

- (1988).
26. PGP 9.5 was isolated from frozen calf brain exactly as described for the human protein [J. F. Doran, P. Jackson, P. A. M. Kynoch, R. J. Thompson, *J. Neurochem.* **40**, 1542 (1983)] except that all buffers contained 10 mM  $\beta$ -mercaptoethanol. The yield of protein from this preparation was 18 mg per kilogram of brain and was identical to that of the published preparations of the human protein. The protein preparation was apparently homogeneous as judged by isoelectric focusing, SDS-PAGE, and PAGE under nondenaturing conditions.
  27. P. J. Duerksen-Hughes, M. M. Williamson, K. D. Wilkinson, *Biochemistry*, in press.
  28. K. D. Wilkinson *et al.*, unpublished data.
  29. G. M. Cole and P. S. Timiras, *Neurosci. Lett.* **79**, 207 (1987).
  30. G. Perry, R. Friedman, G. Shaw, V. Chau, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3033 (1987).
  31. P. G. Galloway, I. Grundke-Iqbal, K. Iqbal, G. Perry, *J. Neuropathol. Exp. Neurol.* **47**, 654 (1988).
  32. C. Bancher *et al.*, *ibid.* **48**, 81 (1989).
  33. P. G. Galloway, *Neurosci. Lett.* **91**, 148 (1988).
  34. S. Love, T. Saitoh, S. Quijada, G. M. Cole, R. D. Terry, *J. Neuropathol. Exp. Neurol.* **47**, 393 (1988).
  35. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
  36. M. J. Cox, R. Shapira, K. D. Wilkinson, *Anal. Biochem.* **154**, 345 (1986).

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**Table 1.** Accumulation of [ $^3\text{H}$ ]IP $_1$  in striate cortical synaptoneurosomes prepared from 5-week-old kittens (11). Results are means  $\pm$  SEM expressed as percentage of basal phosphoinositide turnover.

Agonist	Concentration ( $\mu\text{M}$ )	Percentage of basal turnover	n
Glutamate	10	108 $\pm$ 5	5
	100	168 $\pm$ 4	5
Ibotenate	10	372 $\pm$ 14	5
	100	435 $\pm$ 37	5
NMDA	100	110 $\pm$ 7	3
	300	110 $\pm$ 4	3
AMPA	300	102 $\pm$ 6	3
Carbachol	100	300 $\pm$ 34	5

## A Biochemical Correlate of the Critical Period for Synaptic Modification in the Visual Cortex

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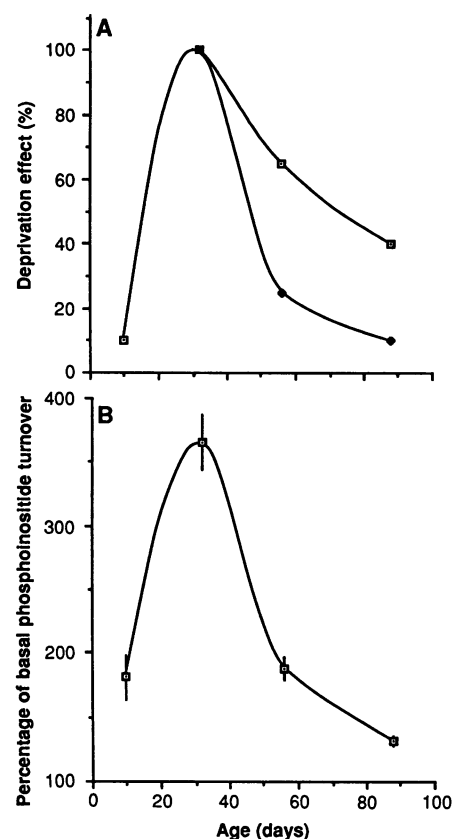
Stimulation of phosphoinositide hydrolysis by excitatory amino acids was studied in synaptoneurosomes of kitten striate cortex at several postnatal ages. Ibotenate and glutamate stimulated phosphoinositide turnover during the second and third postnatal months; *N*-methyl-D-aspartate and DL- $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) were without effect. The developmental profile of ibotenate-stimulated phosphoinositide turnover parallels the postnatal changes in cortical susceptibility to visual deprivation. The transient increase in ibotenate-stimulated phosphoinositide turnover does not occur in visual cortex of kittens reared in complete darkness.

**M**ANIPULATIONS OF THE VISUAL environment during early postnatal life can lead to profound and long-lasting changes in the functional organization of the visual cortex. For example, temporary closure of one eyelid in kittens renders striate cortex unresponsive to stimulation of the deprived eye (1, 2). Responsiveness to the deprived eye can be restored if it is again allowed to view a normal visual environment while the other eyelid is closed (3). This form of synaptic plasticity is limited to a finite period of postnatal development referred to as the "critical" or "sensitive" period (2–5). Estimates of the length of the critical period have varied, but there is general agreement that sensitivity to brief lid closure begins in kittens at about 3 weeks of age, peaks during the 5th week, and then gradually disappears between 12 and 16 weeks (2–5). We report now that this is precisely the period of postnatal development when the excitatory amino acid ibotenate stimulates phosphoinositide hydrolysis in striate cortex. Phosphoinositide turnover leads to the formation of two intracellular second messengers, inositol triphosphate (IP $_3$ ) and diacylglycerol (DAG) (6).

Thus, these data suggest that excitatory synaptic transmission during the critical period is characterized by unique patterns of second messenger activity and that phosphoinositide hydrolysis may play a central role in the experience-dependent modification of visual cortex.

There is evidence that synaptic excitation in the visual cortex depends on activation of excitatory amino acid (EAA) receptors (7). One class of EAA receptor is linked to a phosphoinositase that catalyzes the hydrolysis of phosphatidylinositol-4,5 bisphosphate to form DAG and IP $_3$  (8). Nicoletti *et al.* (9) have showed in rat hippocampus that this metabotropic EAA receptor is activated by glutamate, prefers the agonists ibotenate

and quisqualate, and is unresponsive to *N*-methyl-D-aspartate (NMDA) and kainate. This site is distinct from the traditional quisqualate receptor; for example, it is not stimulated by DL- $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and is not blocked by kynurenic acid. A similar site has been characterized in rat neocortex (10). In both rat hippocampus and neocortex the EAA-stimulated phosphoinositide hydrolysis is low at birth, peaks during early postnatal life, and then declines with increasing age. These observations have prompted speculation that this mechanism is involved in developmental plasticity (9, 10). This hypothesis would be strengthened considerably if it could be shown that



**Fig. 1.** (A) Sensitivity of binocular connections in striate cortex to eyelid suture at different postnatal ages, as estimated by Olson and Freeman by monocular deprivation ( $\square$ ) (2) and by Blakemore and van Sluysers with the "reverse suture" paradigm ( $\diamond$ ) (3). The deprivation effect is the percentage of neurons in area 17 with responses dominated by stimulation of the nondeprived eye [data from figure 1 of (5)]. (B) Phosphoinositide turnover stimulated by 10  $\mu\text{M}$  ibotenate in synaptoneurosomes prepared from kitten striate cortex at different postnatal ages. Data points represent the means  $\pm$  SEM of at least three experiments, expressed as percentage of basal phosphoinositide turnover.

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the developmental transience of EAA-stimulated phosphoinositide turnover correlates with the well-characterized critical period for synaptic modification in the kitten striate cortex. We used a synaptoneurosoma preparation of kitten area 17 to investigate this possibility (11).

We found that both L-glutamate and ibotenate potentially stimulate phosphoinositide turnover in striate cortex from 5-week-old kittens, while AMPA and NMDA (12) were ineffective at concentrations up to 300  $\mu$ M (Table 1). The postnatal changes in ibotenate-stimulated phosphoinositide turnover are correlated with the development of ocular dominance plasticity in kitten striate cortex (Fig. 1). Glutamate-stimulated phosphoinositide turnover had a similar developmental time course, but the correlation with critical period plasticity was not as precise as that for ibotenate (Table 2). However, this result possibly is complicated by postnatal changes in glutamate uptake (13) and, because glutamate is a mixed agonist, also by interactions between the EAA receptor subtypes (14). Nonetheless, the conclusion seems warranted that when the striate cortex is most sensitive to sensory deprivation, it is also most sensitive to stimulation of phosphoinositide hydrolysis by certain excitatory amino acids.

We also routinely measured the stimulation of phosphoinositide turnover by the muscarinic cholinergic agonist carbachol (Table 2). Unlike the excitatory amino acids ibotenate and glutamate, 100  $\mu$ M carbachol was effective at all ages, indicating that the transient increase in stimulated phosphoinositide turnover during the critical period is relatively specific to a mechanism linked to an ibotenate recognition site (15).

We next investigated the development of ibotenate-stimulated phosphoinositide turnover in the striate cortex of animals reared in complete darkness to see if this mechanism requires visual experience for its expression (16). We found that the transient rise in ibotenate-stimulated phosphoinositide turnover at 5 weeks of age did not occur in the striate cortex of dark-reared kittens (Fig. 2A). In contrast, stimulation by carbachol was unaffected by visual deprivation (Fig. 2B). These findings further support the idea that EAA-stimulated phosphoinositide turnover may play a specific role in developmental plasticity because dark rearing postpones the onset of the critical period (17).

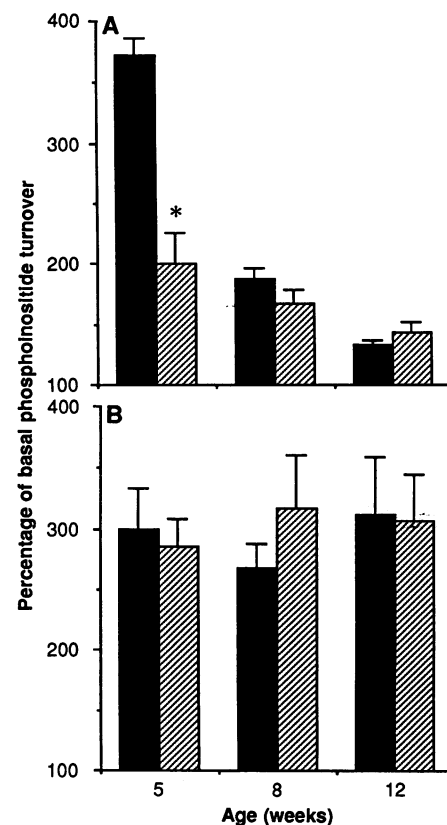
What role could EAA-stimulated phosphoinositide turnover play in the experience-dependent modification of visual cortex? One possibility is that products of phosphoinositide turnover with second messenger activity simply enable the process of synaptic plasticity to proceed during de-

**Table 2.** Accumulation of [ $^3$ H]IP<sub>1</sub> in striate cortical synaptoneurosomes prepared from kittens of different ages. Results are means  $\pm$  SEM of at least three experiments. Basal phosphoinositide turnover was as follows: 10 days, 467  $\pm$  29 counts per minute (cpm); 34 days, 463  $\pm$  45 cpm; 56 days, 300  $\pm$  21 cpm; 87 days, 224  $\pm$  6 cpm; and adulthood, 149  $\pm$  14 cpm. Agonist-stimulated phosphoinositide turnover is expressed as percentage of basal phosphoinositide turnover.

Agonist	Postnatal age				
	10 days	34 days	56 days	87 days	Adulthood
Glutamate					
1 $\times$ 10 <sup>-5</sup> M	114 $\pm$ 7	108 $\pm$ 5	101 $\pm$ 3	95 $\pm$ 6	101 $\pm$ 7
1 $\times$ 10 <sup>-4</sup> M	185 $\pm$ 12	168 $\pm$ 4	120 $\pm$ 7	122 $\pm$ 1	118 $\pm$ 20
1 $\times$ 10 <sup>-3</sup> M	193 $\pm$ 13	241 $\pm$ 9	168 $\pm$ 6	166 $\pm$ 6	133 $\pm$ 21
3 $\times$ 10 <sup>-3</sup> M	196 $\pm$ 13	259 $\pm$ 7	178 $\pm$ 11	177 $\pm$ 1	139 $\pm$ 15
Ibotenate					
1 $\times$ 10 <sup>-5</sup> M	181 $\pm$ 17	372 $\pm$ 14	188 $\pm$ 9	133 $\pm$ 4	103 $\pm$ 10
1 $\times$ 10 <sup>-4</sup> M	212 $\pm$ 17	435 $\pm$ 37	260 $\pm$ 16	175 $\pm$ 1	123 $\pm$ 12
Carbachol					
1 $\times$ 10 <sup>-4</sup> M	325 $\pm$ 4	300 $\pm$ 34	268 $\pm$ 20	312 $\pm$ 46	242 $\pm$ 32

velopment (18); the putative modulators of ocular dominance plasticity, acetylcholine and norepinephrine (19, 20), both potentially stimulate phosphoinositide turnover in the neocortex (21). An alternative hypothesis, inspired by theoretical considerations (22), involves the interaction of second messenger systems linked to NMDA and non-NMDA receptors. It has been proposed that the strengthening of some synapses in striate cortex during development depends on the postsynaptic Ca<sup>2+</sup> conductance mediated by cortical NMDA receptors (23, 24). Synaptic modifications of the type observed in striate cortex could be explained if the second messenger system linked to phosphoinositide metabolism specifically were to promote the weakening of synaptic relations during development (25). According to this idea, input activity that is coincident with strong postsynaptic depolarization, which is a favorable condition for postsynaptic Ca<sup>2+</sup> entry through NMDA receptor channels, would lead to an enhancement of synaptic strength. Input activation coincident with postsynaptic inactivity would stimulate phosphoinositide turnover by means of the non-NMDA receptor without a concomitant postsynaptic Ca<sup>2+</sup> flux, and this would lead to a decrease in synaptic efficacy. Although this hypothesis was formulated largely on theoretical grounds (25), recent work by Palmer *et al.* (14) supports the idea that the second messenger systems linked to NMDA and non-NMDA receptors are antagonistic. They find in the neonatal hippocampus that NMDA inhibits EAA-stimulated phosphoinositide turnover in a Ca<sup>2+</sup>-dependent fashion.

Tests of these ideas will require the development of selective and potent antagonists of this metabotropic glutamate receptor. Nonetheless, whatever the role of EAA-stimulated phosphoinositide turnover proves to be, our data suggest that the nature of excitatory synaptic transmission in



**Fig. 2.** Phosphoinositide turnover in striate cortical synaptoneurosomes prepared from normal (solid bar) and dark-reared (hatched bar) kittens at various ages when (A) stimulated by 10  $\mu$ M ibotenate and (B) stimulated by 100  $\mu$ M carbachol. Values are means  $\pm$  SEM of at least three experiments. \*Difference between normal and dark-reared is statistically significant (*t* test, *P* < 0.002).

striate cortex is unique during the critical period for experience-dependent modifications.

#### REFERENCES AND NOTES

1. T. N. Wiesel and D. H. Hubel, *J. Neurophysiol.* **28**, 1029 (1965).
2. C. R. Olson and R. D. Freeman, *Exp. Brain Res.* **39**, 17 (1980).

3. C. Blakemore and R. C. van Sluyters, *Br. J. Ophthalmol.* **58**, 176 (1974).
4. D. H. Hubel and T. N. Wiesel, *J. Physiol. (London)* **206**, 419 (1970).
5. B. Timney, in *Imprinting and Cortical Plasticity*, J. P. Rauschecker and P. Marler, Eds. (Wiley, New York, 1987), pp. 321–345.
6. M. J. Berridge, *Biochem. J.* **220**, 345 (1984).
7. T. Tsumoto, H. Masui, H. Sato, *J. Neurophysiol.* **55**, 469 (1986).
8. H. Sugiyama, I. Ito, C. Hirono, *Nature* **325**, 531 (1987); F. Sladeczek, M. Récasens, J. Bockart, *Trends Neurosci.* **11**, 545 (1988).
9. F. Nicoletti, M. J. Iadarola, J. T. Wroblewski, E. Costa, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1931 (1986).
10. S. M. Dudek, W. D. Bowen, M. F. Bear, *Dev. Brain Res.* **47**, 123 (1989).
11. Synaptoneurosomes were prepared by the method of Gusovsky and Daly (26) as described for rat neocortex (10). Briefly, kittens of various ages were anesthetized with a small intramuscular dose of a ketamine-xylazine mixture (20 and 2.5 mg/kg, respectively) followed by an intraperitoneal injection of sodium pentobarbital (25 mg/kg) and decapitated. The brains were rapidly removed and cortical area 17 was dissected free. The cortex was weighed and homogenized in 10 volumes of oxygenated (95% O<sub>2</sub>:5% CO<sub>2</sub>) Krebs-Henseleit buffer, pH 7.4 (119 mM NaCl, 4.7 mM KCl, 1.18 mM MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 1.18 mM KH<sub>2</sub>PO<sub>4</sub>, 24.9 mM NaHCO<sub>3</sub>, and 10.0 mM glucose), in a glass-glass homogenizer with four strokes by hand. The homogenate was centrifuged at 1000g for 10 min, and the supernatant was discarded. The pellet was resuspended in 20 volumes of Krebs-Henseleit buffer plus 50 mM Hepes (pH 7.4) and mixed with 1  $\mu$ M [<sup>3</sup>H]inositol (15 Ci/mmol). Aliquots of 320  $\mu$ l were then incubated at 37°C for 60 min to label the inositol lipid pool. Twenty microliters of 200 mM lithium chloride was added 10 min before the addition of agonists for a final incubation volume of 400  $\mu$ l. The tubes were gassed with a ratio of 95% O<sub>2</sub> to 5% CO<sub>2</sub> and capped before each incubation. [<sup>3</sup>H]Inositol was present throughout the 90-min incubation (27). The suspension was then centrifuged and the pellet washed once with fresh buffer and then resuspended in 6% trichloroacetic acid. After centrifugation, the supernatant was collected and mixed with Bio-Rad anion exchange resin AG 1-X8 (1 ml of a 50% slurry in water). The columns were washed four times with 1 ml of water and two times with 1 ml of a mixture containing 200 mM ammonium formate and 100 mM formic acid to elute [<sup>3</sup>H]inositol-1-phosphate (IP<sub>1</sub>) into scintillation vials. All results are from at least three experiments performed in duplicate and expressed as percentage of basal activity. Basal activity is counts per minute of samples from the same run incubated without any transmitter ligands. A condition containing 100  $\mu$ M carbachol was run in every experiment to assess the integrity of the assay system; comparisons between ligands were always done on samples from the same preparations. The synaptoneurosomes were unfiltered as previous reports have shown that this step makes no difference in the results of this assay (26). Also, because IP<sub>3</sub> is rapidly hydrolyzed to IP<sub>1</sub> in this system, accumulation of IP<sub>1</sub> is an accurate measure of overall phosphoinositide turnover (26).
12. NMDA also failed to stimulate PI turnover in synaptoneurosomes prepared from visual cortex of one kitten in which ketamine was omitted from the anesthesia. This finding is in agreement with previous work with rat neocortex (10) in which no anesthesia was used before decapitation.
13. T. R. Riggs, K. G. Pote, H.-S. Im, D. W. Huff, *J. Neurochem.* **42**, 1251 (1984).
14. E. Palmer, D. T. Monaghan, C. W. Cotman, *Mol. Brain Res.* **4**, 161 (1988).
15. That carbachol potentially stimulated phosphoinositide turnover regardless of age also indicates that the developmental changes in ibotenate-stimulated phosphoinositide turnover are not explained by changes in the initial incorporation of [<sup>3</sup>H]inositol into phospholipid. Lipid phase measurements were not performed routinely because preliminary studies confirmed that [<sup>3</sup>H]inositol was incorporated into phospholipid at all ages and that availability of labeled phospholipid did not limit [<sup>3</sup>H]IP<sub>1</sub> accumulation in the assay (27). Nonetheless, [<sup>3</sup>H]inositol incorporation was lower in tissue prepared from visual cortex of older animals, and this is reflected by a lower number of counts per minute under basal conditions (Table 2).
16. Kittens and mother were transferred within a week of birth to a standard breeding cage in a dark room.
17. It is possible that a transient rise in ibotenate-stimulated phosphoinositide turnover occurs before 5 weeks of age. This would be surprising, however, as dark rearing is thought to delay or slow visual cortical development. M. Cynader and D. E. Mitchell, *J. Neurophysiol.* **43**, 1026 (1980); G. D. Mower, C. J. Caplan, W. G. Christen, F. H. Duffy, *J. Comp. Neurol.* **235**, 448 (1985).
18. Because one of the products of phosphoinositide turnover, diacylglycerol, activates protein kinase C, it is interesting that soluble protein kinase C activity is also elevated in striate cortex during the critical period (F.-S. Sheu, T. J. Kasamatsu, A. Routtenberg, *Brain Res.*, in press).
19. T. Kasamatsu and J. D. Pettigrew, *J. Comp. Neurol.* **185**, 139 (1979).
20. M. F. Bear and W. Singer, *Nature* **320**, 172 (1986).
21. R. A. Gonzales and F. T. Crews, *J. Neurochem.* **45**, 1076 (1985).
22. E. L. Bienenstock, L. N. Cooper, P. W. Munro, *J. Neurosci.* **2**, 32 (1982).
23. M. F. Bear, L. N. Cooper, F. F. Ebner, *Science* **237**, 42 (1987).
24. A. Kleinschmidt, M. F. Bear, W. Singer, *ibid.* **238**, 355 (1987).
25. M. F. Bear, in *Recent Advances in Excitatory Amino Acid Research*, E. S. Calvalheiro, J. Lehman, L. Turski, Eds. (Liss, New York, 1988), pp. 393–401.
26. F. Gusovsky and J. W. Daly, *Neuropharmacology* **27**, 95 (1988).
27. In pilot experiments, the time course of IP<sub>1</sub> accumulation stimulated by 10  $\mu$ M ibotenate was studied in synaptoneurosomes prepared from the striate cortex of adult and 4-week-old cats. At both ages, and in agreement with F. Gusovsky, E. B. Hollingsworth, and J. W. Daly [*Proc. Natl. Acad. Sci. U.S.A.* **83**, 3003 (1986)], IP<sub>1</sub> accumulation did not plateau until after 90 min (our usual incubation time). The differences between ibotenate-stimulated phosphoinositide turnover in immature and adult cortex were observed at all incubation times, including those as short as 10 min.
28. We thank W. Bowen for help with the synaptoneurosomes assay, A. Böhner for technical assistance, and B. W. Connors for critically reading the manuscript. Supported by grants from the Office of Naval Research, the National Eye Institute, and the Alfred P. Sloan Foundation.

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## Angiosperm Diversification and Paleolatitudinal Gradients in Cretaceous Floristic Diversity

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The latitudinally diachronous appearance of angiosperm pollen during the Cretaceous is well documented, but the subsequent diversification and accompanying significant changes in floristic dominance have not been assessed quantitatively for a wide range of paleolatitudes. Trend surfaces fitted to within-palynoflora diversity data from 1125 pollen and spore assemblages show that angiosperms first become floristically prominent in low paleolatitude areas (~20°N to 20°S). Non-magnoliid dicotyledons show a similar but slightly delayed pattern of increase and are the principal component of angiosperm diversity from all areas sampled. Monocotyledons and magnoliid dicotyledons are significant primarily in low to middle paleolatitude palynofloras (~50°N to 20°S) during the latest Cretaceous. As angiosperms become increasingly prevalent the importance of most non-angiosperm taxa either decreases or remains unchanged. The only apparent exception is a striking increase in gnetalean diversity concurrent with the initial angiosperm diversification at low paleolatitudes.

ALTHOUGH THE ORIGIN OF ANGIOSPERMS persists as one of the most widely debated issues in evolutionary botany (1–4), the paleobotanical record demonstrates unequivocally that the major early diversification of the group occurred during the mid-Cretaceous (4–10). Through this interval many of the characteristic reproductive features of extant flowering plants appear in the fossil record for the first time (11), and fossil angiosperm leaves and pollen exhibit marked, coincident, patterns of increasing diversity (6–10). By the Cenomanian (earliest Late Cretaceous), at least

four of the eleven extant angiosperm subclasses (Magnoliidae, Hamamelidae, Rosidae, and at least one subclass of Liliopsida), as well as several distinct clades within these groups, had already differentiated (9). Increased abundance of angiosperm fossils parallels this rise in diversity, and together these patterns have been used to infer ecological expansion (6, 7), perhaps as a function of biological attributes of the angiosperm clade (3, 12, 13), and possibly with direct effects on other plant groups (13). Although detailed analyses of local stratigraphic sections provide partial resolution of such ecological effects (7), extrapolation to the level of angiosperms as a whole requires a more inclusive geographic and

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