spectra and R_F values. The vivid green color of these fossil leaves is the result of localized accumulation of this chlorophyll derivative.

The morphological and chemical states of preservation of the Succor Creek specimens provide a basis for making phytochemical correlations between respective fossil and living taxa. The preservational states reported here are comparable to, but significantly different from, those seen in other angiosperm green-leaf localities—for instance, in the Geisel Valley leaf impressions. The presence of chlorophyll derivatives in Geisel Valley (middle Eocene) fossils has been interpreted (*15*) as the result of methyl pheophorbide a accumulation in sites previously occupied by leaves of various taxa. In consequence, such fossils, while providing excellent chemical data, reflect a nonselective concentration of organic constituents (similar to that of an ion-exchange column) derived from many genera rather than one. Such states of preservation preclude phytochemical correlations on the generic level. The fossils collected from the Succor Creek ashfall deposits are cuticular compressions showing a high level of cellular fidelity. The existence of significantly different biochemical profiles between taxa (*Celtis, Ulmus*, and *Zelkova*) make it possible to draw valid chemotaxonomic conclusions. The association of lava flows with lake-bed sediments in the Succor Creek locality suggests that the area had many lakes and streams caused by lava-blocked rivers (*5*). Vegetation that grew peripherally to these lakes caused accumulation of debris in lake bottoms or was buried under volcanic ash. As a result, two very different states of preservation may be found in relatively close proximity. Leaves preserved in shales from Succor Creek do not show the flavonoid profiles reported here.

The possibility of comparing the flavonoid profiles (and other constituents) of known fossil angiosperms with those of extant North American and Asian taxa is suggested by this research and may provide an opportunity for chemophytogeographic interpretations of Tertiary flora.

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Geochemistry and Thermolysis of Flavonoids

Abstract. *The thermal and* pH *stability of selected flavonoids has been determined under simulated geologic conditions. Thermolytic rates and products for various regimes, as determined by gas chromatography–mass spectroscopy, indicate the potential usefulness of flavonoids as thermometric indicators in sediments. The parametric factors affecting flavonoid stability are used to geochemically characterize angiosperm "green leaves" (36 to 25 × 10⁶ years old) from Succor Creek and indicate that these sediments have not experienced temperatures higher than 80°C or extreme* pH *shifts (beyond the range 6.3 to 7.2) during postdepositional maturation.*

The isolation and identification of flavonols, dihydroflavonols, and glycoflavones from Oligocene to Miocene angiosperm leaf fossils (36 to 25 × 10^6 years old) (*1*) suggest that very mild geologic factors were associated with postdepositional maturation of these sediments. Various geophysical and geochemical factors may affect the diagenesis of organic compounds (*2*) and severely alter more labile functional groups (such as OH and COOH) or individual organic constituents. Through model experiments with pure compounds geologic conditions may be simulated and diagenetic pathways of organic compounds may be assessed. (*3*). In an attempt to determine the relative thermal and *p*H stability of various flavonoids, pure standards of dihydrokaempferol, kaempferol, quercetin, and robinin were packed in sealed glass tubes with extracted bentonite clay (*4*) and subjected to various regimes of heat and *p*H (*5*). Bentonite clay was used as the inorganic matrix partly because previous investigators have used this material and it would thus be possible to directly compare the results with published data, but more importantly because this clay is formed from the in situ alteration of volcanic ash. The fossil material that we analyzed from the Succor Creek Flora (*1*) is preserved in a pyroclastic deposit (tephra) similar to bentonite. At various time intervals, the tubes containing organic constituents were opened in a hydrogen atmosphere, and the volatile reaction products were stripped off with a 150°C stream of hydrogen and collected in a liquid nitrogen–cooled vessel. The low-molecular-weight fraction was analyzed by gas chromatography, and nonvolatile reaction products were extracted from the clay and identified by gas chromatography–mass spectroscopy (*6*).

The thermolytic products of a flavanone (dihydrokaempferol) and a flavonol (kaempferol) under alkaline conditions are shown in Fig. 1. Dihydrokaempferol (aromadendrin) occurs widely as the phenol, the 3-*O*-rhamnoside engelitin, the 7-*O*-rhamnoside, and the 7-*O*-glucoside sinensin (*7*), and is thus a useful compound from which to derive a preliminary scheme of flavonoid diagenesis. Under alkaline conditions (*p*H 8.5) the flavanone (A in Fig. 1) undergoes thermal hydrolysis (B → C), with subsequent reduction producing the *p*-hydroxyphenylethanol (G) from *p*-hydroxyacetophenone, and *p*-hydroxybenzyl alcohol (F′) from the hydroxybenzoic acid (D′). The major products of this reaction are phloroglucinol (F) and 2,4,6-trihydroxyacetophenyl (E′). Basic hydrolysis and subsequent oxidation (A and I to L) yield the tetrahydroxyacetophenyl (K) and *p*-hydroxybenzoic acid (L). On the basis of the relative amounts of the products detected, the reaction A to F (or F′) seems preferred under these conditions. Thermal degradation products from the flavanone were not detected until sustained (5 days) temperatures at 100°C were achieved. Dihydrokaempferol yields kaempferol with peroxides and under alkaline conditions (A → H); subsequent thermolysis produces a tetrahydroxyacetophenyl and hydroxybenzoic acid as the major products. Under simulated geologic conditions the reaction A to H was not observed unless peroxide was added to the bentonite clay. The reaction H to L proceeded at 80°C after 4 days; before

this no degradation products were observed. The relative concentrations of the products D′ and F′, F and G, and K and L were found to be reliable indicators of the starting material; that is, the reaction A to C is preferred over A to J, while the flavonol kaempferol degrades exclusively by means of reaction H to L.

Quercetin, a 5,7,3,3′,4′-pentahydroxyflavone, yields product K and dihydroxybenzoic acid under alkaline thermolysis (80°C). The flavanones and flavonols tested were more stable under neutral or slightly acidic conditions (pH = 7.0 or 6.5) than under alkaline conditons; that is, acidic thermolysis was observed only at 120°C, with subsequent phenolic condensations with the aldehydes generated. In the presence of sugars (quercetin 7,4′-diglucosides), phenolic polymerization products were observed which were similar to phenol-formaldehyde resins. The reduction of flavonols (such as quercetin) to anthocyanins by means of vigorous chemical reactions (with methanolic HCl and magnesium) are of theoretical interest under excessive geothermal conditions. Subsequent reduction of anthocyanins (with zinc or magnesium in acidic solution) may result in dimeric products, particularly if the anthocyanins possess a 3-hydroxyl group (for instance, quercetin → cyanidin → 2-bis-flavene structure).

The thermal stability of the kaempferol glycoside robinin (3-robinobioside-7-rhamnoside) under simulated geologic conditions is in agreement with data obtained by other hydrolytic procedures (*8*), which show that under alkaline conditions (pH 8.0) position 3 is very stable. Acid hydrolysis results in intermediate 7-glycoside flavonols and free galactose and rhamnose. Alkaline hydrolysis at 80°C produces the 3-robinobioside kaempferol and rhamnose, while an elevation in temperature results in no appreciable alteration of these products. Acid hydrolysis at pH 5.5 ruptures glycoside linkages, and robinin at 100°C yields phloroglucinol, 2,4,6-trihydroxy- and p-hydroxybenzoic acids, galactose, rhamnose, and small amounts of robinobiose. In generalizing about flavonoids, the mechanism of thermal degradation of polysaccharides may be assumed to involve two reactions: (i) initial dehydration of the molecule and (ii) scission of C-O bonds, either in or between the ring structures (*r*- and *e*-scission, respectively). The *r*-scission type results in almost total yields of CO_2, CO, and H_2O, while *e*-scission yields a levoglycosan end to the polymer and a hydroxylated monomer. From our data, monosaccharides appear more thermally stable than disaccharides.

Quantitative and qualitative changes in sediment chemistry are primarily effected through diagenesis with burial (*9*) and are known to be the result of geothermal gradients (*10*). Several elements of composition and structure contribute to the thermal stability of organic compounds: (i) linearity of chains involving a paraffinic structure, (ii) introduction of double bonds, (iii) the presence of benzene rings, (iv) relatively high molecular weights, (v) cross-linking, and (vi) the absence of oxygen in the molecular backbone. While these aspects of structure are useful guides for determining geothermal stability, exceptions are prevalent; for example, paraffinic structures are weakened by tertiary or quaternary carbons in the chain, while double bonds introduce weakness in other bonds that are in a position β to them. The flavonoid compounds used in thermal stability experiments have features which favor [see (ii) to (iv) above] and inhibit [see (vi)] high-temperature stability. Alterations of aromatic compounds with burial are less pronounced, in general, than those of *n*-alkanes or fatty acids. The relative stability of aromatic structures and their high degree of skeletal specificity provide bases for using them as biochemical markers in paleontological studies (*11*).

On the basis of these data we suggest that the Succor Creek Flora (*12*), which was preserved as the result of pyroclastic ashfall accumulation, experienced neutral to slightly acid conditions during sediment maturation. The isolation of flavonol-3-glycosides and glycoflavones as well as free flavonols and flavanones indicates that the postdepositional history could not have involved temperatures above 80°C or extreme shifts in hydronium ion concentration. Similarly, the isolation of pheophorbide a complexes (degradation products of chlorophyll) and the fatty acid/*n*-alkane profiles, which show strong carbon preference indices reflecting the phytochemistry of referable extant material (*1*), indicate extremely mild geophysical and geochemical conditions during fos-

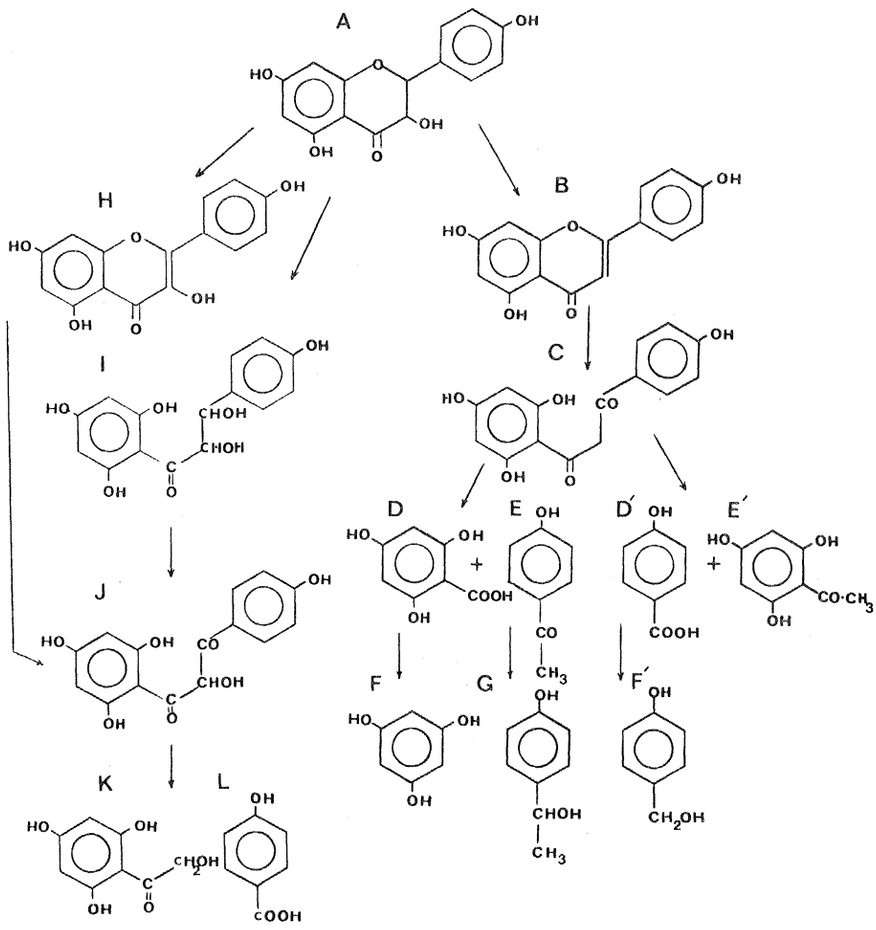

Fig. 1. Thermolytic degradation products of a flavanone, dihydrokaempferol (A), and a flavonol, kaempferol (H), under simulated geologic conditions (packed in extracted bentonite clay and subjected to various thermal and pH regimes). For further details see text.

silization. The thermolytic degradation products of flavonoids under various conditions of *p*H may provide a valuable thermometric tool in geochemical studies of relatively recent sediments.

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4. Bentonite clay was extracted in a Soxhlet apparatus with a mixture of benzene and methanol (3 : 1 by volume). Extracts after 50 hours were gas chromatographed to determine the presence of contaminants.
5. A total of 91 permutations of temperature (from 60° to 180°C in 10° increments) and *p*H (from 5.5 to 8.5, at increments of 0.5 unit) were performed with each standard compound. Preliminary experiments indicated that ranges of 80° to 130°C and *p*H = 6.5 to 8.5 were sufficient to determine the rate and nature of thermolytic alterations.
6. High-resolution mass spectra were obtained on a Perkin-Elmer GEC-AEI MS 904 mass spectrometer. Mixtures of nonvolatile components derived from simulation experiments were separated on Elmer-Perkin preparative (model F-3D) and analytical (model 154) gas chromatographs (the latter incorporates a 1.5 percent SE-30 column).
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HLA Variants of Cultured Human Lymphoid Cells: Evidence for Mutational Origin and Estimation of Mutation Rate

Abstract. *Variants of a diploid lymphoid cell line that show a loss of HLA-B27 antigen occur randomly in time and independently of exposure to the alloantiserum used for their isolation. From these and previous findings of variant stability, inducibility by mutagens, and the absence of linked variation, we conclude that most HLA variants arise by mutation. The mutation rate for HLA-B27 loss is 8 × 10⁻⁷ per cell per generation.*

A variety of methods has been proposed for assessing the carcinogenic and mutagenic potentials of environmental agents (*1*), but few methods have been described for measuring mutations directly in human somatic cells. We have developed a selective system to isolate variants of HLA, the major human histocompatibility complex (MHC), in cultured lymphoid cells (*2*). The MHC is a genetic region of higher organisms about two map units in length containing several loci coding for cell surface alloantigens with functions in self-recognition and defense (*3*). These alloantigens are potentially powerful markers for studying mutagenesis in human cells and for defining the mechanisms which give rise to somatic cell variants. Somatic genetic approaches in turn can be used to study the genetic organization of the MHC and the structure and function of its gene products. We have now isolated and partially characterized over 100 such variants at the HLA A and B loci in two lymphoid lines. Previous evidence excludes certain possible mechanisms of HLA variant formation, and suggests that the variants arise from genetic changes (*2*, *4*–*6*), but the evidence for genetic changes has not been definitive, limiting the usefulness of HLA variants in immunogenetic studies.

An important feature of genetic variation is that it occurs randomly in time and independently of exposure to the agents used for selective isolation of the variants. Determining whether antigenic variants are genetic in origin is important because exposure of cells to antiserum directed against cell surface components, such as that used for selection of HLA variants, can under certain circumstances induce epigenetic changes in antigenic expression (*7*). We now report evidence from fluctuation analysis and reconstruction experiments that HLA variants occur randomly in time and independently of exposure to alloantiserum. From the fluctuation data we have also obtained an estimate of the mutation rate at an HLA locus. These and other quantitative studies of alloantigenic variant formation (*6*) have been facilitated by development of a system in which variants can be isolated by a single exposure to selective conditions (*2*).

Cell line T5-1, an established human diploid line of B lymphoid cell origin (*8*), was used for these studies. It is heterozygous for the linked loci HLA A and B and phosphoglucomutase 3 (PGM3); its HLA haplotypes are Al-B8 and A2-B27 (*6*). Wild-type clones for determining the rate of spontaneous mutation were isolated by plating 3000 T5-1 cells in nutrient agarose; 10 to 14 days later, colonies were picked with a Pasteur pipette, transferred to liquid medium, and grown to 5 × 10⁸ to 2 × 10⁹ cells. Just prior to selection, the modal cell volume and volume distribution of each clone were de-

Table 1. The number of resistant cells in different wild-type clones.

Mean number of cells per clone	Number of cells per sample	Number of samples	Resistant cells per sample*	Mean	Variance†	Ratio of variance to mean	χ^2	*P*	Mean number of resistant cells per clone‡	Mutation rate§
			Experiment 1							
1 × 10⁹	4 × 10⁶	10	9 0 28 0 0 0 2 0 0 0	3.9	76.1	19.5	183.	<.0001	9,750	1 × 10⁻⁶
			Experiment 2							
2 × 10⁹	4 × 10⁶	8	0 2 6 0 4 2 0 8	2.7	6.3	2.3	23.1	<.005	13,500	7.5 × 10⁻⁷
			Experiment 3							
5 × 10⁸	4 × 10⁶	5	0 0 0 1 8	1.8	10.4	5.7	27.1	<.001	2,250	6 × 10⁻⁷

*Each number is for the resistant cells (measured as colonies) from a sample of 4 × 10⁶ cells from a wild-type clone. †Corrected for sampling. ‡Calculated from the average number of resistant cells per sample times the (1/sampling fraction) × (1/cloning efficiency). The average cloning efficiency was 0.10. §Number of mutations per cell per generation [calculated from equation 8 in (*18*)].