



Physical properties of insect cuticular hydrocarbons: model mixtures and lipid interactions

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Cuticular lipids include a diverse array of hydrophobic molecules that play an important role in the water economy of terrestrial arthropods. Their waterproofing abilities are believed to depend largely on their physical properties, but little is known about interactions between different surface lipids to determine the phase behavior of the total lipid mixture. I examined the biophysical properties of binary hydrocarbon mixtures, as a model for interactions between different epicuticular lipids of insects. The midpoint of the solid/liquid phase transition (T_m) for mixtures of *n*-alkanes differing in chain length equaled the weighted average of the T_m s of the component lipids. This was also true for *n*-alkane–methylalkane mixtures. However, alkane–alkene mixtures melted at temperatures up to 17°C above the temperature predicted from the weighted average of component lipid T_m values. Hydrocarbon mixtures did not exhibit biphasic melting transitions indicative of independent phase behavior of the component lipids. Instead, melting occurred continuously, over a broader temperature range than pure hydrocarbons.

Key words: Cuticular lipid; Hydrocarbon; Lipid interactions; Phase transitions; Unsaturation.

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Introduction

Epicuticular lipids provide the primary passive barrier to evaporative water loss in insects and other terrestrial arthropods (Edney, 1977; Hadley, 1994). Thus, understanding functional aspects of surface lipids has been a major objective of researchers interested in insect water balance. Wigglesworth (1945) observed that transpiration increases rapidly above a species-specific “transition” temperature. Following Ramsay (1935), he hypothesized that this phenomenon reflected an actual phase transition of the cuticular lipids. Several studies, using a variety of biophysical techniques, provided evidence consistent with the proposition that the waterproofing abilities of

cuticular lipids depend largely on their biophysical properties, specifically their phase behavior (Beament, 1945; Chefurka and Pepper, 1955; Holdgate and Seal, 1956; Toolson *et al.*, 1979). Structural models of cuticular lipids have been proposed (Beament, 1964; Locke, 1965), but the biophysical properties of surface lipids remain poorly understood.

Cuticular lipids are extremely diverse; over 100 different compounds have been identified from some species (Lockey, 1988; de Renobales *et al.*, 1991; Howard, 1993). Hydrocarbons predominate in most species, but wax esters, ketones, alcohols, sterols, and other compounds have also been identified. The great variety of compounds makes it difficult to predict the biophysical consequences (and, by implication, the waterproofing abilities) of inter- or intra-specific differences in lipid composition. The properties of a few commercially available pure *n*-alkanes have been examined (CRC Handbook, 1992), as well as

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those of some unsaturated and methyl-branched cuticular hydrocarbons (Gibbs and Pomonis, 1995), but very little is known about interactions between lipid components to determine the overall biophysical properties of surface lipids. Do the various components behave independently of each other, so that overall lipid physical properties are a simple weighted average of the properties of each component? Or do interactions between lipid species result in, for example, higher or lower than predicted melting temperatures? In this study, I used Fourier transform infrared spectroscopy (FTIR) to examine the physical properties of two-component hydrocarbon mixtures. These mixtures were intended to serve as simple models for the much more complicated mixtures found *in situ*. To understand how specific structural features affect bulk lipid properties, I analyzed mixtures of *n*-alkanes differing in chain length, mixtures of *n*-pentacosane with methylpentacosanes differing in the position of the methyl branch and alkene-alkane mixtures.

Materials and Methods

Hydrocarbon mixtures

Straight-chain alkanes and alkenes were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin, USA) Methylpentacosanes were a generous gift from J. G. Pomonis. All compounds were $\geq 98\%$ pure, as indicated by gas chromatography. Mixtures were prepared by combining aliquots of hydrocarbons dissolved in hexane. The relative amounts of the component lipids in each mixture were determined by gas chromatography and are expressed as weight fractions. Samples were stored under nitrogen at -20°C until analyzed.

Infrared spectroscopy

FTIR spectroscopy was performed as described by Gibbs and Crowe (1991). Hydrocarbon mixtures were dissolved in hexane, and 5–100 μg were deposited on CaF_2 windows. The windows were placed in a temperature-controlled cell holder in a Perkin-Elmer Systems 2000 FTIR spectrometer. The sample temperature was increased at 2°C intervals from well below the onset of the phase transition to $\sim 10^\circ\text{C}$ above the completion of the transition. Five scans were averaged at each temperature.

The progress of lipid melting was monitored from the frequency of the $-\text{CH}_2-$ symmetric stretching vibration absorbance maximum. This peak shifts from ~ 2849 to $\sim 2853\text{ cm}^{-1}$ as lipids melt. Plots of frequency vs temperature

were fitted to sigmoidal equations. The midpoint of the phase transition (T_m) and the width of the phase transition (ΔT , the difference between the temperatures at which the lipids were 95% and 5% melted) were calculated from the fitted curves.

Results

n-Alkane mixtures

I analyzed three series of straight-chain alkane mixtures, in which the chain lengths of the component hydrocarbons differed by 4, 7, or 10 carbon atoms. The following hydrocarbon combinations were used (chain lengths in parentheses): *n*-pentacosane (25)–*n*-nonacosane (29), *n*-tricosane (23)–*n*-triacontane (30) and *n*-docosane (22)–*n*-dotriacontane (32), respectively. Plots of T_m vs relative proportion of either molecule were linear (Fig. 1). In other words, T_m was equal to the weighted average of the T_m values of the component hydrocarbons. However, phase transitions were broader for mixtures than for pure *n*-alkanes. Pure *n*-alkanes melted abruptly over a $2\text{--}3^\circ\text{C}$ range, whereas mixtures melted over a range of $5\text{--}20^\circ\text{C}$ (data not shown).

n-Pentacosane–methylpentacosane mixtures

I analyzed mixtures of *n*-pentacosane (25 carbons long) with methylpentacosanes having the methyl branch at the 2, 3, 5, 7, 9, 11, or 13 position. The results resembled those obtained for *n*-alkane mixtures; for each series

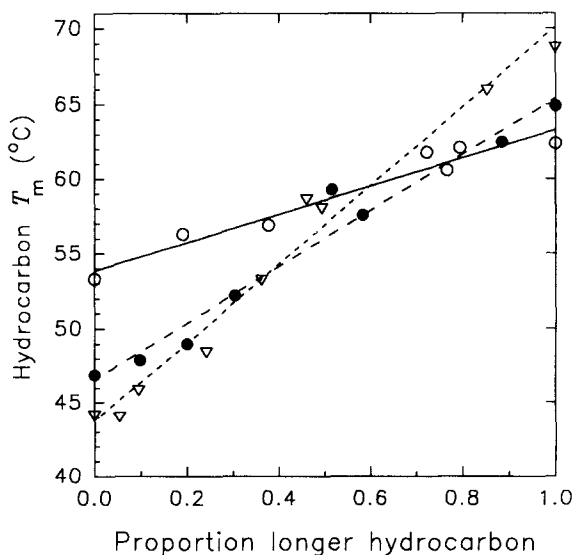


Fig. 1. Melting temperatures of binary mixtures of *n*-alkanes (number of carbon atoms in parentheses). \circ and —, pentacosane (25 carbons) and nonacosane (29). \bullet and ---, tricosane (23) and triacontane (30). ∇ and ---, docosane (22) and dotriacontane (32). Each point represents a single T_m determination. Lines are linear regressions ($r^2 > 0.97$ for all regressions).

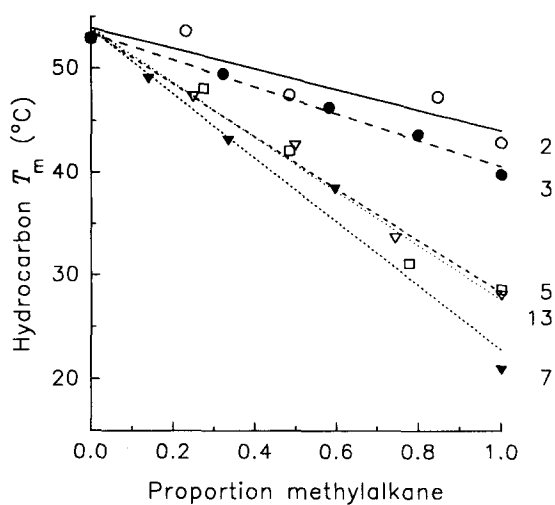


Fig. 2. Melting temperatures for mixtures of *n*-pentacosane with methylpentacosanes differing in methyl branch position. Branches were at 2 (○), 3 (●), 5 (▽), 7 (▼) and 13 (□) positions. Data for mixtures containing 9- and 11-methylpentacosanes were indistinguishable from those for the 7-methyl isomer and have been omitted for clarity. Each point represents a single T_m determination. Lines are linear regressions ($r^2 > 0.92$ for all regressions).

of mixtures, T_m equaled the weighted average of the component T_m s (Fig. 2). These mixtures melted over a 5–23°C range of temperatures (data not shown).

n-Alkane–*n*-alkene mixtures

Only two alkenes were available: (*Z*)-9-tricosene (23 carbons long) and (*Z*)-9-heneicosene (21 carbons). Each was combined with varying amounts of *n*-alkanes of the same chain length. In contrast to the case for alkane–alkane mixtures, combinations of *n*-alkanes with alkenes exhibited a non-linear relationship between T_m and relative proportion (Fig. 3A). Melting temperatures were greater than the weighted average of the component T_m values by as much as 17°C (Fig. 3B). Whereas pure hydrocarbons exhibited sharp phase transitions ($\Delta T < 2^\circ\text{C}$ for *n*-alkanes, 3–8°C for *n*-alkenes), mixtures melted over a 10–30°C range (Fig. 3C).

Discussion

A widely accepted hypothesis holds that the waterproofing abilities of surface lipids are largely a function of their phase state (Wigglesworth, 1945; Noble-Nesbitt, 1991). If this hypothesis is true, then an understanding of lipid interactions is essential to understanding the physiological significance of the diversity of cuticular lipids. In this study, I examined two-component mixtures of cuticular hydrocarbons as a model for lipid interactions *in*

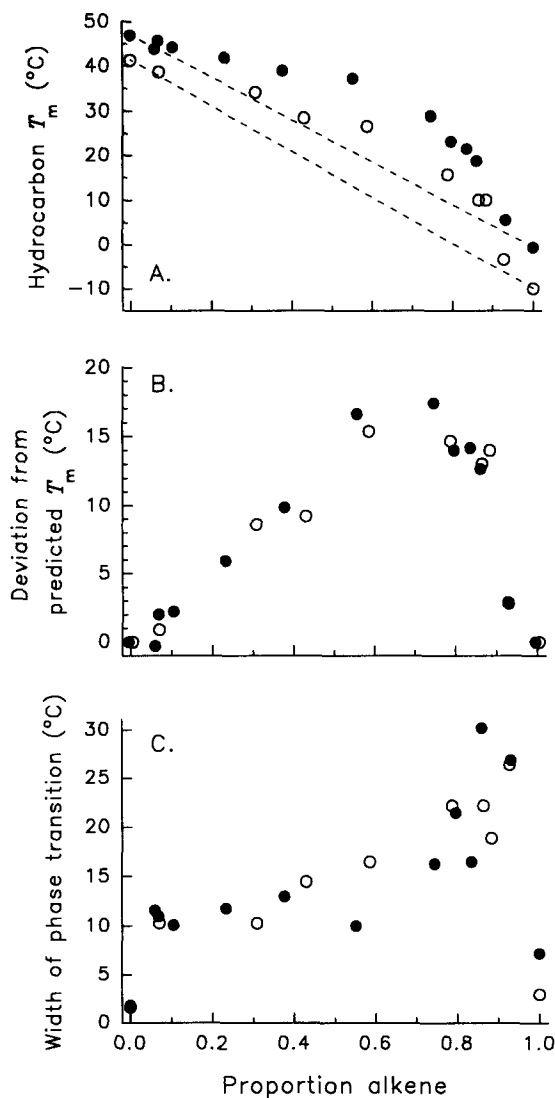


Fig. 3. Phase behavior of mixtures of (*Z*)-9-alkenes with *n*-alkanes having the same chain length. (A) Lipid melting temperatures (T_m). ---, T_m values calculated as the weighted average of the T_m s of the pure components. ○, heneicosene–heneicosane (chain lengths equal 21 carbons); ●, tricosene–tricosane (23 carbons). Each point represents a single T_m determination. (B) Data from A re-plotted as the difference between the measured T_m value and the predicted T_m . (C) Widths of phase transitions (ΔT) for alkene–alkane mixtures, calculated from fitted sigmoidal curves.

situ. Hydrocarbons were chosen because of their quantitative significance in most insects and their availability as pure compounds. The results presented here demonstrate that even these simple mixtures can interact to exhibit physical properties not easily predictable from the phase behavior of the pure components.

One might hypothesize that cuticular lipids do not interact with each other, that is, that each component of the bulk mixture melts separately, at the same temperature as it does in pure form. The linear dependence of T_m on

lipid proportion in alkane mixtures (Figs. 1 and 2) could be construed as supporting this idea. However, if this were the case, one would expect biphasic melting curves for the simple binary mixtures I examined. The data depicted in Fig. 4 contradict this prediction. The open symbols depict melting curves for pure *n*-docosane (22 carbon atoms) and *n*-dotriacontane (32 carbons). The solid line depicts the predicted melting curve for a 1:1 (w/w) mixture of these two alkanes, assuming that each component behaves as it does in pure form. If this were the case, one would observe a step change in frequency at $\sim 44^\circ\text{C}$, corresponding to melting of *n*-docosane. A second step change at $\sim 70^\circ\text{C}$ would indicate melting of *n*-dotriacontane.

The actual melting curve for a mixture containing 50.7% docosane and 49.3% dotriacontane is indicated by the filled triangles in Fig. 4. The mixture did not begin to melt until above 45°C , at which point pure *n*-docosane is completely melted. Melting was complete by 65°C , when pure *n*-dotriacontane is just beginning to melt. Thus, although the midpoint of the phase transition (T_m) can be predicted from the T_m s of the constituent lipids, at any given temperature the biophysical properties of hydrocarbon mixtures are not a simple weighted average of the component properties. I conclude that *n*-alkanes do interact with each other, but so as to broaden the phase transition without affecting the midpoint. A similar analysis holds for *n*-alkane-methyl-alkane mixtures. Melting temperatures were a linear function of percent composition (Fig. 2), and phase transitions were broader for mixtures than for pure alkanes (data not shown). Biphasic melting curves were not observed.

Interactions between *n*-alkanes and alkenes were more complex. Although pure alkenes may melt 50°C lower than alkanes with the same chain length (Fig. 3A), T_m was higher than predicted from a simple linear model for alkane-alkene mixtures. At alkene levels of 60–80%, measured T_m values exceeded predicted by $>15^\circ\text{C}$ (Fig. 3B). Melting points decreased rapidly at higher alkene levels. These effects of alkene proportion on T_m were associated with substantial changes in ΔT . Pure hydrocarbons melted over a relatively narrow ($2\text{--}8^\circ\text{C}$) range, whereas addition of $<10\%$ of a different compound broadened the phase transition to $>10^\circ\text{C}$ (Fig. 3C).

What are the implications of these data for naturally occurring cuticular lipids? It is widely accepted, though by no means rigorously proven, that changes in surface lipid

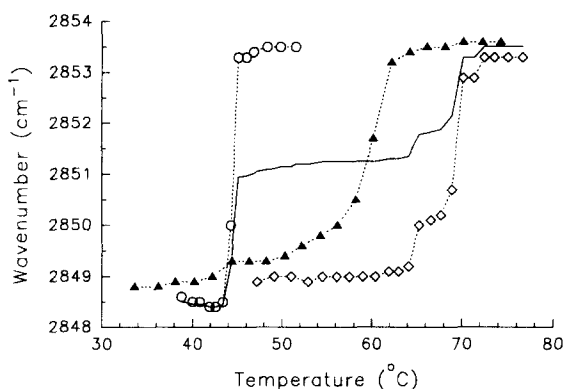


Fig. 4. Lipid melting curves for pure *n*-alkanes (\circ , \diamond) and mixtures: \circ , *n*-docosane; \diamond , *n*-dotriacontane. \blacktriangle , Mixture containing 50.7% docosane and 49.3% dotriacontane ($T_m = 58.1^\circ\text{C}$, $\Delta T = 13.8^\circ\text{C}$). —, the melting curve for a 1:1 (w/w) mixture of docosane and dotriacontane, calculated under the assumption that the phase behavior of each component is completely independent of the other compound.

physical properties will affect cuticular permeability. Indirect support for this view comes from the fact that inter- and intra-specific differences in hydrocarbon chain length have been correlated with water loss rates on numerous occasions (Hadley, 1977, 1978; Toolson and Hadley, 1977, 1979; Toolson, 1982, 1984; Hadley and Schultz, 1987). These differences are easily explainable in terms of the effects of chain length on T_m . The effects of methyl branching have been less well understood. My results indicate that an increase in methyl branching will tend to lower T_m , with internal branching having a greater effect than terminal branching. This hypothesis has been corroborated in a study of thermal acclimation of the grasshopper, *Melanoplus sanguinipes*; individuals reared at lower temperatures exhibit lower T_m s and higher methylalkane levels (Gibbs and Mousseau, 1994).

In *Drosophila* spp., which may contain $>90\%$ cuticular alkenes, the relationship between water loss rates and lipid composition has been more difficult to interpret (Toolson, 1988; Toolson and Kuper-Simbrón, 1989). My analyses of *n*-alkane-alkene mixtures suggest that unsaturation has relatively little effect on T_m , at least not until alkene levels exceed 80–90% of the total surface lipid. Beyond that point, T_m changes rapidly with unsaturation level. If the same situation holds for *Drosophila* surface lipids, relatively small differences in surface lipid saturation may greatly affect surface lipid physical properties. Unfortunately, I was only able to obtain two pure *n*-alkenes. More detailed knowledge of the properties of alkenes and their interactions with

other cuticular lipids awaits the availability of a greater diversity of unsaturated hydrocarbons.

In those insect species studied to date, surface lipids melt over a range of 10–25°C (Gibbs and Crowe, 1991; Gibbs *et al.*, 1991, 1995). The data presented here indicate that these broad phase transitions result from heterogeneity in several factors: chain length, methyl branching, and unsaturation. In fact, addition of <10% of a second hydrocarbon can broaden the phase transition (Fig. 3C). One would predict that widening of the surface lipid phase transition will broaden the transition in cuticular permeability seen in insects. Unfortunately, published studies have lacked the resolution (in both lipid biophysical techniques and permeability measurements) necessary to test this hypothesis. Indeed, it is unclear just how much melting of the surface lipids would be necessary to affect cuticular permeability. Is T_m (the midpoint of the transition) a relevant parameter, or is partial melting sufficient to result in increased transpiration? If so, broadening the surface lipid phase transition could result in increased transpiration at relatively low temperatures. Alternatively, the cuticle could remain relatively impermeable as long as a certain fraction of the lipids remain in a solid state, with transpiration increasing only when surface lipids are almost completely melted.

It might be argued that lipid phase behavior in these isolated mixtures differs from the *in vivo* situation, where hydrocarbons may interact with other cuticular components. Some authors have claimed that cuticular lipids are relatively tightly bound to the cuticle (Beament, 1964). However, most lipids appear to occur in a relatively homogeneous layer on the surface of the cuticle (Noble-Nesbitt, 1991) and are not directly associated with other layers. Toolson *et al.* (1979), using a magnetic resonance technique, found that cuticular lipids exhibited no preferred orientation, which suggests that they do not interact strongly with the cuticle. In addition, lipid phase behavior on exuviae is very similar to that of lipids extracted from exuviae (Gibbs and Crowe, 1991). In summary, the physical properties of isolated lipid mixtures do not differ much from those of lipids *in situ*, and the mixtures analyzed in this study should provide an accurate, albeit simplified, view of the effects of surface lipid variation in insects.

One limitation of this study is that I examined only binary hydrocarbon combinations, containing one or two *n*-alkanes. The limited availability of methylalkanes prevented me

from analyzing methylalkane–alkene mixtures or three-component *n*-alkane–methylalkane–alkene mixtures. Naturally occurring cuticular lipids may also contain non-hydrocarbon lipids. For example, polar lipids of the housefly, *Musca domestica*, tend to lower overall T_m (Gibbs *et al.*, 1995), whereas other types of compounds may increase T_m (e.g., 8,9-pentacosanediol in *Tenebrio molitor* larvae) (Bursell and Clements, 1967; Gibbs and Crowe, 1991). Thus, insects have considerable latitude for adjusting surface lipid-phase behavior. However, it must be noted that cuticular transpiration is only one component of insect water balance. Although surface lipids are clearly important in conserving water, it remains to be seen whether lipid-phase behavior is itself important in the water economy of insects or whether it affects other cuticular lipid functions (e.g., as pheromones).

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