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20. Antiserum was prepared as follows. A Hampshire pig of the genotype $Lpb^{3/4}$ was immunized by eight injections with LDL and seven injections with apoB100 from Lpb^{5} Chester white pigs. The 15 injections were given at 2- to 3-week intervals (*6, 8*). Antiserum to Lpb3 was produced by injecting an $Lpb^{1/5}$ pig with $Lpb^{3/3}$ LDL (six injections at 2- to 3-week intervals). Intact LDL from an $Lpb^{1/3}$ and from an $Lpb^{5/5}$ pig was subjected to discontinuous gel electrophoresis [U. Laemmli, *Nature (London)* **227**, 680 (1970)] with a 3 percent stacking gel and a 3.6 percent resolving gel. The proteins were transferred (10 A-hour) to nitrocellulose paper by a modification of the method of H. Towbin *et al.* [*Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979)]. The paper was first incubated with pig antiserum to Lpb5 or antiserum to Lpb3, and then with antiserum to pig immunoglobulin G conjugated to horseradish peroxidase (Cappel). Protein bands were visualized with 4-chloro-1-naphthol (Bio-Rad). An identical set of lanes was stained with Coomassie R250.
21. LDL from a normal pig was iodinated [M. A. K. Markwell and C. F. Fox, *Biochemistry* **17**, 4807 (1978)] and dialyzed exhaustively against phosphate-buffered saline containing EDTA. Degradation of LDL by cultured pig skin fibroblasts was determined by the method of J. L. Goldstein *et al.* [*Methods Enzymol.* **98**, 241 (1983)] modified as follows: Cells were grown in F-10 media, pig lipoprotein-deficient plasma was used, and silver nitrate was used to precipitate free iodide.
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Lipid Domains in Fluid Membranes: A Quick-Freeze Differential Scanning Calorimetry Study

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The application of rapid-freezing techniques to differential scanning calorimetry (DSC) provides a new approach for understanding the organization of lipids in biomembranes. Use of quick-freeze DSC on membranes of mixed lipid composition supports the existence of nonrandom distributions of lipids (domains) in fluid bilayers. In addition to allowing investigations on the organization of lipids in fluid bilayers, the quick-freeze technique now allows calorimetric studies to be carried out on mammalian membranes which, because of their high cholesterol content, have not been previously amenable to study by DSC.

ONE OF THE MOST IMPORTANT problems in membrane structure is the possible existence of lipid domains—that is, regions of bilayer that differ from one another in lipid composition (*1, 2*). Such domains might have different cholesterol content, fatty acid composition, lipid class, and so forth. Since the activities of intrinsic membrane enzymes are dependent on the types of lipids surrounding them (*3*), the activity of a membrane enzyme would vary depending on which domains they occupy in the bilayer.

Under physiological conditions, biomembranes are in a fluid state. Membrane domains would thus not be expected to be static structures but to be time-averaged. While membrane regions of nonrandom lipid distribution might be brought about by the interactions of lipids with membrane proteins, domains are postulated to be a consequence of the intrinsic mixing properties of lipids. Lipid domains have been shown to exist in crystalline membranes (*4*). Some indication of fluid-fluid immiscibility in at least one artificial lipid mixture has been indicated (*5*). Because of the lack of suitable methodology, it has not been possible until now to establish, let alone to study in depth, the existence of lipid domains in the physiologically relevant fluid bilayer state. Findings establishing the existence of domains in fluid bilayers were obtained with a novel approach that allows investigations of lipid-lipid associations in fluid bilayers by the use of differential scanning calorimetry.

Many of the methods that have most profitably been used to study lipid-lipid interactions use strategies in which the conversion of a bilayer from a crystalline to a fluid state is monitored as a function of temperature. For example, differential scanning calorimetry follows heat absorption as a function of temperature, and many spectroscopic approaches follow alterations in the signals of probe molecules either embedded in or partitioning into the bilayer during changes in temperature. Much valuable knowledge on the interaction of bilayer lipids with one another and with membrane proteins has been obtained in this fashion. This approach has its limitations, however, since information on molecular associations in the fluid state is deduced from observations on the melting of the crystalline state. For example, materials that do not cocrystallize in a crystalline state can be homogeneously mixed in a fluid state. An example of this is seen in the distribution of intramembranous particles as visualized by freeze-fracture electron microscopy (*4*).

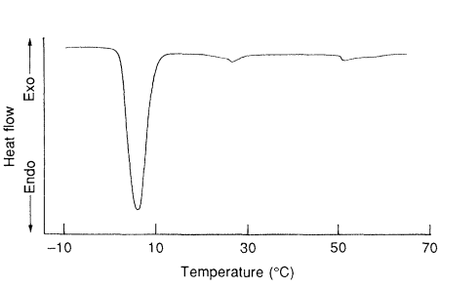

Fig. 1. Thermogram of a quick-frozen sample of homogeneous lipid bilayers in excess water formed from a 1:1 molar mixture of DMPC and DSPC. The sample was quick-frozen from 85°C, loaded as described (*11*), brought up to −30°C in the differential scanning calorimeter, and scanned. The large endotherm is the melting of water. The endotherms resulting from the melting of the lipid bilayers are seen at this sensitivity as slight high-temperature undulations. These endotherms are seen at higher sensitivity in Fig. 2C.

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The success of a quick-quench approach in electron microscopic investigations of biomembrane structure (*4, 6*) suggested that quick-quench methodology might be adapted to techniques such as differential scanning calorimetry. Since quick-freezing techniques preserve bilayer structure (*7*), a useful application of this approach must meet two requirements: (i) a sample must be frozen fast relative to the lateral motion of membrane lipids; and (ii) the lateral motion of lipids in the crystalline state must be slow enough to allow measurement before the system relaxes back to an equilibrium state. If these conditions are satisfied, structural information on the associations of lipids with one another and with proteins in the fluid bilayer might be obtained in a manner analogous to that used in studies of bilayer transitions with conventional differential scanning calorimetry. In the elucidation of the structure of the low-density lipoprotein particle (*8*), for example, the thermal transformations of the cholesterol esters contained in these particles were found to be slow compared to the cooling rates obtainable in a conventional differential scanning calorimeter. This permitted nonequilibrium states of the cholesterol esters to be used in determining the organization of these molecules in the native particle.

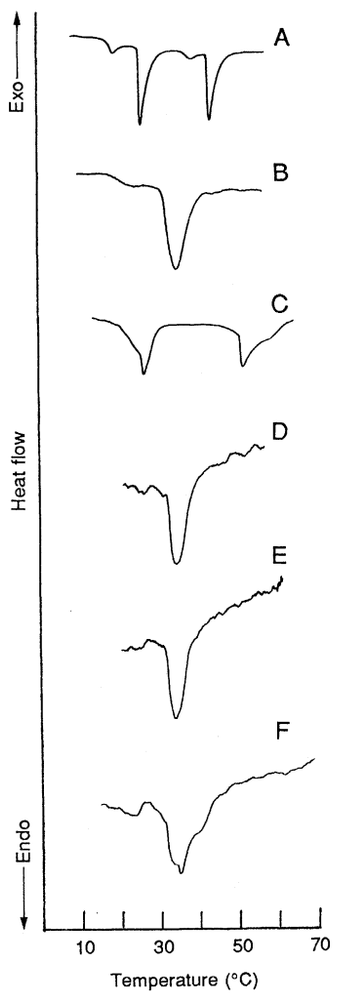

Fig. 2. Thermograms of bilayers in excess water formed from DMPC and DSPC and cooled from 85°C. (A) Conventional scan of a mixture of DMPC bilayers and DSPC bilayers. (B) Conventional scan of homogeneous bilayers containing a 1:1 molar ratio of DMPC and DSPC. (C) Scan of a quick-frozen sample of the preparation used in (B) and scanned immediately after quick-freezing. (D) Conventional rescan of preparation used in (C). (E) Second conventional rescan of preparation used in (C). (F) Scan of a portion of the quick-frozen preparation prepared for (B) but allowed to age for 3 hours under liquid nitrogen before being scanned in the calorimeter.

One of the major difficulties found in adapting very rapid freezing methodologies to differential scanning calorimetry was in developing a means to homogeneously quick-quench samples large (approximately 0.1 mg) relative to that required for freeze-fracture electron microscopy (*9*). Another difficulty was in maintaining the sample at low temperature while loading it into the calorimeter sample pan, hermetically sealing the pan, loading the pan into the calorimeter, and then starting the scanning procedure. In an earlier study, various techniques to accomplish certain aspects of this problem (*10*) were developed. In the work now described, a new approach was employed (*11*).

Figure 1 shows a thermogram of an aqueous dispersion of homogeneous lipid bilayers containing a 1:1 molar ratio of dimyristoylphosphatidylcholine (DMPC) and distearoylphosphatidylcholine (DSPC) quick-frozen from 85°C. The sample was quick-frozen by means of a specially designed apparatus (*11*); the temperature of the sample was increased to −30°C in the calorimeter; and the sample was scanned. The large endotherm occurs at the melting of water. At this sensitivity, the thermal events associated with the melting of the lipid bilayers are seen as undulations in the baseline. This scan is typical of all the data presented here. The thermograms presented in the succeeding figures are the lipid events displayed at higher sensitivity—that is, Fig. 2C is a blow-up of the higher temperature region of this figure. Water when examined alone presents a featureless baseline above the ice melt. Since bilayers were quick-frozen from a fluid state, the possibility that this procedure could put bilayer lipids into a glassy state was considered. Many different types of lipid bilayers of varying lipid composition (with and without cholesterol) as well as native membrane preparations were quick-frozen and scanned up in temperature from −173°C. In no case was there any calorimetric indication that a glass had been formed. For this reason this report deals with the melting of lipids in nonequilibrium and equilibrium crystalline states.

Figure 2A shows a thermogram of a mixture of two types of fully hydrated bilayers in excess water. One type of bilayer was formed from DMPC and the other from DSPC. This mixture was equilibrated to 0°C in the calorimeter in a conventional manner and then scanned. What is seen are two large endotherms resulting from the independent melting of each of the two bilayers. The small endotherms preceding each of the main peaks are those of the premelts associated with transformations occurring in the lattices of each of the two bilayers (*12*). Lowering the temperature of the sample to −173°C in the calorimeter and then scanning resulted in an identical thermogram. Repeated scans of this sample from 0°C again gave identical endotherms. In contrast, Fig. 2B is the thermogram of a single type of bilayer containing a 1:1 molar ratio of both the phospholipids used in the previous experiment. Its thermal behavior is in agreement with that reported in earlier calorimetric studies (*13*). The large endotherm results from the melting of the two co-crystallized phospholipids. As in Fig. 2A, repeated scans of this preparation cooled in a conventional manner from 85°C to either −173° or 0°C gave identical thermograms.

Figure 2C shows the thermogram of a preparation of the homogeneous bilayers used in Fig. 2B quick-quenched from 85°C. Two separate peaks appear at approximately the melting temperatures seen for the pure lipid bilayers (Fig. 2A). If this preparation is left in the calorimeter, cooled in a conventional manner from 85°C, and then scanned, the thermogram obtained (Fig. 2D) is the same as that for a conventionally manipulated sample (Fig. 2B). A further rescan of the sample cooled to −173°C again gives the same endotherm (Fig. 2E). These experiments suggest that in the fluid bilayer there is fluid-fluid immiscibility between the two phospholipids. Presumably during the very rapid quick-freezing process, the bilayer crystallizes so rapidly that the lateral mobility of the lipids is too slow to allow co-crystallization into the equilibrium state. Upon scanning, the two endotherms that appear correspond to the melting of separate domains of relatively homogeneous lipid species. Successive conventional rescans of the same sample performed without removing the sample from the calorimeter showed conventional behavior.

That the dual peaks seen in Fig. 2C are the result of very rapid freezing and not of exposure to liquid nitrogen temperatures is confirmed by the experiment shown in Fig. 2E, in which the sample was brought down

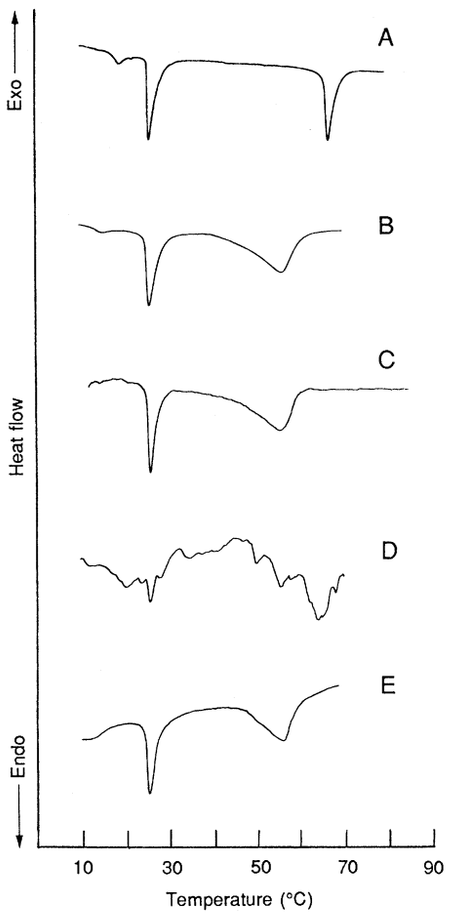

Fig. 3. Thermograms of bilayers in excess water formed from DMPC and DAPC and cooled from 85°C. (A) Conventional scan of a mixture of DMPC bilayers and DAPC bilayers. (B) Conventional scan of homogeneous bilayers containing a 1:1 ratio of DMPC and DAPC. (C) Fast-freeze preparation of these bilayers run immediately after fast-freezing. (D) Quick-freeze preparation of these bilayers scanned immediately after quick-freezing. (E) Conventional rescan of preparation used in (D).

to liquid nitrogen temperature from 85°C in a conventional manner. Further, a fast-freezing procedure in which the encapsulated sample used in Fig. 2B was equilibrated to 85°C, then rapidly immersed in liquid nitrogen, loaded at low temperature, and scanned resulted in a thermogram identical to that obtained in Fig. 2B. Since this manner of freezing is very rapid compared to that obtainable in a calorimeter, it appears that only extremely rapid freezing procedures are sufficient to capture bilayer lipid organizational heterogeneity. Figure 2F shows another aspect of kinetic factors associated with quick-frozen crystalline lipid bilayers. Both Fig. 2C and Fig. 2F are initial scans of portions of the same quick-frozen sample. The difference is that after the sample was initially quick-frozen, it was divided into several portions and loaded into several calorimeter pans. The thermogram shown in Fig. 2C is that of material scanned immediately, whereas the other portions were stored under liquid nitrogen. The thermogram shown in Fig. 2F is that of one of these reserved portions scanned 3 hours after it was quick-frozen. It is a degenerate form of Fig. 2C. Samples held under liquid nitrogen for still longer time periods gave thermograms identical to those shown in Fig. 2, B, D, and E. It appears then, that even in the crystalline state at liquid nitrogen temperatures the lateral mobility of bilayer lipids may be sufficient to allow an equilibrium distribution to be reached in a matter of hours after quick-freezing. Diffusion coefficients for lipids in crystalline bilayers with defects have been measured to be around 10^{-11} cm^2/sec at 0° to 20°C (*14*). Even if, at liquid nitrogen temperatures, diffusion coefficients were several orders of magnitude lower, a randomization of lipids to an equilibrium distribution would be expected to occur in a few hours.

Calorimetry on other quick-frozen mixed lipid preparations also gives thermograms different from those obtained by conventional means. Figure 3 shows thermograms of bilayers containing DMPC and diarachidoylphosphatidylcholine (DAPC). In each of these experiments, the samples were cooled from 85°C before scanning. Figure 3A is a conventional scan of a mixture of two types of bilayers composed of either DMPC or DAPC. These two phospholipids are sufficiently different in melting temperature that when present in the same bilayer they do not cocrystallize in an ideal manner. Thus, as seen in the scan of a conventionally cooled preparation (Fig. 3B), there are two major endotherms. The lower temperature endotherm corresponds to the melting of crystalline domains composed primarily of DMPC. The higher temperature endotherm corresponds to the melting of crystalline domains consisting primarily of DAPC. Figure 3C is a thermogram of a sample of the above preparation fast-quenched from 85°C. It is identical to the conventionally cooled preparation. When, however, a sample is quick-quenched and scanned in the calorimeter, the result is as shown in Fig. 3D. Here a complex thermogram is seen composed of multiple peaks. Upon rescanning in the normal manner, a conventional thermogram is obtained (Fig. 3E).

Several other lipid mixtures have been examined by this procedure. In all cases the thermograms obtained were reproducible but differed from conventional scans. Preliminary quick-freeze measurements have been made on cholesterol-rich synthetic bilayers and the cholesterol-rich human red blood cell membrane. When either of these are scanned in a conventional manner, the lipid transition is suppressed by the presence of bilayer cholesterol (*15–17*). With the quick-quench procedure, distinct but variable endotherms characterized by high heats of melting were obtained. The ability to observe endotherms in cholesterol-rich bilayers provides a means of understanding bilayer organization in biological membranes that previously were not amenable to investigation. The variability in the quick-freeze scans of cholesterol-rich bilayers may be due to sample handling or to insufficiently rapid freezing. Improvements in methodology should resolve this problem.

The development of quick-freeze differential scanning calorimetry allows the investigation of bilayer lipid organization in the physiologically relevant fluid state. The ability to obtain patterns for cholesterol-rich membranes will facilitate investigations of the organization of biological membranes with a high cholesterol content.

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11. The sample is placed on a thermoregulated surface mounted on an inverted pneumatically driven piston. After thermal equilibration, the piston is triggered. The piston rapidly moves down a guide until it hits a stop. At this point, the sample flies off the sample surface onto the bottom portion of an aluminum calorimeter pan nested in a well in a brass holder. This pan holder rests in a brass block maintained at −173°C. After this quick-freezing procedure, the pan holder containing the bottom portion of the calorimeter pan with sample is withdrawn from the quenching block and inserted into another brass block also maintained at liquid nitrogen temperature. A precooled pan cover is inserted onto the pan and the unit is hermetically sealed by a precooled dye. The major difficulty in this operation is the successful sealing of the calorimeter pan at liquid nitrogen temperatures. This step is necessary since the heat of evaporation of water is sufficiently great that it obscures low-energy thermotropic lipid events. Once sealed, the pans are placed in a liquid nitrogen bath and transferred to a differential scanning calorimeter (Dupont 1090) equilibrated to −173°C. At no time from quenching until loading into the calorimeter does the temperature of the sample rise above −173°C. After the sample is loaded into the calorimeter, the temperature of the sample is increased to the desired starting level and the scanning is begun. Another procedure used in this study, called fast-freezing, merely entails taking a sample sealed in a calorimeter pan preequilibrated to a given temperature and quickly placing it under liquid nitrogen. This fast-frozen sample is loaded into the calorimeter in the manner described previously. While this procedure allows much faster quenching of a sample than is possible in the calorimeter itself, it is not nearly as rapid as the quick-freeze procedure. Samples were scanned in the

calorimeter at a rate of 5 Celsius degrees per minute. Typical sample sizes were about 8 ml of an aqueous 10% lipid dispersion. All lipids were suspended in distilled water. Silicon oxide was used as a reference.
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Early Cretaceous Angiosperm Leaves from Southern South America

EDGARDO J. ROMERO AND SERGIO ARCHANGELSKY

Early angiosperm leaves from the Aptian (113 to 119 million years ago) Baqueró Formation of Patagonia have been found in a fossil flora dominated by more than 100 species of gymnosperms and pteridophytes. They may be the first early Cretaceous angiosperm leaves to be reported from southern South America and one of the few reported in the Southern Hemisphere. The leaves are large, lobate, craspedodromous, and dentate (A-1 teeth) and have ramified tertiary veins and random fourth-order venation. Several of these features have been found in coeval and younger strata elsewhere, but not in the same combination. They were probably a marginal component of the flora.

RECENT YEARS HAVE WITNESSED the discovery or reevaluation of Cretaceous fossil fruits, flowers, cuticles, leaf imprints, and pollen grains that have provided insights into the early evolution of angiosperms. Critical reevaluation has resulted in rejection of most reports of pre-Cretaceous angiosperms (*1*), establishing the oldest unequivocal record of the group in the Barremian (*1–4*).

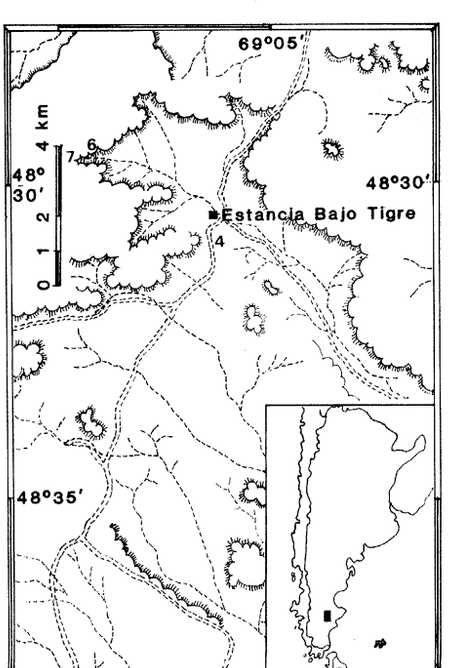

Fig. 1. Map of the fossil locality.

Descriptions of several Albian and Cenomanian flowers (*5*) support the idea that the main lines of evolution within the angiosperms were being differentiated at that time. Early angiosperm leaves are rare. Barremian or Aptian remains have been described only from the Soviet Union and eastern North America (*6–11*).

Characteristic Mesozoic floras occur in several formations in Patagonia. Among them, the floras preserved in the Baqueró Formation (*12*), reported as Barremian to Aptian in age, have been intensively studied. Younger floras, dominated by angiosperms, are those from Mata Amarilla and Cerro Dorotea formations, probably of Coniacian to Maastrichtian age (*13*). We now describe the first angiosperm leaf imprints in the Baqueró flora—the first description of early Cretaceous angiosperm leaves from South America and one of the few in the Southern Hemisphere (*14*).

The Baqueró Formation crops out in an extensive area in Santa Cruz Province, Argentina (*12*) (Fig. 1). It consists mainly of tuffaceous, fluvial, and minor lacustrine deposits, together with paleosols. It is divided into two members, with the lower containing a rich assortment of fossil compressions. In the Estancia Bajo Tigre area the formation is 140 m thick and contains kaolin and laminated silts with plant remains at the base. Plant fossils are usually concentrated in lenses. Angiosperm leaves were found first by one of us (S.A.) at site 7 (*12*), but were not reported. More and better preserved angiosperm leaves were collected in 1983. At site 7 (*12*) two levels (named after the most abundant genus) have been recognized: (i) *Brachyphyllum*, with only fragments of angiosperm leaves, and (ii) *Ptilophyllum*, where the best specimens of angiosperms occur. At this level, angiosperms are found in poorly laminated brown tuffs 15 to 20 cm thick containing limonite veins; these poorly laminated tuffs rest on laminated brown tuffs that include abundant organic matter, twigs, roots, and coal fragments. These lie in turn on a conspicuous layer with abundant *Ptilophyllum* remains.

The angiosperm leaves are covered by small gypsum crystals that fill the areoles bordering the veins. Fine details are obscure, with only the primary and secondary veins well marked. Cuticles are not present on the leaves. Apart from the species described here, abundant fragmentary leaves have been found in the same bed. Considering them all reveals the presence of other angiosperm species, some with entire margins and others with more complicated teeth. However, their poor preservation (Fig. 2F) does not warrant a description.

The other plants described so far from the Bajo Tigre area are all ferns and gymnosperms (*12, 15*). The only angiosperm pollen grain reported from the formation is the small, monosulcate *Clavatipollenites hughesii* (*16*).

Originally, the age of this formation was considered to be Barremian or Aptian (*12*). Recently, this unit was assigned a late Barremian to early Aptian age on the basis of its showing of a transition from *tectifera-corrugatus* to *Antulsporites-Clavatipollenites* pollen zones (*17*).

Five fairly complete specimens of angiosperm leaves described here have simple, symmetrical laminae (*18*), are pinnately lobed, and are 7 to 14 cm long and 6 to 13 cm wide (mesophyll size class). Fragmentary remains show that the leaves may also be palmately lobed, with two to three pairs of small lobes. Probably both types and intermediate forms were present in the fossil species. The apex of each lobe is obtuse; the leaf base is badly preserved, but is probably truncate or cordate. The margin is dentate and serrate, with obtuse, simple, regular, A-1 (convex-convex) teeth separated by angular sinuses. The principal vein of each tooth is a branch of a tertiary vein that enters the tooth slightly eccentrically. No accessory veins were observed. Nor was any glandular-

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