C69F and C178F, chain-elongation activity gradually shifted from predominant synthesis of C_{15} FPP to exclusive synthesis of C_{10} GPP. Activity in the [^{14}C]DMAPP assay was readily detected for the C219F chimera and became progressively greater for C243F and CPPase. The F219C protein was inactive in both assays. Activity for irregular products was low in all of the chimeras in the F → C series. Trace amounts of materials were seen in all of the TLC traces, with the F178C chimera giving somewhat more C_{10} product than the others.

Product analysis. The TLC radioassay did not resolve individual components within each group of C_5, C_{10}, and C_{15} diphosphates. Gas chromatographic (GC) and GC mass spectrometric analysis of alcohols obtained after hydrolysis of the diphosphates with alkaline phosphatase showed that C_{10} and C_{15} chain-elongation alcohols were the exclusive products from FPPase-M with selective, but not exclusive, formation of the *E* double-bond isomers when the enzyme was incubated with IPP and DMAPP (Fig. 4 and table S2) (*18*). A similar incubation with CPPase-M gave four C_{10} products: chrysanthemol (COH) (*15*) from cyclopropanation, lavandulol (LOH) (*17*) from branching, and geraniol and nerol (*19*) from chain elongation. In the series C69F → C243F, irregular monoterpenes were first detected as minor products with the C98F chimera, which gave LOH and an unexpected isomer, maconelliol (MOH) (*2*), from a cyclobutanation reaction. Synthesis of irregular monoterpenes became the dominant reaction for the C243F chimera, where LOH was favored over COH by a ratio of ~3:1. For CPPase-M, the major product was COH, which was only favored over LOH by a ratio of ~2:1. In the F69C → F178C series, irregular products (LOH and COH) were only detected for the F69C chimera (table S2). The proportion of chain-elongation products to irregular products increased as the concentration of IPP increased relative to that of the allylic substrate, reflecting competition between the isomeric substrates for binding.

Except for CPPase-M and the C243F and F69C chimeras, reactions proceeded slowly when DMAPP was the sole substrate. In addition to COH, LOH, and MOH, small amounts of planococcol (POH) (*9*), an isomer of MOH, were detected in extracts from incubations with the C98F and C147F chimeras, indicating that these two enzymes also synthesized planococcyl diphosphate (PPP) (Fig. 4 and table S3). Thus, most of the proteins that we studied synthesized irregular monoterpenes. The exceptions were FPPase-M and C178F, which only catalyzed chain elongation, and F219C, which was inactive in all of the assays. Several of the enzymes synthesized at least one compound generated by the cyclopropanation, branching, and cyclobutanation reactions in addition to chain-elongation products. The WT *A. tridentata* ssp. *spiciformis* CPPase was

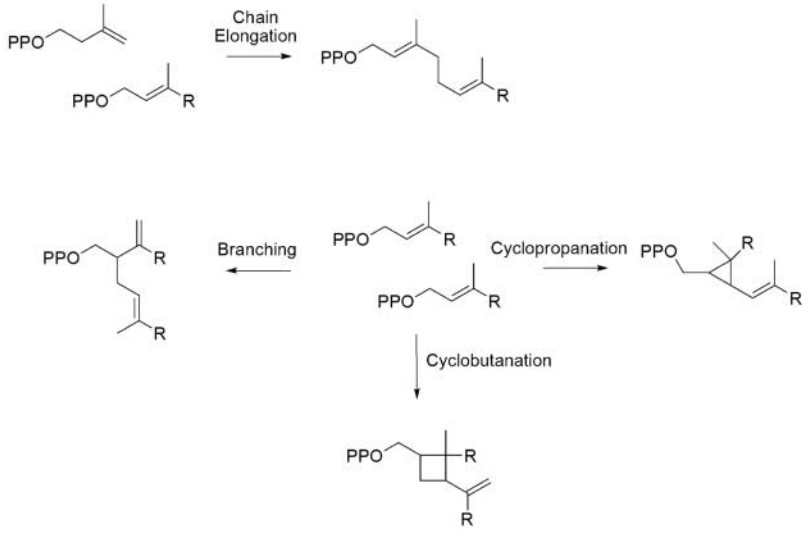

Fig. 2. Building reactions in the isoprenoid biosynthetic pathway. PPO, diphosphate; R, $CH_2(CH_2CH=C(CH_3)CH_2)_nH$, where n = 0, 1, 2, 3, and so forth.

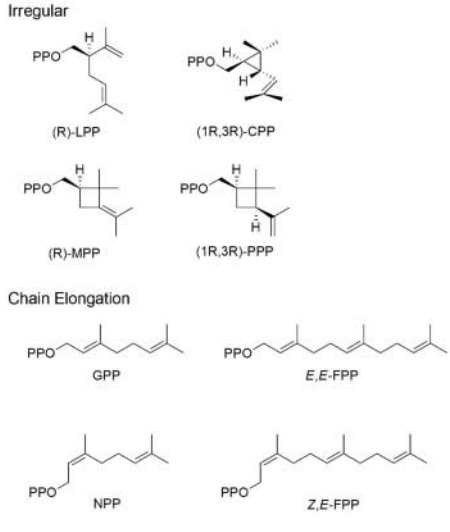

Fig. 3. Products from incubations with IPP and DMAPP. NPP, neryl diphosphate.

slightly more selective for CPP than CPPase-M. The WT CPPase from *C. cinerariaefolium* was slightly more selective for cyclopropanation relative to branching and chain elongation than the sagebrush enzymes.

Stereochemistry. The absolute stereochemistries of the irregular products from the enzyme catalyzed reactions were established by hydrolyzing the isoprenoid diphosphates to the corresponding alcohols and comparing their GC retention times by coinjection with authentic samples on a chiral column (figs. S3 and S4). In each case, a single enantiomer [(1*R*,3*R*)-COH (*19*), (*R*)-LOH (*20*), (*R*)-MOH (*2*), and (1*R*,3*R*)-POH (*9*)] was found. The absolute stereochemistries of the alcohols isolated from our enzyme-catalyzed reactions were identical to those of the corresponding alcohols isolated from natural sources.

Overview. The isoprenoid pathway uses only four different coupling reactions to build the carbon skeletons of its metabolites from the fundamental five-carbon building blocks IPP and DMAPP. Metabolites built by chain elongation and cyclopropanation are broadly distributed in nature, and the enzymes that catalyze the coupling reactions have been identified and characterized. Metabolites from branching and cyclobutanation are narrowly distributed, and the biosynthetic enzymes and the corresponding genes have not been identified.

CPPase and the FPPase/CPPase chimeras synthesize single enantiomers of CPP, LPP, maconelliyl diphosphate (MPP), and PPP. The absolute stereochemistries of the cyclopropane ring in CPP, the chiral center in LPP, and cyclobutane rings in MPP and PPP require that C1 of the dimethylallyl unit destined to alkylate the C2-C3 double bond of the other dimethylallyl unit is located on the *Re* face of the C2-C3 double bond. If one assumes that formation of PPP proceeds through a "least motion" reaction coordinate, the absolute configuration of the C3 cyclobutane carbon dictates that the enzymes bind the two molecules of DMAPP in an "endo" orientation (fig. S5).

Thus far, only a single set of enantiomers has been reported for the naturally occurring irregular isoprenoid compounds formed by branching, cyclopropanation, and cyclobutanation reactions. The absolute stereochemistries of the alcohols obtained by hydrolysis of CPP, LPP, MPP, and PPP in our studies are identical to those of COH, MOH, and POH isolated from natural sources. Likewise, the topologically related 1*R*,2*R*,3*R* enantiomers of presqualene diphosphate (*21*) and prephytoene diphosphate (*22*) are the only known naturally occurring stereoisomers of these two important metabolites. Work with recombinant squalene synthase indicates that the relative orientation of the two molecules of FPP, which are condensed to form presqualene diphosphate, is identical to the "endo" orientation predicted for DMAPP (*4*). The stereochemical similarities between the compounds synthesized by the chimeric proteins and those synthesized by their respective WT enzymes in nature are consistent with a scenario in which the naturally occurring enzymes evolved from a common ancestor that provided the original template for the orientation of the bound substrates. Genes for the cyclopropanation enzymes evolved twice, presumably from an *E*-selective chain-elongation template: once at the very beginning of cellular evolution for biosynthesis of squalene and phytoene, and subsequently much later for biosynthesis of CPP.

The rather conservative changes that we made in the catalytic site suggest that the elongation and irregular isoprenoid products are formed by a similar chemical mechanism with branches leading to the individual products. A comprehensive mechanism for the four coupling steps during biosynthesis of irregular isoprenoid compounds, based on what is known about the stereochemistries of chain elongation, cyclopropanation, branching, and cyclobutanation and the mechanism of chain-elongation reaction, is shown in Fig. 5. The chain-elongation reactions catalyzed by FPPase proceed by a

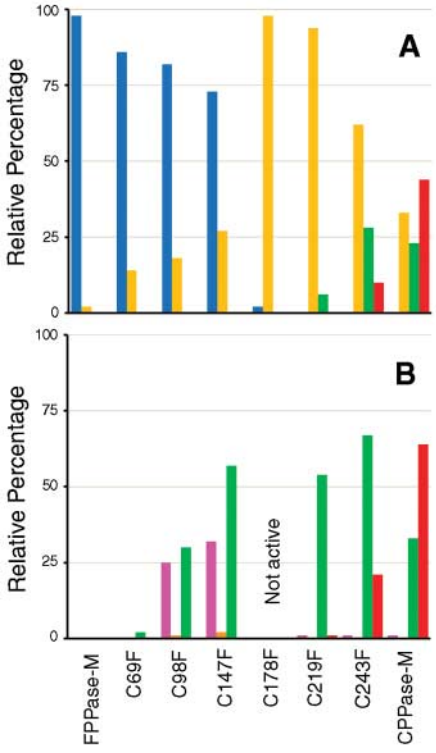

Fig. 4. Relative percentage of products from incubations with FPPase-M, CPPase-M, and the C → F series of chimeras. FPP, blue; GPP, gold; LPP, green; CPP, red; MPP, pink; PPP, orange. Diphosphate products were hydrolyzed by treatment with alkaline phosphatase, and the relative percentages of the corresponding alcohols were determined by GC. (**A**) Incubation for 2 hours at 30°C with IPP (200 μM) and DMAPP (200 μM). (**B**) Incubation for 12 hours at 30°C with DMAPP (3 mM).

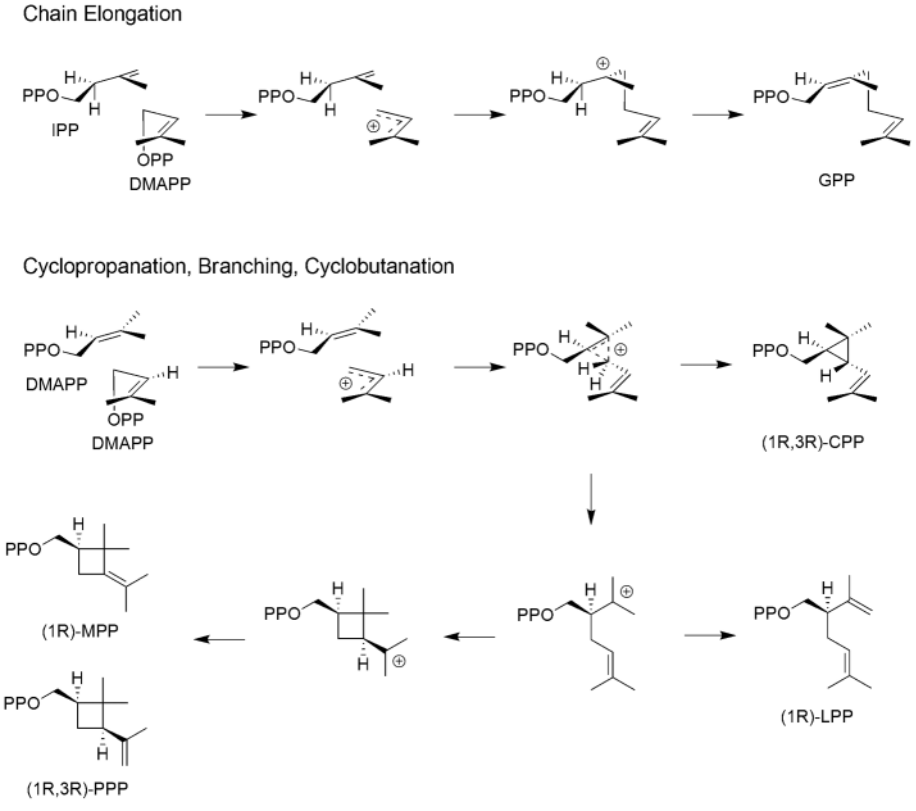

Fig. 5. A dissociative electrophilic alkylation mechanism for chain elongation, cyclopropanation, branching, and cyclobutanation.

dissociative electrophilic alkylation of the double bond in IPP by the allylic cations generated from DMAPP or GPP (*23*). By analogy, for biosynthesis of irregular monoterpenes, we suggest that a related dissociative electrophilic alkylation of the double bond in DMAPP by the dimethylallyl cation results in a protonated cyclopropane intermediate. This species can be deprotonated to give CPP or rearrange to a tertiary cation, which can in turn be deprotonated to give LPP. Alternatively, the tertiary cation can cyclize to give a cyclobutylcarbinyl cation that can then be deprotonated to give MPP or LPP. Formation of any specific product would be controlled by the ability of the enzyme to stabilize a specific intermediate along the reaction coordinate through dipolar and electrostatic interactions and to facilitate the selective removal of protons. The stereochemistries of the products result from the conformations of the two bound substrate molecules before the reaction. Only minor changes in the relative positions of the substrates are required to accommodate the formation of the different products.

This scenario provides an attractive mechanism for the evolution of the isoprenoid pathway through gene duplication and random mutagenesis of the duplicate genes to give new proteins, one of which is constrained to retain its original function, whereas the other is free to acquire a new activity. The isoprenoid fold first seen in the *E*-selective chain-elongation enzyme avian FPPase (*12*) has also been found in the cyclopropanation enzyme squalene synthase (*13*) (sterol biosynthesis) and several different terpenoid cyclases (*14*) along with aspartate-rich motifs involved in binding allylic diphosphate substrates, indicating that the enzymes evolved from a common ancestor. Phylogenetic correlations suggest that the cyclopropanation enzyme phytoene synthase (carotenoid biosynthesis) also has an isoprenoid fold. Our discovery that chimeric enzymes from FPPase and CPPase catalyze branching and cyclobutanation reactions suggests that WT enzymes with these activities also share this common ancestor.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/316/5821/73/DC1
Materials and Methods
Figs. S1 to S5
Tables S1 to S3
Electron Impact (EI) Mass Spectra
Chemical Ionization (CI) Mass Spectra
References

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Schemas and Memory Consolidation

Dorothy Tse,[1]* Rosamund F. Langston,[1]* Masaki Kakeyama,[2] Ingrid Bethus,[1] Patrick A. Spooner,[1] Emma R. Wood,[1] Menno P. Witter,[3] Richard G. M. Morris[1]†

Memory encoding occurs rapidly, but the consolidation of memory in the neocortex has long been held to be a more gradual process. We now report, however, that systems consolidation can occur extremely quickly if an associative "schema" into which new information is incorporated has previously been created. In experiments using a hippocampal-dependent paired-associate task for rats, the memory of flavor-place associations became persistent over time as a putative neocortical schema gradually developed. New traces, trained for only one trial, then became assimilated and rapidly hippocampal-independent. Schemas also played a causal role in the creation of lasting associative memory representations during one-trial learning. The concept of neocortical schemas may unite psychological accounts of knowledge structures with neurobiological theories of systems memory consolidation.

The concepts of "mental schema" and "mental models" as frameworks of knowledge are now well established (*1*, *2*), with implications for story recall, deductive inference, and education (*3*, *4*). For example, the memory of grammatically correct but semantically unusual prose passages is substantially better when subjects have an activated and relevant mental framework with which to understand them (*5*). An everyday experience for working scientists is remembering complex new information in an academic seminar. Our ability to do so depends as much on our possession of an appropriate mental schema as on the communicative skill of the speaker in logically conveying his or her message. In the absence of such mental frameworks, we are unable to follow scientific inferences in a talk and have the phenomenological experience of being functionally amnesic for its content a surprisingly short time later.

Curiously, this fundamental idea about memory has had little impact in neuroscience. Selective activation of a specific region within the posterior parietal cortex occurs in human subjects when, having been given relevant pictorial information earlier, they correctly interpret unusual textual information that would otherwise be incomprehensible (*6*). Animal studies have rarely considered the issue of what the animal itself brings in the way of knowledge to a learning situation, with the exception of studies of spatial and relational memory (*7*–*9*). This is partly because most experiments are conducted with "experimentally naïve" animals, and also because the creation of a mental schema is difficult to map precisely onto concrete neuroscience concepts such as anatomical connectivity or synaptic plasticity. The present experiments test the idea that the schema concept is directly relevant to the neural mechanisms of systems memory consolidation (*10*–*12*).

Experiments on schema learning. We trained rats to learn several flavor-place associations concurrently, using different flavors of food (flavor cues) and sand wells (place cues) located within a familiar testing environment called an "event arena" (*13*). The task was to learn which

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