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PLANT-DETERMINED VARIATION IN CARDENOLIDE CONTENT AND THIN-LAYER CHROMATOGRAPHY PROFILES OF MONARCH BUTTERFLIES, *Danaus plexippus*[1] REARED ON MILKWEED PLANTS IN CALIFORNIA

3. *Asclepias californica*[2,3]

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Abstract—Variation in gross cardenolide concentration of the mature leaves of 85 *Asclepias californica* plants collected in four different areas of California is a positively skewed distribution ranging from 9 to 199 μg of cardenolide per 0.1 g dry weight with a mean of 66 μg/0.1 g. Butterflies reared individually on these plants in their native habitats contained a normal distribution of cardenolide ranging from 59 to 410 μg of cardenolide per 0.1 g dry weight with a mean of 234 μg. Cardenolide uptake by the butterflies was a logarithmic function of plant concentration. Total cardenolide per butterfly ranged from 143 to 823 μg with a mean of 441 μg and also was normally distributed. Populational variation of plant cardenolide concentrations occurs within subspecies, but the northern subspecies *A. c. greenei* does not differ significantly from the southern *A. c. californica.* Generally higher concentrations occur in

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[2]Apocynales: Asclepiadaceae.
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butterflies from northern populations and in females. No evidence was adduced that cardenolides in the plants adversely affected the butterflies. Low cardenolide concentrations in the leaves and the absence of cardenolides in the latex characterize both *A. californica* and *A. speciosa,* but not *A. eriocarpa.* Thin-layer chromatography in two solvent systems isolated 24 cardenolide spots in the plants, of which 18 are stored by the butterflies. There was a minor difference in the cardenolide spot patterns due to geographic origin of the plants, but as in our previous studies, none in the sexes of the butterflies. Unlike *A. eriocarpa* and *A. speciosa, A. californica* plants lack cardenolides with *Rf* values greater than digitoxigenin. Overall, the cardenolides of both *A. californica* and *A. speciosa* are more polar than those in *A. eriocarpa. A. californica* plants contain cardenolides of the calotropagenin series including calotropin, calactin, and uscharidin, and the latter is metabolically transformed by monarch larvae to calactin and calotropin. Cardenolides of this series also occur in *A. vestita,* and *A. cordifolia* from California, the neotropical *A. curassavica,* and the African *Calotropis procera, Gomphocarpus* spp., and *Pergularia extensa;* they therefore cross established taxonomic lines. *A. californica* is the predominant early season milkweed in California and may be important in providing chemical protection to the spring generation of monarchs in the western United States. *A. speciosa, A. eriocarpa,* and *A. californica* each imparts distinctive cardenolide fingerprints to the butterflies, so that ecological predictions are amenable to testing.

Key Words—*Danaus plexippus,* Lepidoptera, Danaidae, monarch butterflies, *Asclepias californica,* Asclepiadaceae, milkweeds, ecological chemistry, plant–insect interactions, chemical ecology, chemical defense, chemotaxonomy, coevolution, thin-layer chromatography, cardenolide fingerprints, cardenolides, calotropagenin glycosides, calactin, calotropin, uscharidin.

INTRODUCTION

This paper is the third in our series presenting a multifaceted analysis of cardenolides in monarch butterflies sequestered from seven species of larval foodplants of the genus *Asclepias* (Asclepiadaceae) in California. Our purposes, explained in more detail in our studies on *Asclepias eriocarpa* Benth. (Brower et al., 1982) and on *A. speciosa* Torr. (Brower et al., 1984), are to investigate quantitative variation in the cardenolide contents of the butterflies as they relate to the milkweed plants growing in their natural habitats, to adduce evidence about the chemistry and emetic properties of the cardenolides and, by means of thin-layer chromatography, to establish plant-determined cardenolide fingerprints of the butterflies. We now report on cardenolides in monarchs collected as larvae on four populations of two subspecies of *Asclepias californica* Greene.

METHODS AND MATERIALS

Geographical and Ecological Distribution of Asclepias californica

Asclepias californica is an endemic California milkweed common in the foothills and lower mountainous areas of the southern half of California but is absent from the lowlands of the Central Valley. Two subspecies are distinguished by floral morphology (Woodson, 1954), and their distributions are shown in Figure 1 based on Woodson (1954) and Lynch (1977), as well as new data summarized from several herbarium collections (Lynch, as cited in Brower et al., 1982). *A. californica* subsp. *greenei* Woodson occurs to the north in central California and is geographically isolated by several mountain ranges from the southern *A. californica* subsp. *californica* Greene (Munz and Keck, 1959). The nearest the two approach each other is approximately 20–30 km southeast of area 3 (Figure 1) in Tulare and Kern counties where they are separated by the divides of the Sierra Nevada and Greenhorn Mountains.

In the Coast Ranges, the northern limit of *A. c. greenei* is in the vicinity of Mt. Diablo in Contra Costa County. [Lynch, 1977, determined that a record reported from Lake County by Woodson, 1954, was based on a misidentified herbarium specimen of *Asclepias cordifolia* (Benth.) Jepson.] Populations in the western Coast Ranges extend southwards to the Santa Lucia Ranges of northern San Luis Obispo County where they usually occur on south-facing slopes in chaparral clearings or in foothill woodlands at elevations ranging from 250 to 1400 m. The Coast Ranges and Sierra Nevada populations of *A. c. greenei* are isolated from each other by the grasslands and marshes of the Central Valley. In the Sierra Nevada, extensive populations occur along the foothills of the eastern border of the San Joaquin Valley from Mariposa County, southwards to Kern County and the Greenhorn Mountains. Also occurring primarily on south-facing slopes, these populations extend from valley grassland communities at 150 m up to foothill woodland, chapparal, and *Pinus ponderosa* pine forest communities at 1000 m. The northernmost populations of *A. c. californica* are located in western Inyo County along the eastern slopes of the Sierra Nevada and in Kern County along the Kern River. This subspecies extends southward to San Diego County along the slopes of the Paiute and Tehachapi ranges into the Transverse and Peninsular ranges. Although Woodson (1954) extends *A. c. californica* southwards into northern Baja California, we have observed no specimens or citations to verify its distribution south of San Diego County.

Populations in these ecologically diverse areas occur in a variety of plant communities. These include chaparral and foothill woodlands in

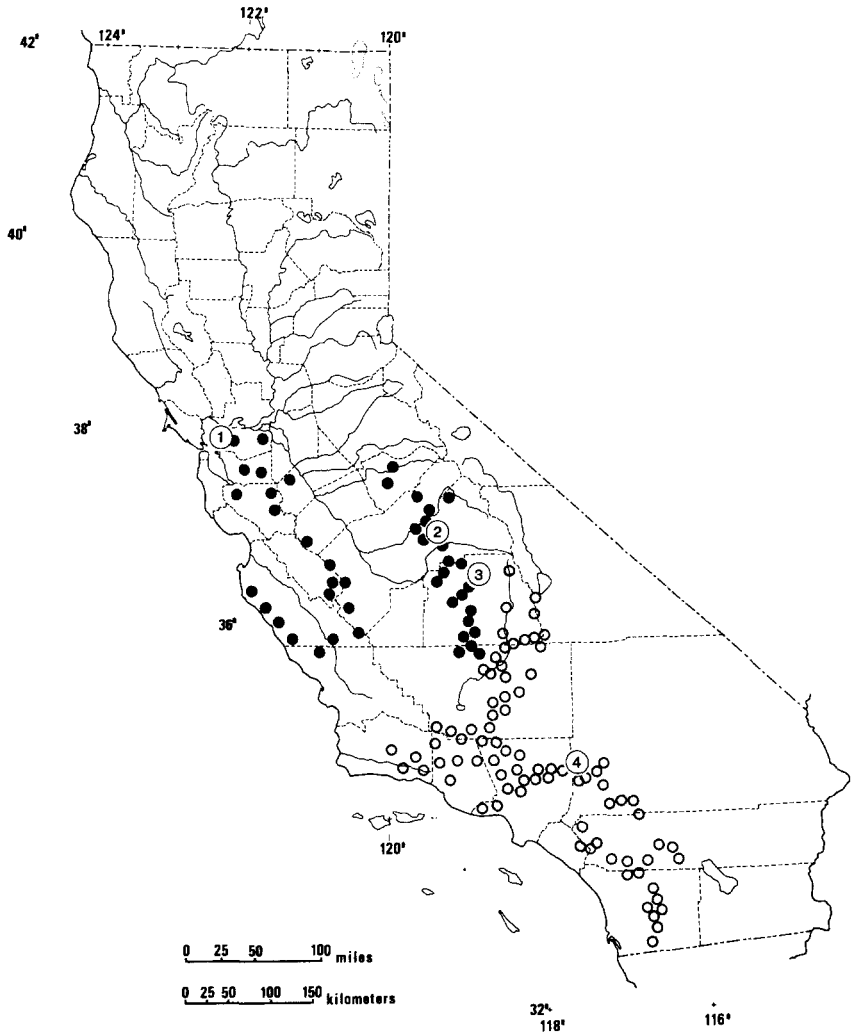


FIG. 1. The known geographic distribution of the milkweed *Asclepias californica* in California and the collection sites of the material analyzed in the study. The numbered open circles are the four areas where the 85 pairwise samples of monarch butterflies and their corresponding individual *A. californica* foodplants were collected. The solid black circles indicate populations of *A. californica* subsp. *greenei* and the open circles indicate those of *A. californica* subsp. *californica*. Area 1 (Mt. Diablo) is located in the central Coast Ranges, area 2 (Pine Flat Reservoir) is in the foothills of the southern Sierra Nevada, area 3 (Sequoia National Park) is in the lower montane zone of the southern Sierra Nevada, and area 4 (Wrightwood) is on the eastern margin of the San Gabriel Mountains which are part of the Transverse Range northeast of Los Angeles.

Santa Barbara, Ventura, Los Angeles, Orange, and San Diego counties and pinon–juniper woodlands, sagebrush scrub–chaparral transitions, and foothill to woodland–Jeffrey pine forest transitions in parts of Kern, Inyo, Los Angeles, Riverside, and San Bernardino counties. The southern subspecies extends beyond this habitat and also occurs on flats and gentle slopes with easterly and westerly exposures. Elevations for the known populations of *A. c. californica* range from 300 to 2200 m (Lynch, 1977).

Locations, Methods, and Dates of Sample Collections

We collected immature monarchs (*Danaus plexippus plexippus* L., western U.S.A. population) from milkweeds over an extensive area of California during the summer of 1975 and the spring and summer of 1976. Whereas previously (Brower et al., 1982, 1984), most individually matched plant–butterfly samples were obtained by collecting fifth-instar larvae and chrysalids directly from the wild plants, most of the butterflies utilized in this study were obtained by bagging wild eggs or young larvae on individual plants in nylon net bags and allowing them to mature therein until subsequently collected as fifth instars or chrysalids. Lack of sufficient numbers of breeding monarchs in some areas required that we transfer to selected plants eggs or young larvae obtained either in the wild or by confining one or more females in netting over a milkweed plant. Most transferred eggs did not survive, whereas most first- or second-instar larvae did.

Figure 1 indicates the four areas from which we obtained a total of 85 paired butterfly and plant samples. Those from area 3 were obtained during the summer of 1975, while those from the rest were collected during the spring and summer of 1976. We selected the areas to represent a diversity of biological communities across the range of both subspecies of *A. californica.* Geographical and altitudinal data were confirmed using various California regional maps (Anon. 1972a,b, 1974, 1975) and U.S. Geological Survey topographical maps (Anon., 1965, 1967, and 1968a,b).

Area 1 (Mt. Diablo) is in the central Coast Ranges in Contra Costa County on the southwesterly facing hillsides of Mt. Diablo at an elevation of 415 m. The community is open foothill woodland vegetation with a heavily grazed herbaceous layer composed largely of introduced annual grasses and forbs. Because we found no immatures here, we transferred eggs and early instar larvae collected on *A. californica* plants in area 2 on April 23 and bagged them on the Mt. Diablo plants. We then collected them on May 7, 1976. The twenty paired leaf–butterfly samples are Nos. 724, 727, 733–735, 737–739, 741, 742, and 745–754.

Area 2 (Pine Flat Reservoir) is in the foothills of the southern Sierra Nevada in Fresno County. This area is in the Sierra National Forest located

approximately 18 km north of Piedra on Trimmer Springs Road along the northern shore of Pine Flat Reservoir. Numerous plants were found on the south-facing slopes at an elevation of 400 m. These slopes are vegetated by heavily grazed European grasses interspersed with foothill woodlands dominated by digger pine (*Pinus sabiniana* Dougl.), blue oak (*Quercus douglasii*) H. and A., and interior live oak (*Q. wizlizenii*) A. DC. (Barbour and Major, 1977). We found many first- and second-instar larvae here on April 20, 1976, and bagged and subsequently collected them (as well as completely wild individuals) on May 15 797A, 798, 800, 802, 804, 807–809, 812, 813, 816, 821–825, 827, 828, and 830).

Area 3 (Sequoia National Park) is in the montane zone of the southern Sierra Nevada in Tulare County, northeast of Three Rivers at Potwisha Campground in Sequoia National Park, at an elevation of 750–850 m. The vegetation was transitional between the Sierran foothill woodland and an open ponderosa pine forest. We found numerous plants in partial clearings on the steep southwesterly facing slopes above the campground and obtained 26 paired samples including wild Nos. 43, 44B, and 45 on June 11, 1975; Nos. 86–89 on June 27; Nos. 175–176 and 184–185 on July 8; and Nos. 259–260 on July 19. Bagged eggs and first- or second-instar larvae produced Nos. 178 and 181–183 collected on July 8, and Nos. 261–266 and 268–270 on July 19.

Area 4 (Wrightwood) is located at an altitude of 1850 m on the southwestern facing slopes of Swartout Valley on the eastern slope of the San Gabriel Mountains of the Transverse Ranges in western San Bernardino County. This area is immediately north of the San Andreas fault and has vegetation which is transitional between a pinon pine (*Pinus monophylla* Torr. and Frem.) -juniper woodland (*Juniperus californica* Carr.) and Jeffrey pine forest (*Pinus jeffreyi* Grev. and Balf. in A. Murr.), with few grasses and forbs. Other dominant species included sagebrush *Artemesia tridentata* Nutt., California buckwheat *Eriogonum fasciculatum* Benth., and flannel bush *Fremontodendfron californicum* (Torr.) Cov. More than 100 *A. c. californica* plants were found scattered over the steep open hillside. Twenty paired samples were obtained by bagging first or second instar larvae on May 16, 1976. (We transferred these larvae from wild *A. eriocarpa* plants growing in the vicinity of area 2.) Collections made on June 6, 1976, are sample Nos. 914, 916, 918, 919, 921, 923, 924, 929, 931, 933, 934, 936–941, 943, 946, and 947.

Analytical Procedures

Gross Cardenolide Content. During the summer of 1977, we determined the gross cardenolide concentrations of the 85 butterflies (44 males and 41 females) and the leaves from the respective individual plants upon which they had been reared from the four areas. All butterfly samples

were reared on separate plants. Each sample was spectroassayed by the same procedures used in Brower et al. (1982) which give results in micrograms of cardenolide (equivalent to digitoxin) per 0.1 g dry weight.

Thin-Layer Chromatography. The cleanup of *A. californica* plant and butterfly extracts utilized the procedure described in Brower et al. (1984). Prior to cleanup, the plant extracts had a mean cardenolide concentration of 3.49×10^{-5} *M* (95% confidence limits = 0.17, $N = 5$, SD = 0.14), and the cleaned extracts 1.70×10^{-5} M (95% confidence limits = 0.19, $N = 5$, SD = 0.15) giving a cardenolide recovery efficiency of 49%. Respective values for the butterfly samples were 6.95×10^{-5} M (95% confidence limits = 0.06, $N = 5$, SD = 0.05) and 5.86×10^{-5} M (95% confidence limits = 0.12, $N = 5$, SD = 0.10), giving a recovery efficiency of 84%. The conditions for TLC development in two separate solvent systems [chloroform–methanol–formamide (90:6:1) = CMF, and ethyl acetate–methanol (97:3) = EAM], visualization, and photography of developed chromatograms were also as described previously. We carried out the TLC analyses in April–May 1979. Our TLC protocol spotted extracts of three male and three female butterflies and their respective plants on each of eight plates with two plates for each of the four areas, giving a total of eight plates with 48 butterfly–plant pairs. The rationale for selecting the samples and the methods of quantifying the $R_{\text{digitoxin}}$ and spot intensity (SI) values were as described previously. Eight of the plant and one of the butterfly samples contained insufficient cardenolide to replate in the EAM system.

We ran commercial digitoxin and digitoxigenin as reference standards along with calactin, calotropin, and uscharidin on each of the plates. As in the *A. speciosa* study, we found that interfering plant substances resulted in lower cardenolide recoveries and therefore weaker TLC spot profiles than expected, especially in those channels in which less than an estimated 50 μg of total cardenolide were spotted.

The mean $R_{\text{digitoxin}}$ (R_d) value for digitoxigenin in the CMF system was 2.12 (range = 1.89–2.29, SD = 0.11, $N = 16$) which is intermediate to that found in the *A. speciosa* (1.89) and *A. eriocarpa* (2.67) studies. These differences contribute to difficulty in quantitative comparisons of *Rd* values in different TLC runs, but do not affect the relative positions of the cardenolides in the TLC regions below and above digitoxin. In addition to quantifying the relative positions of the various spots, comparisons of TLC patterns are also based on the mean and standard deviations of each spot's intensity value (SI) as well as each spot's probability of occurrence (PO).

Statistical Analyses

Cardenolide Concentrations, Total Cardenolide, and Dry Weights. Statistical analyses were performed via the University of Florida Northeast Regional Data Center utilizing SAS, Release 79.6 (Anon., 1976). We

analyzed dry weights of the butterflies, cardenolide concentrations, and the total cardenolide contents of the butterflies, and cardenolide concentrations of the plants. Proc. Univariate and Freq. Plot Normal tests were used to examine frequency distributions and to test for normality (Kolmogorov D statistic). Data sets which proved to be not normally distributed ($P < 0.05$) were transformed ($\log_{10}$) which resulted in normality. We carried out two-way analyses of variance (two-way ANOVAs) separately on the plant and butterfly data to determine the significance of the influence of sex, area, and their interaction upon cardenolide concentration. Because of unequal numbers in the cells, we used the general linear models procedure (GLM). The minimum number of observations in any cell was nine (Table 1). Type IV sum of squares statistics were used if there was one or more significant interactions in the data, whereas type II sum of squares were used if there was none. We used Duncan's multiple range tests to compare the significances of the differences between mean values for the geographic areas with $P \leq 0.05$.

To relate cardenolide concentrations, total cardenolide contents, and dry weights of individual butterflies to the cardenolide concentrations of their respective plants, we ran regressions of the butterfly data (Y, the dependent variable) against the plant concentrations (X, the independent variable). Plots of residual values further examined the data for possible relationships not explained either by the linear or logarithmic models. Linear regressions of the butterfly data (concentrations and total cardenolide per butterfly) against the $\log_{10}$ values of the plant concentration data best conformed (highest r^2 values) to the relationship $Y = b(\log X) + a$ (see also Brower et al., 1984). We carried out each regression in two steps. The first considered the overall relationship of Y to X and calculated r^2, the slope, and the intercept. The significance of the difference of the slope and Y intercept values from zero is based on the t statistic. The second set of regressions broke down the dependence of the butterfly data on the cardenolide concentrations in the plants by sex, by area, and by their interactions. Where appropriate, sequential retesting dropped insignificant interactions to assess the significance of main effects per se.

TLC $R_{digitoxin}$ *and Spot Intensity Values.* As pointed out previously (Brower et al., 1982, 1984), the reliability of cardenolide fingerprinting depends upon a high degree of correlations between R_d values of the respective cardenolides in the plants and butterflies. While our TLC spot comparison methodology predetermines the correspondence of the respective plant and butterfly spots for each plant butterfly pair, the SAS linear regression program allows for the simultaneous testing of the dependence of the R_d values for all butterfly spots on the R_d values of their respective plant spots as affected by geographic area, sex, and interplate variation in the entire sample. We ran these same regressions on the spot intensity data.

TABLE 1. SUMMARY OF MEANS AND GRAND STANDARD DEVIATIONS OF CARDENOLIDE CONCENTRATIONS OF 85 WILD-COLLECTED INDIVIDUAL *Asclepias californica* PLANTS AND MONARCH BUTTERFLIES REARED THEREON[a]

	Plant material[b]			Butterfly material[b]		
	Males	Females	Means[c]	Males	Females	Means[c]
Area 1: central Coast Ranges, Mt. Diablo ($N = 10, 10$)	111.3	121.1	116.2	259.4	316.3	287.9
Area 2: southern Sierra Nevada, foothills, Pine Flat Reservoir ($N = 9, 10$)	30.2	56.7	43.5	163.3	211.8	187.6
Area 3: southern Sierra Nevada, Montane, Sequoia Nat. Pk. ($N = 15, 11$)	60.4	65.0	62.7	257.9	255.8	256.9
Area 4: San Gabriel mountains, Wrightwood ($N = 10, 10$)	39.5	43.7	41.6	188.2	200.7	194.5
Grand means[d]	61.0	71.5	66.1	223.0	246.4	234.3
Grand SDs[d]	42.0	48.7	45.4	70.5	75.6	73.1
Grand Ns	44	41	85	44	41	85
Ranges	9–199			59–410		

[a]Collections are from the four areas shown in Figure 1. Data are μg (equivalent to digitoxin) per 0.1 g dry weight of butterfly or plant material.
[b]The plant material corresponds to the butterflies according to the sex of the butterfly; the butterfly material represents the butterflies reared on their respective plants.
[c]The means above the grand means are the mean male value added to the mean female value divided by 2.
[d]Based on all 44 males, 41 females, and their respective plants.

Because we did not spot samples from each area on all eight plates, there were too few area replicates to provide sufficient degrees of freedom in the regression model to estimate the simultaneous effects of plate, area, sex, and their interactions on the main butterfly–plant regression. To rectify this, we ran two initial regressions: the first dropped area as an independent variable, and the second dropped plate. These two regressions showed that sex had no significant main or interactive effects with either plant R_d, plate (or area), or with any two- or three-way interactions in the regressions (P values ranged from >0.09 to <0.74). We then dropped sex and reran the regression with plate nested within the area variable (Tables 8A and 9A). In

future studies, a more robust statistical analysis can be achieved by running samples of all areas and both sexes on each plate.

RESULTS

Gross Cardenolide Concentrations

Quantitative Variation in Plants. The pattern of variation is shown in Figure 2. All 85 plants contained cardenolide and ranged from 9 to 199 μg/0.1 g with a grand mean of 66.1 μg and a grand standard deviation of 45.4 μg (Table 1). This variation encompasses approximately one order of magnitude, and in terms of cardenolide as a percent of dry weight represents from 0.01 to 0.20% (mean = 0.07%). The curve over the histogram in Figure 2 displays the expected normal distribution calculated by the z statistic (Steel and Torrie, 1960) and shows that the variation is positively skewed (moment of skewness = 0.91; mean and median = 66.1 and 54.0; $D = 0.130$; $N = 85$; $p < 0.01$), i.e., the majority of plants contain less than the average concentration of cardenolide. However, the $\log_{10}$ transformed data do not depart significantly from normality: mean and median = 1.719 and 1.740; $D = 0.068$, $P > 0.15$. Further statistical analyses of the plant concentration data are therefore based on the $\log_{10}$ transformed data.

Inspection of the means in Table 1 suggests differences both in the cardenolide concentrations of the plants from the four geographic areas and in the plants fed on by the males vs. the females. The two-way ANOVA (Table 2A) indicates that the area difference is highly significant ($P < 0.0001$) but that the difference in the plants according to the sex of butterfly that fed upon them is not and that the interaction is also not significant. The order of the cardenolide concentrations from highest to lowest in the four areas is area 1, 3, and 2 $\simeq$ 4. Duncan's test indicates that plants in area 1 (116 μg/0.1 g) are significantly higher than all the other areas. Concentrations in area 3 (62 μg/0.1 g) differ from area 2 (44 μg/0.1 g), but not from area 4 (42 μg/0.1 g), and area 2 and 4 also do not differ significantly from each other. The grand variance of the cardenolide concentration of the plants upon which the females were reared is not significantly different than those on which the males were reared (for the s^2 female/s^2 male, $F = 0.964$; $df = 40, 43$; $0.25 < P < 0.50$).

These data indicate significant populational variation in the gross cardenolide concentrations of *Asclepias californica* plants. The central Coast Range population has substantially higher concentrations than the three other populations, two of which in turn differ among themselves but without any obvious geographic pattern. Most importantly, there are no significant differences based on the two subspecies.

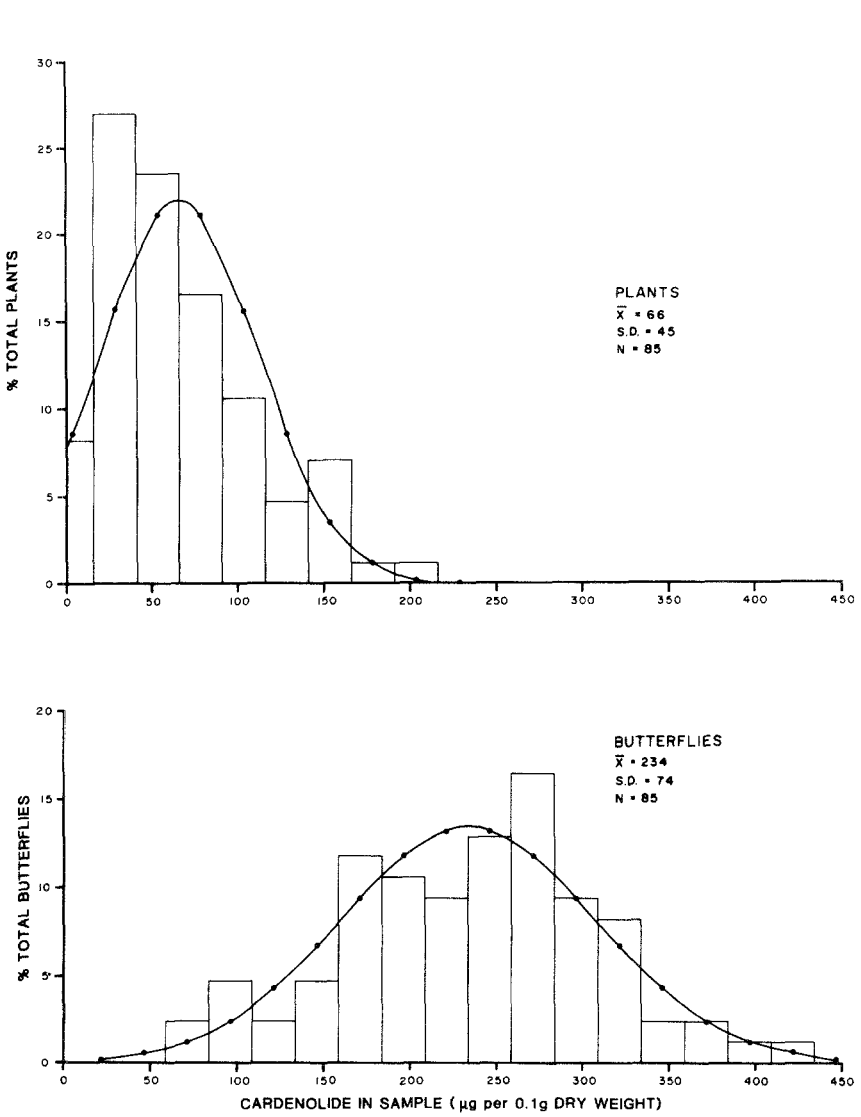


FIG. 2. Frequency distributions of the gross cardenolide content (as μg per 0.1 g dry weight, equivalent to digitoxin) of 85 *Asclepias californica* plant samples and 85 adult monarch butterflies reared thereon. All individual butterflies were collected as fifth-instar larvae or chrysalids on the respective individual milkweed plants growing in their natural environments from the four geographic areas shown in Figure 1. The width of each bar is 25 μg. The curves above the histograms are the calculated normal distributions.

TABLE 2. TWO-WAY ANOVA OF CARDENOLIDE CONCENTRATION DATA IN TABLE 1.

Source of variation	*df*	SS	MS	*F*	*P*
A. Plant material ($r^2 = 0.40$)[a]					
Model	7	3.51795	0.50256	7.45	<0.0001
Error	77	5.19549	0.06747		
Corrected total	84	8.71344			
Area	3	3.28745		16.24	<0.0001
Sex	1	0.11847		1.76	>0.18
Sex × area	3	0.13054		0.64	>0.59
B. Butterfly material ($r^2 = 0.37$)[b]					
Model	7	169895	24271	6.57	<0.0001
Error	77	284427	3694		
Corrected total	84	454322			
Area	3	145271		13.11	<0.0001
Sex	1	15065		4.08	=0.047
Sex × area	3	13058		1.18	>0.32

[a]Type II sum of squares, data are $\log_{10}$ of μg/0.1 g.
[b]Type II sum of squares, data are μg/0.1 g.

Quantitative Variation in Butterflies. All 85 butterflies contained cardenolide. The concentration range for the four geographic areas was 59–410 μg/0.1 g with a grand mean of 234.3 μg/0.1 g (Table 1). This range spans approximately one order of magnitude and in terms of cardenolide per gram dry weight represents from 0.06 to 0.41% (mean = 0.23%), i.e., from about 2 to 7 (mean = 3.5) times that in the plants.

As seen in Figure 2, the pattern of variation in cardenolide concentration in the butterflies is normally distributed (mean and median = 234.3 and 238.0, respectively; $N = 85$, $D = 0.059$; $P > 0.15$). The grand variance of the 66 males does not differ significantly from that of the 45 females ($F = 1.15$, $df = 40, 43$, $0.75 > P > 0.55$). The grand butterfly standard deviation is 75.6 μg, i.e., 1.55 times that of the plants they were reared upon (Table 1, Figure 2). In other words, the range of variation in the butterflies is substantially increased over that of the plants, as well as normalized.

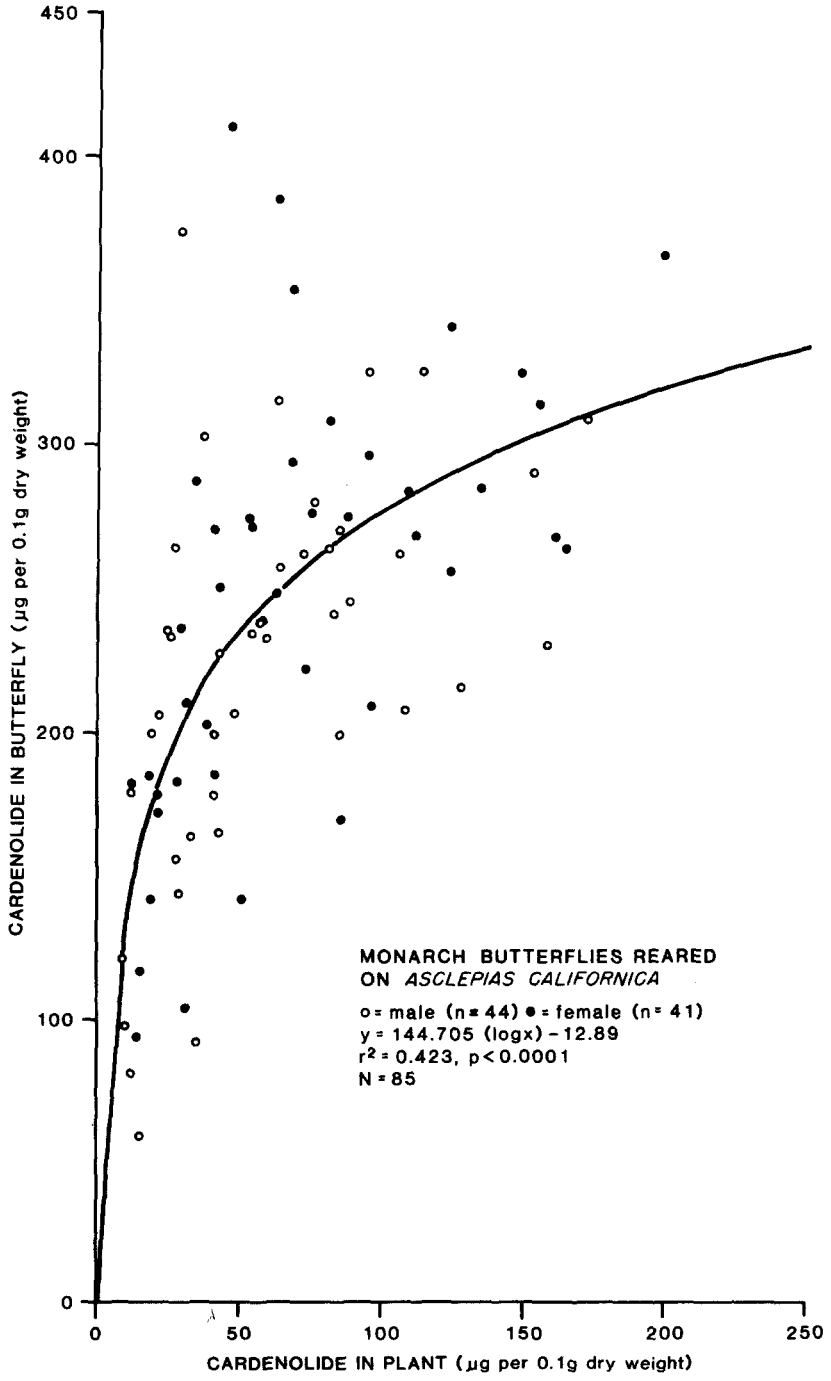
The two-way ANOVA of the two sexes and four areas indicates that the butterfly concentrations differ significantly by both sex and by area, but without a significant interaction (Table 2B). Overall, females have higher cardenolide concentrations in three of the four areas. Duncan's comparison of the four geographic areas indicates that butterflies from area 1 (288 μg/0.1 g) and area 3 (257 μg/0.1 g) have significantly higher concentrations than those from area 4 (194 μg/0.1 g) and area 2 (189 μg/0.1 g). Areas 1 and 3 do not differ significantly, nor do areas 4 and 2. The order of the mean cardenolide concentrations in the butterflies from highest to lowest (area 1,

3, 4, and 2) is similar to that of the plant concentrations (area 1, 3, and $2 \simeq 4$).

Cardenolide Concentrations of Butterflies as Function of Cardenolide Concentrations of Plants. The mean cardenolide concentration in the butterflies is 3.54 times that of their respective plants (grand mean ratio) and the overall range in the butterflies (59–410 μg/0.1 g) is shifted substantially upwards from that of the plants (9–199 μg/0.1 g). Figure 3 plots butterfly concentrations against the respective plant concentrations for both males and females and the main regression model (Table 3A) confirms the logarithmic relationship $Y = 144.705 \log_{10}X - 12.89$ ($r^2 = 0.423$; $P < 0.0001$). The slope is significantly different from 0 at the $P < 0.0001$ level, whereas the intercept is not ($P > 0.69$). Plotting of the residual values in both the linear and log regressions substantiated this logarithmic relationship. The regression equation predicts that butterflies which ate plants

TABLE 3. LINEAR REGRESSION ANALYSES OF CARDENOLIDE CONCENTRATIONS (μg/0.1 g DRY WT) IN BUTTERFLIES (DEPENDENT VARIABLE) VS. CARDENOLIDE CONCENTRATIONS ($\log_{10}$ μg/0.1 g DRY WT) IN THEIR RESPECTIVE PLANTS, ACCORDING TO FUNCTION $y = b\ (\log_{10}x) + a$.

Source of variation	*df*	SS	MS	*F*	*P*
A. Overall regression pooling sex and geographic areas ($r^2 = 0.423$; type IV sum of squares)					
Plant concentration	1	192390	192390	60.96	<0.0001
Error	83	261932	3156		
Corrected total	84	454322			
Estimated value of parameters		SE	*T* for H=O		*P*
$a = Y$ intercept $= -12.889$		32.24	−0.40		>0.69
b = slope $= 144.705$		18.53	7.81		<0.0001
Equation for the line: $Y = 144.705\ (\log_{10}x) - 12.89$					
B. Butterfly concentrations on plant concentrations by area, by sex, and for all interactions ($r^2 = 0.533$; type II sum of squares)					
Model	15	242052	16137	5.25	<0.0001
Error	69	212270	3076		
Corrected total	84	454322			
Log plant conc	1	67181		21.84	<0.0001
Area	3	6236		0.68	>0.57
Sex	1	262		0.09	>0.77
Log plant × area	3	4113		0.45	>0.72
Log plant × sex	1	88		0.03	>0.86
Sex × area	3	995		0.11	>0.95
Log plant × sex × area	3	832		0.09	>0.96


450
400
300
200
100
0
0
50
100
150
200
250
CARDENOLIDE IN BUTTERFLY (µg per 0.1g dry weight)
CARDENOLIDE IN PLANT (µg per 0.1g dry weight)
MONARCH BUTTERFLIES REARED
ON ASCLEPIAS CALIFORNICA
o = male (n = 44) ● = female (n = 41)
y = 144.705 (logx) - 12.89
r² = 0.423, p < 0.0001
N = 85

containing as little as 2 μg/0.1 g would contain approximately 31 μg/0.1 g of cardenolide, those which fed upon 50-μg plants would contain 233 μg/0.1 g, those on 100-μg plants, 276 μg/0.1 g, and those on 300-μg plants, 346 μg/0.1 g, etc. The second regression analysis of these data (Table 3B) indicated that the high dependence of the butterfly concentration on the plants is unaffected by the sexes, the four geographic areas, or their interactions (all P values > 0.57).

Dry Weights of Butterflies

The dry weights of the butterflies (Table 4) are normally distributed (grand mean = 0.190 g, median = 0.193 g), $D = 0.055$; $P > 0.15$) and the variances of the two sexes do not differ significantly ($F = 1.54$; df = 65, 44; $0.25 > P > 0.10$). Two-way ANOVA indicates a significant difference of the means both by sex and by geographic area, but no significant interaction (Table 5A). The average dry weight of the males is 0.196 g and the females 0.183 g (Table 4), i.e., the males on average weigh 1.07 times the females. Duncan's test indicates a significant difference among the geographic areas grouped as follows: area 4 (0.197 g) and area 3 (0.196 g), area 1 (0.190 g), and area 2 (0.173 g). Areas 3 and 1 do not differ from each other, but both areas 4 and 3 differ from area 2.

Dry Weights of Butterflies as Function of Cardenolide Concentrations in Plants and Butterflies. The same linear regression model as in Table 3A was run to relate the dry weights of the butterflies (Y) to the $\log_{10}$ cardenolide concentrations of the plants (X). The overall model indicated no correlation ($r < 0.0003$; $F = 0.0211$ $P > 0.89$). The butterflies' overall dry weights likewise showed no correlation with the cardenolide concentration of the butterflies ($r^2 < 0.03$, $F = 2.48$, $P > 0.11$).

Total Cardenolide in Butterflies.

The data for all four areas are normally distributed (mean = 441.2 μg; median = 459.6 μg; $D = 0.080$; $P > 0.15$). Total cardenolide per butterfly ranged from 143 to 823 μg with a standard deviation of 144 μg (Table 4). The variances of the males and females do not differ significantly ($F = 1.43$; $0.25 > P > 0.10$). The males contain an average of 431 μg and the females 452 μg. The two-way ANOVA indicates a highly significant difference

FIG. 3. Gross cardenolide concentrations of adult monarch butterflies (Y axis) as a function of the gross cardenolide concentrations of their larval foodplants (X axis). Each of the 85 data points represents one corresponding individual butterfly-plant rearing experiment. Open circles are males and solid circles are females. The line is derived from the regression equation $Y = b(\log_{10} X) + a$, transformed back to standard coordinates.

TABLE 4. SUMMARY OF DRY WEIGHTS AND TOTAL CARDENOLIDE CONTENT (EQUIVALENT TO DIGITOXIN) OF 85 ADULT MONARCH BUTTERFLIES REARED ON *A. californica* PLANTS POOLED FROM FOUR GEOGRAPHIC AREAS IN TABLE 1.

		Dry weights (g)			Total cardenolide (μg)		
Sample size		Means	SD	Range	Means	SD	Range
Male	44	0.196	0.030	0.128–0.287	431	131	143–684
Females	41	0.183	0.026	0.127–0.235	452	157	151–823
Both	85	0.190	0.029	0.127–0.287	441	144	143–823

among the areas but neither the sex nor the interaction is significant (Table 5B). Duncan's test of the geographic differences indicates that butterflies from area 1 (543 μg) and area 3 (493 μg) do not differ significantly; those from area 4 (384 μg) and area 2 (323 μg) also do not differ significantly, but those from the first two differ from the latter two. The order of the mean total cardenolide per butterfly from highest to lowest in the four areas (area 1, 3, 4, and 2) is identical to the butterfly concentrations and is similar to the mean plant concentrations (area 1, 3, and $2 \simeq 4$).

Total Cardenolide in Butterflies as Function of Cardenolide Concentrations in Plants. Regression analyses (Table 6A) indicated that total

TABLE 5. TWO-WAY ANOVAS OF DRY WEIGHTS AND TOTAL CARDENOLIDE CONTENT OF BUTTERFLIES BY FOUR AREAS AND TWO SEXES[a].

Source of variation	*df*	SS	MS	*F*	*P*
A. Dry weight of butterflies ($r^2 = 0.17$)					
Model	7	0.01134	0.00162	2.19	<0.044
Error	77	0.05689	0.00074		
Corrected total	84	0.06823			
Area	3	0.00677		3.05	<0.034
Sex	1	0.00331		4.48	<0.038
Sex × area	3	0.00075		0.34	>0.80
B. Total cardenolide per butterfly ($r^2 = 0.38$)					
Model	7	667421	95346	6.93	<0.0001
Error	77	1075158	13963		
Corrected total	84	1742579			
Area	3	613558		14.65	<0.0001
Sex	1	17711		1.27	>0.26
Sex × area	3	44398		1.06	>0.37

[a]Type II sum of squares (data are normally distributed).

TABLE 6. LINEAR REGRESSION ANALYSES OF TOTAL CARDENOLIDE (μg) PER BUTTERFLY (Y AXIS, DEPENDENT VARIABLE) VS. CARDENOLIDE CONCENTRATIONS ($\log_{10}$ μg/0.1 g DRY WT.) IN THEIR RESPECTIVE PLANTS, ACCORDING TO FUNCTION $Y = b(\log_{10} X) + a$

Source of variation	*df*	SS	MS	*F*	*P*
A. Overall regression pooling sex and geographic areas ($r^2 = 0.403$; type IV sum of squares)					
Plant concentration	1	702932	702932	56.12	<0.0001
Error	83	1039646	12526		
Corrected total	84	1742579			
Estimated value of parameters	SE		*T* for H=O		*P*
a = Y intercept = − 31.33	64.23		− 0.49		>0.62
b = slope = 276.60	36.92		7.49		<0.0001
Equation for the line: $Y = 276.60 (\log_{10} X) - 31.33$					
B. Total cardenolide per butterfly on $\log_{10}$ plant concentrations by area, by sex, and for all interactions ($r^2 = 0.543$; type II sum of squares)					
Model	15	945870	63058	5.46	<0.0001
Error	69	796709	11547		
Corrected total	84	1742579			
Log plant concentration	1	243380		21.08	<0.0001
Area	3	16069		0.46	>0.71
Sex	1	1051		0.09	>0.76
Log plant concentration × area	3	15027		0.43	>0.73
Log plant concentration × sex	1	1119		0.10	>0.75
Sex × area	3	20538		0.59	>0.62
Log plant concentration × sex × area	3	18500		0.53	>0.66

micrograms of cardenolide per butterfly is highly dependent on the $\log_{10}$ plant concentrations ($Y = 276.60 \log_{10} X - 31.33$; $r^2 = 0.403$; $P < 0.0001$), and the slope is significantly different from 0 at the $P < 0.0001$ level, whereas the intercept does not differ significantly from zero. This regression equation predicts that butterflies which fed upon plants containing as little as 2 μg/0.1 g would contain approximately 52 μg of cardenolide, those on 50-μg plants would contain 439 μg, those on 100-μg plants would contain 522 μg, and those on 300 μg plants would contain 654 μg, etc.

The second regression (Table 6B) indicated that the only significant predictor of butterfly concentrations derives from the plant concentrations ($P < 0.0001$): neither the sex of the butterflies, their geographic location nor the interaction has a significant effect (all P values > 0.62). The order of the mean total cardenolide per butterfly in the four areas (area 1, 3, 4, and 2) is similar to that of the mean plant concentrations (Area 1, 3, and $2 \simeq 4$).

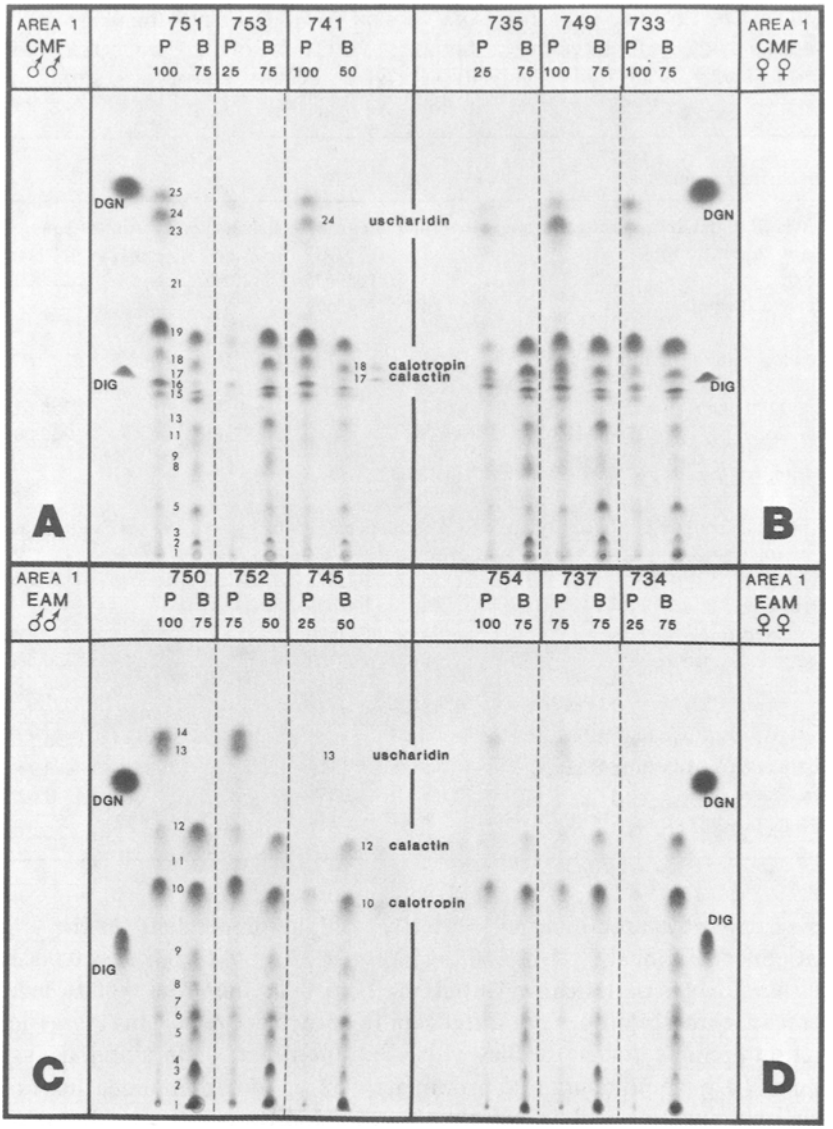


FIG. 4. Photographic reproduction of the thin-layer chromatographic profiles of the cardenolides stored by adult monarch butterflies from their *Asclepias californica* larval foodplants in California. Sections A–D of the plate each show the TLC profiles of three pairs of plants and corresponding individual male or female monarch butterflies reared on these plants form the Mt. Diablo (area 1) population. Five standards were run on each plate including digitoxin (DIG), digitoxigenin (DGN), uscharidin, calactin, and calotropin. Sections A and B were run in the chloroform–

TLC Cardenolide Profiles of Plants and Butterflies: Chloroform–Methanol–Formamide (CMF) System

Figure 4A and B is a photograph of one of the eight TLC plates run in the CMF system and shows six individual plants and the three male and three female butterflies reared thereon from area 1. Table 7 summarizes the means and standard deviations for both $R_{\text{digitoxin}}$ (R_d) and spot intensity (SI) values for all the spots in the 48 plant and butterfly samples from the four areas. Table 7 also gives each spot's probability of occurrence (PO), based on the proportion of plants or butterflies in which each spot occurred, regardless of intensity. Although several spots were sometimes weak or absent in the individual plant or butterfly channels, the standard deviations of the R_d values of all spots are low, i.e., resolution of the cardenolides is good.

Figure 5 is based on the mean R_d and SI values from Table 7 and depicts the average cardenolide profile for the CMF system for the plants and butterflies representing most of the geographic range of *Asclepias californica.* The shapes and sizes of the spots are drawn from a representative TLC plate and are generalized in the figure. Twenty-five discernible cardenolides of varying intensities were resolved in this TLC system. Of these, 24 occurred in the plants and 18 occurred in the butterflies. Spot 10 was not present in any of the plants nor was spot 12 in any of the butterflies.

Figure 5 also shows the R_d values for digitoxin, digitoxigenin, calactin, calotropin, and uscharidin. The mean migration distance for 16 digitoxin spots (two on each of the eight plates) was 43.75 mm, with a range of 38.0–47.5 mm, and a SD of 2.77 mm. Corresponding values for 16 digitoxigenin spots were: 92.40 mm, 86.5–97.0 mm, and a SD of 2.78 mm. The mean $R_{\text{digitoxin}}$ value for digitoxigenin was 2.12, with a range of 1.89–2.29, and a SD of 0.11. Corresponding $R_{\text{digitoxin}}$ values for eight uscharidin spots were 1.86, 1.74–1.98, SD = 0.08; for eight calotropin spots were 1.06, 1.02–1.11, SD = 0.03; and for eight calactin spots were 0.98, 0.97–0.99, SD = 0.01. (One each of these three standards was spotted on the center of each plate.)

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methanol–formamide system which separates a total of 25 spots. Twenty-four of these (all except spot 10) occur in the plants, and 18 (Nos. 1–19, excepting spot 12) occur in the butterflies. Because of the low gross concentrations of cardenolides in these examples, several spots are not visualized (but see the generalized drawing in Figure 5). Sections C and D show a second set of six sample pairs run in the ethyl acetate–methanol system which separates up to 14 cardenolides in the plants and up to 12 in the butterflies. Spots 17 and 18 in the CMF system and spots 12 and 10 in the EAM system correspond, respectively, to calactin and calotropin, and spots 24 and 13 in the two respective systems correspond to uscharidin.

TABLE 7. SUMMARY OF MEANS AND STANDARD DEVIATIONS FOR $R_{digitoxin}$ VALUES AND SPOT INTENSITIES FOR 25 CARDENOLIDES AND PROBABILITY OF THEIR OCCURRENCE IN 48 PAIRED PLANTS AND BUTTERFLIES.[a]

	Means				Standard deviations							
	$R_{digitoxin}$ values		Spot intensity values		$R_{digitoxin}$ values		Spot intensity values		Probability of spot		Subsample sizes	
Spot No.	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly
25	2.05		1.94		0.12		1.10		0.71		34	
24	1.91		2.10		0.10		1.22		0.83		40	
23	1.78		1.40		0.10		0.52		0.21		10	
22	1.64		1.00		0.04		0.00		0.15		7	
21	1.51		1.17		0.06		0.41		0.13		6	
20	1.46		1.00		0.00		0.00		0.02		1	
19	1.19	1.19	2.62	4.21	0.06	0.05	1.34	0.94	0.77	1.00	37	48
18	1.06	1.04	2.16	3.47	0.05	0.04	1.07	1.06	0.77	0.94	37	45
17	0.97	0.96	2.24	2.64	0.04	0.02	1.09	1.14	0.60	0.81	29	39
16	0.93	0.92	2.53	2.91	0.03	0.03	1.34	1.31	0.67	0.73	32	35
15	0.89	0.89	2.05	3.18	0.03	0.03	1.08	1.17	0.40	0.81	19	39
14	0.83	0.83	1.26	3.14	0.03	0.04	0.65	1.32	0.40	0.79	19	37
13	0.72	0.73	1.13	3.04	0.05	0.04	0.46	0.87	0.48	1.00	23	48
12	0.67		1.25		0.06		0.50		0.08		4	
11	0.64	0.64	1.11	2.58	0.03	0.04	0.32	1.07	0.38	0.90	18	43
10		0.62		2.42		0.05		1.02		0.50		24
9	0.52	0.56	1.10	2.00	0.02	0.05	0.32	1.05	0.21	0.67	10	32
8	0.48	0.50	1.18	1.92	0.05	0.05	0.39	0.96	0.38	0.81	17	39
7	0.42	0.44	1.00	1.42	0.03	0.04	0.00	0.61	0.15	0.40	7	19
6	0.35	0.35	1.00	1.11	0.03	0.02	0.00	0.31	0.15	0.77	7	37
5	0.25	0.27	1.19	3.75	0.02	0.02	0.48	0.98	0.56	1.00	27	48
4	0.16	0.20	1.00	1.50	0.01	0.01	0.00	0.67	0.06	0.25	3	12
3	0.12	0.14	1.18	2.90	0.02	0.01	0.40	1.16	0.23	0.88	11	42
2	0.07	0.08	1.03	3.73	0.02	0.01	0.18	1.25	0.63	1.00	30	48
1	0.00	0.00	1.11	2.31	0.00	0.00	0.33	1.00	0.19	0.94	9	45

[a] Based on chromatograms of 48 plants and corresponding butterflies reared thereon (chloroform–methanol–formamide TLC system).

Plants. As pointed out in the methods, the overall SIs in the cleaned plant extracts often were less than anticipated from the spectroassay results obtained with the crude plant extracts. This may have been due, at least in part, to recording a higher concentration of cardenolides (by a factor of about two) in the crude extracts than in the cleaned extracts. There are two possible explanations for this: (1) interfering noncardenolide coextractives

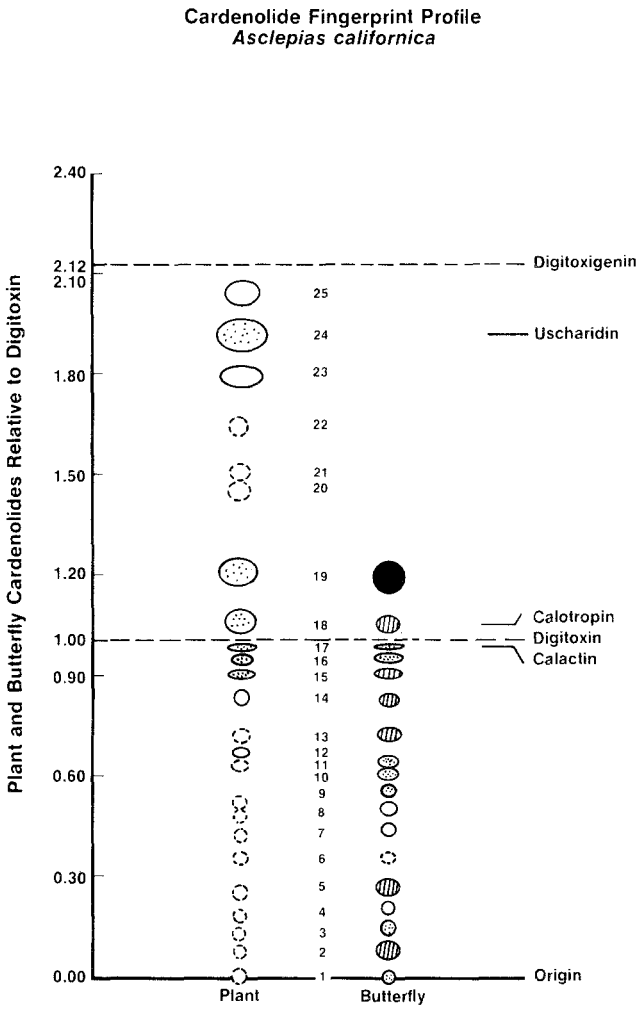

FIG. 5. The cardenolide fingerprint profile for freshly emerged monarch butterflies reared as larvae on *Asclepias californica* based on the CMF (chloroform–methanol–formamide) TLC system. The diagram shows mean $R_{digitoxin}$ and spot intensity values for 24 plant and 18 butterfly cardenolide spots and is based on the data in Table 8 for 48 plants and the 48 respective butterflies (24 males, 24 females) reared thereon. Spot intensities are represented as follows: 1.00 to <1.25, dotted circle; 1.25 to <2.00, closed circle; 2.00 to <3.00, stippled circle; 3.00 to <4.00, hatched circle; and 4.00–5.00, black circle. Fifteen of the 18 spots in the butterflies (all but Nos. 4, 6, and 7) have spot intensity values $\geq$1.50, probabilities of occurrence $\geq$0.50, and are highly diagnostic of the cardenolide fingerprint profile of freshly emerged monarch butterflies which have fed on *Asclepias californica*. Mean R_d values for calactin, calotropin, uscharidin, and digitoxigenin were, respectively 0.98, 1.06, 1.86, and 2.12.

produced a false-positive response in the spectroassay and were removed by the lead acetate cleanup, or (2) some cardenolide was lost during the cleanup. The first explanation is more plausible because the contribution of this responding background, which may occur to some extent in many *Asclepias* species, appears to be exaggerated at low plant concentrations. Seiber et al. (1982) reported 84% cardenolide recovery for another sample of *A. californica* plants which had a mean gross cardenolide concentration of 115 μg/0.1 g—roughly twice that found in this study. Also, our cleanup procedure was identical for both plant and butterfly samples so that, had we lost significant amounts of cardenolide during cleanup, the percentage losses should have been similar for both sample types. In fact, the average recovery for *A. californica* butterflies was 84%—similar to that for plant and butterfly samples from *A. eriocarpa* (Brower et al., 1982) and *A. speciosa* (Brower et al., 1984).

As a result of low concentrations, 13 of the 24 spots in the plants were frequently not detected on the TLC plates and had PO values of less than 0.40 (spots 1, 3, 4, 6–9, 11, 12, and 20–23; Table 7). This leaves 11 spots as particularly diagnostic: 2, 5, 13–19, 24, and 25. The 24 plant spots occur in two distinct R_f regions, those below digitoxin ($N = 16$, or 67%) and those above digitoxin ($N = 8$, or 33%). Unlike both *A. speciosa* and *A. eriocarpa* plants, no spots in *A. californica* occur above digitoxigenin in this TLC system.

Butterflies. The mean SI values for the butterflies (Table 7) are in all instances greater than their corresponding plant SIs. This undoubtedly accounts for their uniformly higher PO values. Thus 16 of the 18 spots (all but 4 and 7) have PO values of greater than 0.40. Moreover, only one of the 18 spots (No. 6) has an SI value of less than 1.25. This leaves 15 of the 18 spots as particularly diagnostic of the butterfly cardenolide pattern. Of these, spots 18 and 19 occur above and the remaining 13 occur below the digitoxin standard.

As was true of *A. eriocarpa* and *A. speciosa,* all the butterfly cardenolides occur in two TLC regions: 16 (89%) of them have R_f values below digitoxin, two (11%) above digitoxin, and none occurs above digitoxigenin. Overall, the butterflies are capable of storing cardenolides within approximately 58% of the R_d range of those found in the plants ($= R_d$ plant spot 19/R_d plant spot 25).

Plant–Butterfly TLC Relationships: Effects of Sex, Geography, and Plate

Because of the low PO values of some of the spots, particularly in the plants, this analysis could use only 317 of the 816 possible spot pairs (48 plant–butterfly pairs × the 17 spots in common). The numbers of matching pairs is shown in the footnote to Table 8. The analyses of SI values (Table 9)

TABLE 8. LINEAR REGRESSION ANALYSES OF $R_{digitoxin}$ VALUES OF 17 BUTTERFLY CARDENOLIDES (Y) AS FUNCTION OF $R_{digitoxin}$ VALUES OF RESPECTIVE PLANT CARDENOLIDES (X).[a]

Source of variation	*df*	SS	MS	*F*	*P*
A. Dependence of butterfly R_ds on plant R_ds as affected by four geographic areas (8 TLC plates nested therein), $r^2 = 0.966$)[b]					
Model	15	44.6374	2.9758	5808	<0.0001
Error	301	0.1542	0.0005		
Corrected total	316	44.7916			
Plant R_d	1	24.4700		47764	<0.0001
Area	3	0.0026		1.67	>0.172
Plate (area)	4	0.0046		2.24	>0.065
Plant R_d × area	3	0.0018		1.18	>0.315
Plant R_d × plate (area)	4	0.0080		3.91	<0.005
B. Dependence of butterfly R_ds on plant R_ds alone ($r^2 = 0.996$)[c]					
Model	1	44.612	44.61	78246	<0.0001
Error	315	0.179	0.0006		
Corrected total	316	44.791			
Estimated value of parameters		SE	*T* for H=O		*P*
a = *Y* intercept = 0.016		0.003	5.79		<0.0001
b = slope = 0.981		0.004	279.72		<0.0001
Equation for the line: $y = 0.981x + 0.016$					

[a] Visualized in the chloroform–methanol–formamide TLC system in 48 samples of plants and respective butterflies reared thereon. The regressions are based on 317 plant–butterfly spot pairs as follows: spot 1 = 9 pairs; 2 = 30; 3 = 11; 4 = 2; 5 = 27; 6 = 5; 7 = 2; 8 = 17; 9 = 9; 11 = 18; 13 = 23; 14 = 17; 15 = 18; 16 = 26; 17 = 29; 18 = 37; 19 = 37.
[b] Type IV sum of squares.
[c] Type II sum of squares.

used this same data subset. Initial regression analyses (see Methods and Materials) indicated that neither the slope nor the intercept of the regression of butterfly values on plant values for either R_d or SI are affected by the sex of the butterflies.

$R_{digitoxin}$ *Values.* Regression analysis with the plate variable nested within the area variable (Table 8A) indicates a highly significant dependence of butterfly R_d on plant R_d ($r^2 = 0.996$, $P < 0.0001$). There is also a significant interaction ($F = 3.91$, $P < 0.005$) involving butterfly and plant R_d values in the different plates as nested within the areas. The overall regression model therefore indicates that the slope of the regression line is slightly different for different areas because of different migration rates on the plates in the four separate TLC runs. This experimental variable is difficult to control, but fortunately its magnitude, even though statistically significant, is not great

TABLE 9. LINEAR REGRESSION ANALYSES OF SPOT INTENSITY VALUES OF 17 BUTTERFLY CARDENOLIDES (Y) AS FUNCTION OF SPOT INTENSITY VALUES OF RESPECTIVE PLANT CARDENOLIDES (X)[a]

Source of variation	*df*	SS	MS	*F*	*P*
A. Dependence of butterfly SIs on plant SIs as affected by four geographic areas (8 TLC plates nested therein), $r^2 = 0.184$)[b]					
Model	15	96.2285	6.4152	4.52	<0.0001
Error	301	427.0522	1.4188		
Corrected total	316	523.2808			
Plant SI	1	45.8543		32.32	<0.0001
Area	3	7.8345		1.84	>0.13
Plate (area)	4	9.6552		1.70	>0.14
Plant SI × area	3	17.0013		3.99	<0.009
Plant SI × plate (area)	4	6.0490		1.07	>0.37
B. Dependence of butterfly SIs on plant SIs alone ($r^2 = 0.085$)[b]					
Model	1	44.57	44.57	29.33	<0.0001
Error	315	478.71	1.52		
Corrected total	316	523.28			
Estimated value of parameters		SE	*T* for H=O		*P*
$a = y$ intercept = 2.763		0.131	21.14		<0.0001
b = slope = 0.355		0.066	5.42		<0.0001
Equation for the line: $y = 0.355x + 2.763$					

[a] Visualized in the chloroform–methanol–formamide TLC system in 58 samples of plants and respective butterflies reared thereon. The regression is based on the same plant–butterfly spot pairs as in Table 8.
[b] Type II sum of squares.

(see variation in R_d values for digitoxigenin, above). We next reran the regression model for the main effect alone (Table 8B) which gives the same high r^2 value (0.996). The estimated main regression equation is $Y = 0.981X + 0.016$, i.e., there is a virtual one-to-one correspondence of the plant and butterfly cardenolide R_d values.

SI Values. The overall relationship of butterfly SIs (Y) to plant SIs (X) is $Y = 0.355X + 2.763$ (Table 9B). The butterfly SIs showed considerably less correspondence with the plants than did the R_d values. The low r^2 value (0.184) for the first test (Table 9A) indicates only a weak (but statistically significant, $P < 0.0001$) correspondence of butterfly SIs on their respective plant SIs, and there was also a significant interaction of plant intensity × area ($P < 0.009$). Neither area per se, plates nested within the areas, nor the plant SI × plate (area) interaction was significant. The dependence of butterfly SIs solely on plant SIs (Table 9B) is also weak ($r^2 = 0.085$).

The A. californica *CMF Fingerprint Profile.* As summarized in Table 7 and Figure 5, the butterflies contain 18 of the 24 cardenolides present in the *A. californica* plants as resolved in the CMF solvent system. Fifteen of these (all but spots 4, 6, and 7) have SI values ≥ 1.50 and PO's ≥ 0.50. These 15 spots therefore are particularly diagnostic of the cardenolide fingerprint profile of freshly emerged monarch butterflies which have fed on *A. californica.*

Bioconcentration of Cardenolides in CMF System. Examination of SI values in Table 7 and Figure 5 confirms the overall quantitative bioconcentration of cardenolides by the butterflies and also suggests that it is greater for some spots, particularly those below an R_d value of approximately 0.85 (i.e., below spot 15) than for the others. Thus butterfly–plant SI value ratios greater than 2.0, from the highest to the lowest ratios, are those for spots 2, 5, 13, 14, 11, 1, and 3. These data do not indicate a consistently increasing trend of bioconcentration from higher to lower R_d values.

TLC Cardenolide Profiles of Plants and Butterflies: Ethyl Acetate–Methanol (EAM) System

As in the *A. eriocarpa* and *A. speciosa* studies, resolution of fewer cardenolides occurred in this TLC system than in the CMF system. We therefore did not quantitatively analyze the data as in Tables 7–9. Examples of three male and three female butterflies from area 1 (Mt. Diablo) and the six plants they were reared upon are in Figure 4C and D. A maximum of 14 spots in the plants and 13 in the butterflies were resolved in this system. Some of the butterflies from area 3 had an additional spot (9a) above spot 9 in this system.

Spot 10 corresponds to calotropin, spot 12 to calactin, and spot 13 to uscharidin. Calotropin occurred at consistently high intensities in the plants, whereas calactin and uscharidin were resolved at generally lower intensities. Spot 11 was present in only a few plant samples. Spots 1–9 occurred below digitoxin and matched the *Rd* values of the corresponding spots in the butterflies, as indicated in Figures 4C and D. However, due to the low gross concentrations in the plant material, these spots frequently were not resolved. In the butterflies nine spots (1, 3–7, 9, 10, and 12) were consistently resolved and usually of high intensity. Spot 10 (calotropin) was the darkest and most consistently resolved, followed by spot 12 (calactin). Spot 11 occurred weakly in a few butterflies. Neither spot 13 (uscharidin) nor 14 occurred in the butterflies. As in the CMF system, most of the spots of lower *Rf* than digitoxin were more concentrated in the butterflies than in the plants. Except for one extra spot at an *Rd* of approximately 1.00 in some of the butterflies from area 3, no obvious differences occurred in the TLC profiles of the 39 plant and 47 butterfly samples studied in the EAM system.

Conclusions from Both TLC Systems

The TLC data indicate that the majority of cardenolides present in the leaves of *A. californica* plants are stored by the butterflies. Spots 20–25 of the CMF system and spots 13 and 14 of the EAM system occur in the plants but not in the butterflies and the evidence suggests that CMF spot 24 and EAM spot 13 are uscharidin which is metabolized in the butterflies to calactin and calotropin (see Figures 4 and 5, and Discussion). As in the *A. eriocarpa* and *A. speciosa* studies, the cardenolide profiles in both TLC systems are highly consistent in the plants, both sexes of butterflies, and throughout California.

DISCUSSION

Quantitative Variation of Cardenolides in Plants and Butterflies. As was found both in the *A. eriocarpa* and *A. speciosa* studies, large variation occurred in the mean cardenolide concentrations of the 85 *A. californica* plants (9–199 μg/0.1 g), in the butterflies reared on these plants (59–410 μg/0.1 g), and in the total cardenolide per butterfly (143–823 μg). The leaves of *A. californica* have a mean concentration of 66 μg/0.1 g which is 73% of *A. speciosa* leaves (90 μg/0.1 g) and only 16% of *A. eriocarpa* leaves (421 μg/0.1 g). However, the butterflies reared on *A. californica* have higher mean concentrations (234 μg/0.1 g) than do butterflies reared on *A. speciosa* (179 μg/0.1 g), but both have lower concentrations than do those reared on *A. eriocarpa* (317.6 μg/0.1 g). *A. californica*-reared butterflies are also similar to those reared on *A. speciosa* in that in the process of sequestration and storage there is an increase of both the mean and the variance of their cardenolide concentrations compared to their plants, whereas they decrease both parameters when reared on *A. eriocarpa*. In other words, the shapes of the plant and butterfly histograms are reversed from those in the *A. eriocarpa* study (compare Figure 2 in this and the *A. speciosa* study with Figure 3 of the *A. eriocarpa* study). The higher concentrations of the *A. californica* butterflies compared to their plants may actually be even greater than 3.5-fold because of overestimation of the plant concentrations (see Methods and Materials).

Our evidence indicates significant populational variation in the gross cardenolide concentrations of *Asclepias californica* plants. The central Coast Range population has substantially higher concentrations than the three other populations, two of which in turn differ among themselves. However, there is no significant difference based on the two subspecies. These results differ from those previously established for both *Asclepias eriocarpa* (Brower et al., 1982) and *A. speciosa* (Brower et al., 1984) in which variation

in the cardenolide contents of the plants over an even wider geographic range was comparatively minor.

The fact that the populations of the distinct subspecies of *A. californica* show no consistent concentration differences suggests an evolutionary conservatism in the gross cardenolide contents of the plants and also implies that ecological factors outweigh genetically based divergence within geographically isolated populations. One such ecological determinant may be the time of the year that the plant material is collected. Thus in another sample of *A. californica* leaves from area 2 on July 25, 1979, Seiber et al. (1982) determined a mean concentration of 115 μg/0.1 g compared to our determination of 43.5 μg/0.1 g in the May 15, 1976, sample. Nelson et al. (1981) also found substantial temporal variation within leaf concentrations of *A. eriocarpa* over the course of one growing season.

The higher mean concentrations in female compared to male monarchs reared on *Asclepias eriocarpa* (Brower et al. 1982), on several other *Asclepias* species (Brower and Glazier, 1975; Brower et al., 1975; c.f. Cohen, 1983), and in collections from wild United States populations (Brower et al., 1972; Brower and Moffitt, 1974) was again found in butterflies reared on *A. californica*. However, the trend of higher concentrations in the butterflies to the north and lower concentrations to the south suggested in the *A. speciosa* study is not sustained by our data on *A. californica*.

Relationship of Cardenolide Concentrations and Total Cardenolide in Butterflies to Cardenolide Concentrations in Plants. Both concentrations and total cardenolide in the butterflies are proportional to the logarithm of the cardenolide concentrations in the plants, i.e., the butterflies significantly increase their cardenolide contents when feeding on low-concentration plants and reach an upper capacity when reared on plants containing high concentrations. This was also true for monarchs reared on *A. speciosa* but not *A. eriocarpa*. The difference occurs because *A. eriocarpa* plants all contain sufficiently high cardenolide concentrations that the lower portion of the uptake curve does not pertain (see also Brower et al., 1984).

The regression analyses also determined that concentration and total cardenolide in the butterflies are solely dependent on the concentrations of the cardenolides in the plants: no significant effects derive from storage differences based either on plants from different geographic areas or from male and female butterflies.

The data also suggest that monarchs are more adept at sequestration of cardenolides from *A. californica* and therefore able to store substantially more cardenolide than when they are reared on correspondingly low-concentration *A. speciosa* plants (compare Figure 3 of both studies).

Inferred Emetic Potencies of Monarchs Reared on A. californica. While we did not determine the emetic potencies of the butterflies reared on

A. californica in this study, we have elsewhere shown that cardenolides of the calotropagenin series (Roeske et al., 1976; Brower et al., in preparation) have a much higher emetic potency than those in *A. speciosa* (Brower et al., 1984). Thus *A. californica* may well be a very important milkweed for providing protection to the founding generation of monarchs during spring in the western United States.

Dry Weights of Butterflies and Relationships to Plant and Butterfly Cardenolide Concentrations. The average dry weight of the males in this study was slightly but significantly higher (1.07) than the females. Lighter females also characterized both the *A. eriocarpa* and *A. speciosa* studies. The butterflies' overall dry weights showed no correlation either with the cardenolide concentration in the plants ($r^2 < 0.0003$, $F = 0.02$, $P > 0.89$) or with the cardenolide concentration in the butterflies ($r^2 < 0.03$, $F = 2.48$, $P > 0.11$). The dry weight data therefore provide no evidence for a metabolic cost of either cardenolide ingestion or storage.

Relationship of Cardenolide Chemistry and Storage in A. californica. Coincidence of TLC R_f values in two solvent systems strongly indicates that spots 23, 18, and 17 in the CMF system and spots 13, 10, and 12 in the EAM system are uscharidin, calotropin, and calactin, respectively. Spot 25 in CMF may be voruscharin, although we were unable to obtain confirmation in the EAM system (Fig. 6). These four cardenolides are members of a series derived from the genin calotropagenin. Much is known of the chemistry of the calotropagenin cardenolides (Bruschweiler et al., 1969). They occur in the neo-tropical *A. curassavica* L. (Reichstein et al., 1968; Singh and Rastogi, 1969), in the African *Calotropis procera* L. (Hesse and Reicheneder, 1936; Hesse et al., 1939), *Gomphocarpus* spp. (Roeske et al., 1976; Cheung et al. 1983), and in *Pergularia extensa* (Roeske et al., 1976). Aspects of their uptake and storage have been studied in detail for monarch butterflies (Reichstein et al., 1968; Roeske et al., 1976; Seiber et al., 1980), the grasshopper, *Poekilocerus bufonius* Klug (von Euw et al., 1967), and in *Aphis nerii* (Malcolm, 1981). The evidence is that calactin and calotropin are the principal cardenolides stored when monarchs feed on plants containing calotropagenin glycosides. The 3′-keto glycoside uscharidin and its 3′-spiro derivatives uscharin and voruscharin are not stored per se by monarchs, but give way instead to calactin and calotropin (isomeric 3′-OH derivatives) by metabolism in the feeding larvae (Marty and Krieger, 1984). A similar metabolic and storage scheme has been proposed for an analogous yet distinct series of epoxy cardenolides in *A. eriocarpa* (Brower et al., 1982) wherein the 3′-keto glycoside labriformidin and its 3′-spiro derivative labriformin are metabolized to desglucosyrioside (3′-OH derivative) which is stored by monarchs. Because the larval metabolites are more polar than their parent cardenolides in the plants, the overall cardenolide TLC profile of monarchs is distinct from the plants, particularly in favoring cardenolides of lower R_f. The tendency of monarchs

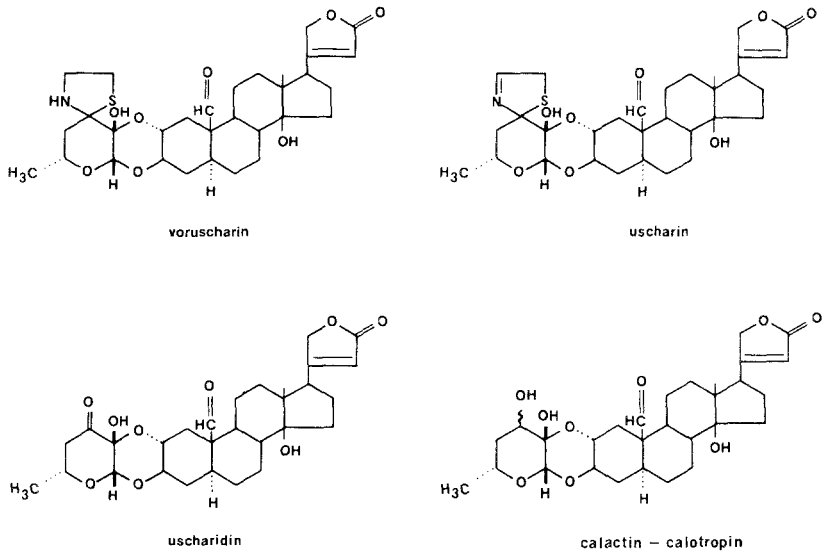

FIG. 6. Structures of five cardenolides determined by TLC analyses to occur in *Asclepias californica* plants. Uschariden, calactin, and calotropin matched perfectly with standards in both TLC systems. Voruscharin and uscharin assignments were not confirmed by cochromatography with standards. These five cardenolides occur together in *Asclepias curassavica* and several other species of Asclepiadaceae.

to store the more polar plant cardenolides, including calactin, calotropin, and desglucosyrioside, as well as compounds of even lower R_f, without metabolic alteration reinforces the trend towards an enrichment of lower R_f cardenolides in the butterflies relative to their larval foodplants.

A. californica plants and monarchs reared upon them conform to this general pattern. CMF spots 24 (uschariden), 25 (possibly voruscharin), and 20–23 are almost certainly metabolized to calactin, calotropin, and perhaps other cardenolide products which, along with cardenolides stored without metabolic alteration, constitute the cardenolide profile of freshly emerged adults. Because the calotropagenin series occurs in *A. vestita* and *A. cordifolia* (Seiber et al., 1982) and probably also in *A. cryptocerus* S. Wats. and *A. solanoana* Woodson (Roeske et al., 1976), the general storage pattern reported here for *A. californica* will likely hold for monarchs reared on these other *Asclepias* species. However, this commonality does not exclude the possibility of distinguishing among the cardenolide patterns for butterflies reared on other calotropagenin-containing *Asclepias* species. For example, *A. californica* plants and butterflies both contain a major TLC spot (spot 19, CMF) which does not occur in *A. curassavica*, although both plant species have calactin, calotropin, and other calotropagenin glycosides. This

spot 19 did not correspond to any standards in our possession and thus was not identified.

Conversion of high to lower R_f cardenolides also occurs in monarchs reared on *A. speciosa* (Brower et al., 1984) and the African milkweed, *Gomphocarpus physocarpus* E. Mey (Roeske et al., 1976; Brower et al., 1982). The general picture thus emerges that monarchs directly store plant cardenolides of moderate to high polarity, but chemically convert very nonpolar cardenolides to those of the moderate polarity range which they then store.

Chemotaxonomic Relationships of Calotropagenin Cardenolides. Woodson (1954) placed *Asclepias californica* in his subgenus 6, Solanoa, which also contains *A. solanoana* and *A. cryptocerus.* The first of these is a highly restricted and specialized California endemic, while the second is more widely distributed and occurs on the edges of the Great Basin from California northwards to Oregon, eastwards to western Colorado, and southwards to Arizona. As pointed out above, all three appear to contain cardenolides of the calotropagenin series. However, calatropogenin cardenolides also characterize the unrelated neotropical *A. curassavica,* the African *C. procera, Gomphocarpus* spp., and two other endemic California milkweeds, *A. vestita* Hook and Arn. and *A. cordifolia* (Benth.) Jepson (Seiber et al., 1982, 1983).

We conclude that cardenolides of the calotropagenin series cut widely across taxonomic lines established by Woodson (1954). Consequently, they either are of little use as chemotaxonomic character or, more likely, the infrageneric taxonomy of *Asclepias* needs reevaluation. Proof of the usefulness of cardenolide profiles in *Asclepias* taxonomy must await the analyses of more of the 108 North American species.

Comparison of Fingerprint Profiles of A. californica, A. speciosa, *and* A. eriocarpa. Twenty cardenolides were resolved in the *A. eriocarpa* plants and/or butterflies in the CMF system, 24 were resolved in *A. speciosa,* and 25 in *A. californica.* Monarchs store 16 cardenolides from *A. eriocarpa,* 21 from *A. speciosa,* and 18 from *A. californica. A. californica* plants have no cardenolides above digitoxigenin in this TLC system and thereby differ from both *A. eriocarpa* and *A. speciosa* plants which have at least two cardenolides above digitoxigenin. In both TLC systems for all three plants, the butterflies stored no spots above digitoxigenin, and in the CMF system the ratio of the R_d range of the spots in the butterflies divided by that of the plants is similar, i.e., 0.62 in *A. eriocarpa,* 0.76 in *A. speciosa,* and in 0.58 in *A. californica.*

Butterfly cardenolide profiles more closely resemble those in the corresponding *A. californica* and *A. speciosa* foodplants than in *A. eriocarpa.* For *A. speciosa,* we ascribed this to the virtual lack of cardenolides in the latex, and this situation also pertains in *A. californica* (Seiber et al., 1982).

In contrast *A. eriocarpa* plants have latex with very high concentrations of the less polar cardenolides, particularly those with an *N*, *S*-spiro ring at C-3′, which monarch larvae are readily able to metabolize and store as indicated above. Thus, in both *A. californica* and *A. speciosa*, only the plant tissue cardenolides are available for uptake and storage by the monarchs, whereas in *A. eriocarpa* cardenolides are additionally available from the latex.

Comparison of the TLC fingerprint profiles (CMF system) of butterflies reared on the three plants can be made with Figure 5 of this paper, Figure 6 in Brower et al. (1982), and Figure 5 in Brower et al. (1984). Major differences shared by both *A. californica* and *A. speciosa* in contrast to *A. eriocarpa* are the greater number of polar cardenolides below digitoxin (16 in *A. speciosa* and 17 in *A. californica* vs. 11 in *A. eriocarpa*). *A. speciosa* butterflies alone have the very prominent spot 10 at $R_d = 0.60$. Although the TLC profile of butterflies reared on *A. californica* is generally similar to that when they are reared on *A. speciosa*, the two profiles differ in several diagnostic ways. Overall, the TLC profile of *A. californica*-reared monarchs is much more uniform in appearance. Spot 18 ($R_d = 1.04$) is major in *A. californica* but has no counterpart in either *A. speciosa* or *A. eriocarpa*. Spots 13–17 in *A. californica* generally coincide with spots in the same general R_d region as those in *A. speciosa*. However, all five are of uniformly high SIs with high POs, whereas in *A. speciosa* they have generally lower SI and PO values. Importantly, although these five spots are roughly coincident in the CMF system, they are chemically distinct in the two milkweeds. Thus in *A. californica* they are calotropin, calactin, and other calotropagenin glycosides, whereas in *A. speciosa* they are epoxy cardenolides related to desglucosyrioside.

Distinction of wild-captured monarch butterflies which fed as larvae on *A. californica*, *A. eriocarpa* (Brower et al., 1982), and *A. speciosa* (Brower et al., 1984) by means of their cardenolide profiles should be clear-cut in the CMF TLC system if no major changes in cardenolide composition occur during the aging of adult monarchs. A recent investigation by Cohen (1983) in fact supports this: no qualitative differences were found in a population of adults of mixed ages which fed on *A. curassavica* in south Florida. These findings bode auspiciously for the utilization of cardenolide fingerprints of monarch butterflies for ecological studies (see also Brower, 1984a).

Ecological Implications. As far as is known, the repopulation of the entire western North American range of the monarch butterfly is dependent upon the reproduction of monarchs which survive the winter in dense aggregations along the California coastline from north of San Francisco to north of Los Angeles (Downes, in Williams et al., 1942; Williams, 1958; Urquhart, 1960; Urquhart et al., 1965, 1970). These colonies begin breaking up from approximately the middle of February through the middle of March with

both the timing and rate of breakup dependent upon local geographic and climatic conditions (Hill et al., 1976; Tuskes and Brower, 1978; Chaplin and Wells, 1982). Extensive research on the spring remigration is lacking, but the evidence suggests that increasingly fecund females fly in a predominantly easterly direction and disperse across the Coast Ranges, the Central Valley, and on into the Sierras and perhaps also into the Great Basin before they die by early summer (Urquhart, 1960; Urquhart and Urquhart, 1977; Brower, 1977; Brower and Huberth, 1977).

Dr. Arthur M. Shapiro's records for 12 years of the first-sighted spring monarchs in the Central Valley (Sacramento, Yolo, and Solano counties) are as follows: for 1972 through 1975: 6, 23, 13, and 3 March; for 1976, 9 February; and for 1977 through 1983, 21, 12, 18, 15, 16, 13, and 8 March. These observations, together with ours on the phenology of California milkweeds (see also Lynch, 1977), indicate that the spring remigration is well underway before any milkweed species have sprouted in the central Coast Ranges, i.e., in those areas closest to the overwintering colonies. Although females can probably oviposit on any *Asclepias* species they encounter (Brower, 1984b), it seems most likely that most remigrants will reach the eastern slopes of the Sierras at about the time the *A. californica* populations are sprouting (see Figure 1). Owing to its early appearance, large size, and abundance, *A. californica* may well be the most important milkweed for monarchs in establishing their spring generation in the western U.S.A. The large numbers of young caterpillars we saw in area 2 in mid-April 1976 is consistent with this interpretation. *A. cordifolia* also is a spring and early summer montane species utilized by monarchs. Although it is more widespread than *A. californica*, its populations tend to be less abundant and more patchy, and it also sprouts slightly later than *A. californica* (Brower et al., in preparation).

If the remigrating monarchs do in fact oviposit predominantly on *A. californica*, then the cardenolides stored from this plant may be of critical importance in providing chemical protection to each new spring generation of monarch adults in western North America. On the other hand, by midsummer both *A. californica* and *A. cordifolia* largely dry out and therefore can be of only marginal importance for monarchs of the last summer generation and therefore of the overwintering populations.

Now that we have established that *A. speciosa*, *A. eriocarpa*, and *A. californica* have distinctive cardenolide fingerprints, these and other ecological predictions are amenable to testing by comparing fingerprints of individuals from the spring and summer breeding populations with those of the fall overwintering populations.

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