the positive and negative ions were made by simply reversing the polarity of the battery and hence reversing the radial electric field in the sampling tube of the instrument, so the data should have a high relative accuracy and the trends in the profiles should be accurately portrayed even though the absolute conductivities may be in error.

The results in the lee of the lead indicated that the conductivities of ions of both polarities were somewhat larger than the baseline values. Also, at 70 cm above the ice, the conductivity of the negative ions was greater than that of the positive ions. The conductivity values decreased rapidly with height and became comparable to the baseline values at about 120 cm.

The fluid in the leads was an ice slush composed of water and pieces of ice with no visible bubble activity. This observation suggests a mechanism of ion formation at the leads which is different from the mechanism of surf electrification (*4*), or is composed of a number of mechanisms, some producing negative ions and some positive ones. Surf electrification originates from the bursting of bubbles at the sea surface and results in the production of a predominantly positive charge, although a similar mechanism in fresh water often results in the production of a negative space charge (*5*). It is possible that some very small bubbles do burst in the ocean-water portion of the open leads and produce excess positive ions. The production of the excess negative ions may possibly be a result of the breakup of microbubbles and the release of gas either during the melting process or during the freezing of new water (*6*). Most significant, however, are the indications that the leads are acting as sources of atmospheric ions.

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Variation in Cardiac Glycoside Content of Monarch Butterflies from Natural Populations in Eastern North America

Abstract. *A new spectrophotometric assay has been used to determine the gross concentration of cardiac glycoside in individual monarch butterflies. Adults sampled during the fall migration in four areas of eastern North America exhibited a wide variation in cardiac glycoside concentration. The correlation between spectrophotometrically measured concentrations and emetic dose determinations supports the existence of a broad palatability spectrum in wild monarch butterflies. The cardiac gylcoside concentration is greater in females than in males and is independent of the dry weight of the butterflies; contrary to prediction, both the concentration mean and variance decrease southward. The defensive advantage of incorporating cardiac glycosides may be balanced by detrimental effects on individual viability.*

Recent studies in ecological chemistry have demonstrated that larvae of monarch butterfiies, *Danaus plexippus* L., sequester cardiac glycosides from milkweed plants (Asclepiadaceae) (*1–3*). This ability confers unpalatability on the insects by making them emetic to avian predators such as the blue jay, *Cyanocitta cristata bromia* Oberholser. Furthermore, the birds react to the vomiting by rejecting numerous subsequent monarchs on sight alone, so that a single emetic experience confers considerable immunity on the prey species (*4*). It has been inferred from these findings together with the well-established emetic properties of digitalis and other cardiac glycosides (*5*) that the cardiac glycosides alone are responsible for the emetic response.

Wild monarch butterflies have been shown to exhibit an emesis dimorphism in which some individuals cause vomiting and others do not. The frequency of emetic butterflies varies in different populations. For instance, 24 percent of a sample of monarchs collected in Massachusetts during the 1969 fall migration proved emetic when force-fed individually to blue jays. Other populations varied in this respect, as few as 10 percent to as many as 90 percent of the butterflies being emetic (*3*, *6*).

It is possible that these populations are dimorphic because some larvae fed on poisonous milkweeds while others fed on nonpoisonous milkweeds such as *Gonolobus rostratus* (Vahl), Roemer and Schultes (*1*). It is also possible that the wild populations have a palatability spectrum (*2*) based on a continuous variation in the amounts of ingested cardiac glycosides, in turn dependent on the variation in kind and amounts of these substances present in the available wild milkweed food plants. According to the latter hypothesis, some of the nonemetic butterflies would contain cardiac glycosides, but in amounts insufficient to cause emesis. Such a finding could explain the results of Duffey (*7*), whose qualitative tests indicated the presence of cardiac glycosides in an unstated number of wild monarchs from Ontario and a single individual from Manitoba.

To explore quantitatively the possible variation in cardenolide content, we have developed a spectrophotometric assay to determine the gross concentration of cardiac glycosides present in individual monarch butterflies. Butterflies were removed from frozen storage (−20°C), dried for 16 hours in a forced draft oven at 60°C, and then ground individually to a fine powder with a mortar and pestle. Portions of the ground powders (0.100 g, about half of one butterfly) were individually weighed into 5-ml volumetric flasks, and about 4.5 ml of 95 percent ethanol was added to each flask. The flasks were then incubated at 70° to 78°C in a water bath shaker for 1 hour to extract the cardiac glycosides. The solutions were cooled to 20°C, brought to volume (5.00 ml), shaken vigorously by hand, and centrifuged at low speed for about 5 minutes in the volumetric flasks in a table centrifuge. The supernatant fluid (extract) was taken for determination of glycoside content.

The cardiac glycosides were determined with a modification of a procedure (*8*) based on the reaction of 2,2′,4,4′-tetranitrodiphenyl (TNDP). Be-

Table 1. Means ($\bar{x}$) and variances (s^2) of the cardiac glycoside concentrations (absorbance) per 0.1 g of dried male and female monarch butterflies. The butterflies were collected in fall 1970 from migratory populations in four geographic areas of eastern North America.

Sex	Ontario			Massachusetts			Maryland			Florida			Totals		
	$\bar{x}$	n	s^2	$\bar{x}$	n	s^2	$\bar{x}$	n	s^2	$\bar{x}$	n	s^2	$\bar{x}$	n	s^2
Male	0.193	38	.010	0.194	71	.008	0.138	25	.007	0.102	80	.004	0.153	214	.009
Female	0.202	40	.012	0.203	51	.009	0.161	31	.006	0.145	21	.008	0.185	143	.009
Totals	0.197	78	.011	0.198	122	.009	0.151	56	.007	0.111	101	.006	0.166	357	.009

cause TNDP is not available commercially, we prepared it by vigorous nitration of 2,2′-dinitrodiphenyl (*9*). Our experimental arrangement canceled the absorbance due to plant and animal pigments but still permitted accurate detection of the reaction absorbance. The blue color results from a complex (probably a charge transfer complex) between an anion on the butenolide portion of the cardiac glycosides and TNDP.

Absorbance measurements were made at 20°C in 1-cm stoppered cells in a Perkin-Elmer model 402 spectrophotometer (25-mm slit width) at 575 nm (*10*). The spectrophotometer was equipped with a water-cooled multiple sampling accessory that allowed four samples and references to be measured simultaneously. The sample cell contained 0.30 ml of 95 percent ethanol, 0.50 ml of butterfly extract, and 1.00 ml of a solution (0.15 percent, weight to volume) of TNDP in 95 percent ethanol. The reference cell contained 1.30 ml of 95 percent ethanol and 0.50 ml of the butterfly extract. To start the reaction 0.20 ml of 0.100*N* sodium hydroxide was added to each cell. Both cells were shaken gently and immediately placed in the spectrophotometer. Absorbance was monitored until a constant value was reached (about 40 minutes). We used calotropincalactin (*11*) as a standard and the reaction gave a strictly linear dependence of plateau absorbancy on glycoside concentrations of 2.5×10^{-4} to $4.7 \times 10^{-9}M$ [six dilutions; correlation coefficient, $r = .99926$ (*12*)]. Extracts from butterflies raised in the laboratory and fed on *Gonolobus rostratus*, which lacks cardiac glycosides (*1*), gave no significant absorbance; the mean and standard deviation for six males in this case were 0.015 and 0.007, and for six females they were 0.013 and 0.011.

Samples of wild monarch butterflies were collected during the 1970 fall migration from four different geographic areas in eastern North America. These were Salmon Point, Ontario, on the north shore of Lake Ontario; Hockanum, Massachusetts, in the Connecticut Valley near Northampton; Baltimore, Maryland; and Flamingo, Florida, the most southerly mainland site available, on the southern edge of Everglades National Park (*13*). At the outset of our experiment, we made three predictions: (i) that there would be quantitative variation in cardiac glycoside content in each of the four populations, (ii) that the mean cardiac glycoside content of the monarchs would increase from north to south, and (iii) that the variance of the cardiac glycoside content would also increase southward. Our reasoning for the second and third predictions was based on the fact that the number of milkweed species in the genus *Asclepias* increases southward (*14*).

The results show that there is a broad spectrum of gross cardiac glycoside content in these eastern migrating monarch butterfly populations (Table 1 and Fig. 1). The range of ab-

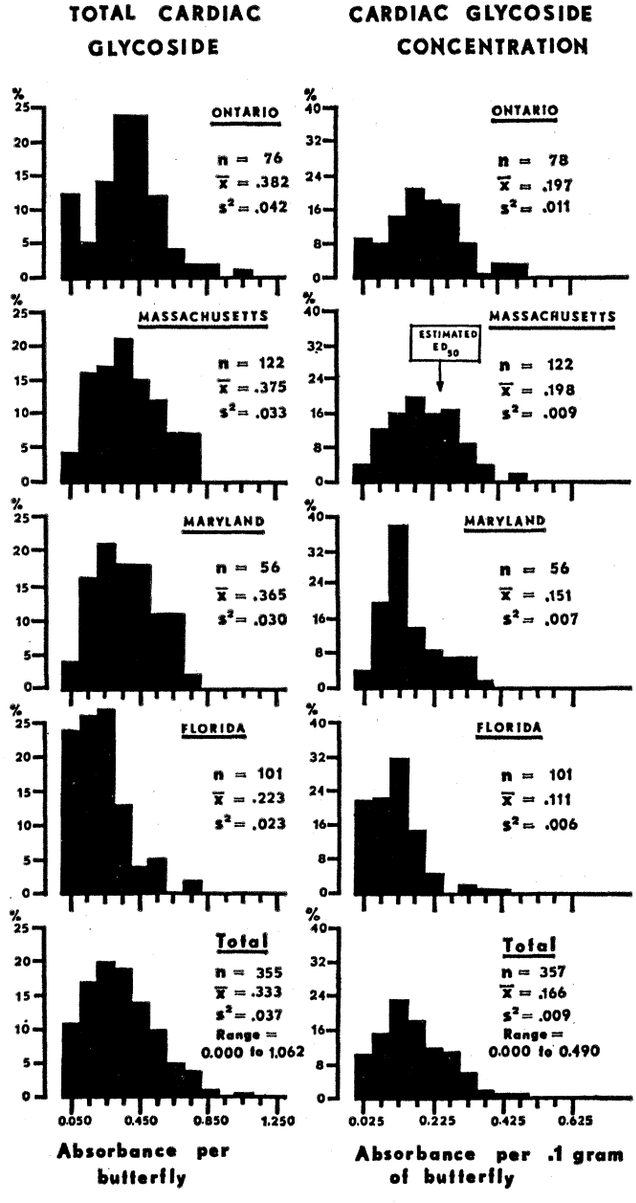

Fig. 1. Natural variation in cardiac glycoside content of wild monarch butterflies collected during the 1970 fall migration from four geographic localities in eastern North America. The ordinate is percentage of total sample, $\bar{x}$ is the mean absorbance, and s^2 is the variance. Absorbance was measured at 575 nm per 0.1 g of butterfly and calculated per total butterfly, according to the procedures described in the text. A sample absorbance of 0.200 corresponds to a $1.53 \times 10^{-5}M$ solution of calotropincalactin, or 8.15 μg of cardiac glycosides per milliliter of extract. For the Massachusetts sample, butterflies with an average weight of 0.20 g and with an absorbance of 0.230 per 0.1 g contain one ED_{50} unit for the average blue jay predator. A broad palatability spectrum is thus indicated in wild monarch butterflies.

Table 2. Two-way analysis of variance for Table 1 (Program 1380 BE/ST4, Wang Laboratories, 1970).

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F* ratio	*P*
A (sex)	0.033	1	0.033	4.202	.025–.05
B (geographic area)	0.302	3	0.100	12.833	$<.001$
AB (interaction)	0.014	3	0.004	0.614	$>.50$
Within cell	2.741	349	0.007		

sorbance per 0.1 g of butterfly is from 0.000 to 0.490. Because the average dry weight of all the monarchs studied was 0.206 g and the spectrophotometric assay required only 0.1 g, it was possible to bioassay the second half of the same materials. This was done for the Massachusetts sample only; the residual powders from the 33 butterflies with the highest concentrations of cardiac glycosides were mixed together, as were those from the second highest group (65 individuals). The absorbances of samples from each of these groups were 0.280 and 0.185, respectively. Using our standard bioassay technique (*2*), we determined the dosages of powdered butterfly material necessary to produce emesis with a probability of 50 percent (emetic dose 50, or ED_{50}) in blue jays. For the first group the ED_{50} was 0.152 g per 100 g of bird (*15*). This is equivalent to 1.55 emetic doses per butterfly per bird, based on an average blue jay weight of 85 g and an average butterfly weight of 0.2 g. We estimated approximately 0.7 emetic dose per butterfly per bird for the second group (*16*). Thus, for the Massachusetts materials an absorbance of 0.23 per 0.1 g of butterfly probably corresponds to about one ED_{50} unit.

From Fig. 1 we conclude, at least for the Massachusetts sample, that about 60 percent of the wild monarch butterflies contain less than one emetic unit (including some without any cardiac glycosides), and about 40 percent contain more than one emetic unit. It will be necessary to use larger samples to determine the number of emetic units present in the butterflies with the highest concentrations of cardiac glycosides, but these probably contain between five and ten emetic units. Our results provide strong support for the contention (*17*) that the palatability of wild monarch butterflies to vertebrate predators ranges in a spectrum from completely acceptable to wholly unacceptable.

These results confirm our first prediction, but contradict predictions (ii) and (iii); both the cardiac glycoside concentration in the monarch butterflies and the variation in that concentration decreased from north to south. Table 1 is a summary of the cardiac glycoside concentrations for both sexes, for the four geographic areas. The results of a two-way analysis of variance are given in Table 2. The concentration of cardiac glycosides is greater in females than in males for all four samples, and this difference between the sexes was also found in laboratory monarchs. We reared individuals from a single stock of butterflies (Oregon B-3) at Amherst College and fed them only greenhouse-grown *Asclepias curassavica* L. plants that were propagated from seeds collected from a single field in Trinidad, West Indies. The average absorbance per 0.1 g (dry weight) was 0.479 for 15 females and 0.367 for 15 males ($t = 4.28$; degrees of freedom, 28; $P < .001$).

Although the glycoside concentrations in the monarch samples from Ontario and Massachusetts do not differ significantly from each other, the concentrations in the Maryland and Florida samples are progressively lower. This is true for both sexes. The interaction between sex and geographic area is not significant; in both sexes the glycoside content tends to decrease in the same way southward. Table 1 also shows that the variances decrease southward for both sexes, except in the case of Florida females, for which the higher value is probably due to the small number involved. The variance of the total Ontario sample is significantly greater than that of the Florida sample (*F* ratio, 1.83; degrees of freedom, 76 and 99; $P < .025$).

A two-way analysis of variance of the weights of the butterflies indicates no significant difference between the sexes and no systematic variation from north to south, although the Maryland individuals are heavier than the others. The mean weight of all the males is 0.205 g and the variance is 0.002 ($n = 214$). The corresponding values for the females are 0.207 g and 0.003 ($n = 141$). The mean weights for both sexes from the four areas, from north to south, are 0.201, 0.191, 0.254, and 0.200 g. Thus, there is no tendency for weight to decrease southward. Furthermore, except for the heavy Maryland females ($r = -.404$, $P < .025$), there is no significant correlation (*12*) between the cardiac glycoside concentrations and the weights of the butterflies.

As the samples were composed of migrating nonresident butterflies, the further south they were taken the greater was the likelihood of their representing admixtures from an increasing geographic area. The unexpected southward decrease in both mean cardiac glycoside content and variation could be an ecological clue suggesting that selection may be operating against high cardiac glycoside content in the migrating insects. If this proves true, then the evolutionary problem (*4*) of maintaining the variation in unpalatability of monarch butterfly populations might be explained on the basis of a selective balance, with high concentrations of cardenolide conferring greater protection against predators, but resulting in decreased viability. The fact that both wild and laboratory-reared males have lower cardiac glycoside concentrations than females is not inconsistent with this hypothesis.

On the other hand, it may be that southern milkweeds contain cardiac glycosides of a higher toxicity, so that the butterflies need not absorb as much to be emetic. That monarchs and another insect may be selective in their uptake of cardiac glycosides has been suggested (*11*, *18*). Further combined chemical and biological assays are planned to explore this alternative.

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9. Ten grams of 2,2′-dinitrodiphenyl (Aldrich Chemical Co., Milwaukee, Wisconsin) were dissolved in 100 ml of red fuming nitric acid (density 1.6, Baker). The mixture was heated to 80°C, 100 ml of concentrated sulfuric acid and an additional 100 ml of red fuming nitric acid were added, and the mixture was refluxed for 1 hour. After it had cooled to room temperature, the reaction mixture was poured onto ice and water, and the product was collected by suction filtration. After six recrystallizations from boiling acetic acid, 3.37 g (29 percent yield) of pale yellow prisms were obtained, melting point 166.5° to 167.0°C (capillary, Thomas Hoover). The reported values of the melting point are 165°C [H. C. Gull and E. E. Turner, *J. Chem. Soc.* **1929**, 491 (1929)] and 165° to 167°C [(*8*); F. Ullmann and J. Bielecki, *Ber. Deut. Chem. Ges.* **34**, 2174 (1901)].
10. Absorbances were determined at 575 nm, rather than 620 nm, the maximum absorbing wavelength for the reaction, to facilitate comparisons with plant extracts at a later time. Chlorophyll interferes less with the absorbance at 575 nm than with that at 620 nm. The F peak (536 nm) of a holmium oxide filter was used to monitor the variation of the spectrophotometer at intervals throughout the experiment ($n = 39$; average absorbance, $\bar{x} = 0.313$; S.D. = 0.008).
11. The pure, crystalline calotropin-calactin mixture consisted of two isomeric cardiac glycosides (molecular weight 532.61) and was obtained from T. Reichstein. We assumed that all the cardiac glycosides in the butterflies have nearly the same extinction coefficients on reaction with TNDP. These two glycosides have been isolated from monarch butterflies reared on *Asclepias curassavica* and also occur in the leaves of this plant [T. Reichstein, *Naturwiss. Rundsch.* **20**, 499 (1967)].
12. Program 1316 EB/ST3 Rev. 1, 700 Series program library, vol. 9. Wang Laboratories, Inc., Tewksbury, Massachusetts, 1971.
13. The Ontario sample was collected between 31 August and 1 September 1970, the Massachusetts sample between 19 and 26 September 1970, the Maryland sample between 4 and 26 September 1970, and the Florida sample between 27 November and 1 December 1970.
14. According → R. E. Woodson [*Ann. Mo. Bot. Gard.* **41**, 1 (1954)], there are 12 species of *Asclepias* in northeastern North America and 21 in the Southeast. Monarchs reared on two of the latter, *A. curassavica* and *A. humistrata* Walt., are known to be extremely emetic (*1–3*).
15. Thirteen blue jays had the following dosages and responses: 0.174 g, two birds vomited; 0.157 g, four birds vomited, two did not; 0.142 g, one bird vomited, three did not; 0.129 g, one bird did not. Three additional birds at the 0.129-g dosage also did not vomit, and three birds at the 0.192-g dosage did. The 95 percent confidence levels of the ED_{50} are 0.142 to 0.163 g.
16. The ED_{50} test for the less concentrated sample broke down, probably because of the large amount of powder needed to provide a sufficient dose of toxin. More than 0.300 g of powder had to be force-fed, and apparently such large amounts of dry material interfere with absorption of the toxin. Of 14 birds tested, two vomited within 1 hour, and three within 2 hours. With lesser amounts of more toxic materials, vomiting has almost always occurred in the first hour.
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An Isomorphous Heavy-Atom Derivative of Crystalline Formylmethionine Transfer RNA

Abstract. *An isomorphous osmium derivative of crystalline yeast initiator transfer RNA has been prepared and interpreted to 6-angstrom resolution. The coordinates of the heavy atoms have been determined by Patterson and "direct" methods applied to the difference coefficients of the centric projections, followed by least-squares refinement. There is one dominant site per asymmetric unit, consistent with the finding by neutron-activation analysis that there is approximately one osmium atom per molecule of transfer RNA. The osmium derivative appears to be a normal substrate for enzymatic aminoacylation.*

A complete understanding of transfer RNA (tRNA) structure will almost certainly require a crystallographic solution based on the method of heavy-atom isomorphous replacement. We report the preparation of a useful "single-site" osmium derivative of yeast formylmethionine tRNA (yeast Os-tRNA^{fMet}) which has been interpreted in crystallographic terms. Initial chemical studies indicate that one osmium atom is bound irreversibly to the tRNA molecule at a site that does not interfere with enzymatic aminoacylation.

Crystals of yeast initiator tRNA (yeast tRNA^{fMet}) were reacted with a 100-fold excess of osmium by soaking in $\simeq 0.3$ ml of stabilizing supernatant solution (*p*H 5.50) composed of 2.90*M* $(NH_4)_2SO_4$, 0.005*M* cacodylate and Mg^{2+}, 0.002*M* spermine, 0.002*M* K_2OsO_4 (Alfa Inorganics), and 0.062*M* pyridine. The crystals gradually became colored, and appeared blue when viewed down the hexagonal axis and amber when viewed normal to the unique axis. The color change appeared to be complete within 2 weeks. No loss of color was noted when the Os-tRNA^{fMet} crystals were soaked for several months in an osmium-free supernatant solution.

Table 1. Neutron-activation analysis of crystalline yeast Os-tRNA^{fMet}. Sample (a) was a crystal soaked for at least 7 days in osmium-free supernatant solution, then dissolved in water. Sample (b) received the same treatment as sample (a) but was also passed over G-25 Sephadex that was equilibrated with a solution of 0.05*M* $(NH_4)_2SO_4$, 0.005*M* Mg^{2+}, and 0.005*M* cacodylate, *p*H 5.6. Sample (c) received the same treatment as sample (a) but was also dialyzed against a solution of 0.2*M* NaCl, 0.001*M* ethylenediaminetetraacetic acid, 0.005*M* formic acid, and 1 percent ethylene glycol at *p*H 4.0; then passed over G-25 Sephadex that had been first equilibrated with the same solution. Sample (d), the control, was a dissolved crystal that was not exposed to osmium. The A_{260} unit has been defined (*5*) and is taken to equal 2.0 nmole of yeast tRNA^{fMet}. The precision of osmium analysis at this level is 3.3 percent. (The analysis was performed by G. Reed.)

Sample	A_{260} units	Osmium	
		10^{-7} g	Gram-atoms per mole of yeast tRNA
a	0.94	3.84	1.1
b	0.35	1.35	1.0
c	0.60	2.24	1.0
d	2.50	0.00	0.0

The stoichiometry of the complex was established by neutron-activation analysis. Table 1 shows that one osmium atom was bound per molecule of tRNA—even after the tRNA was "denatured" and subjected to gel filtration over G-25 Sephadex that had first been equilibrated with a denaturing buffer containing 1 percent (by volume) ethylene glycol to scavenge Os VI (*1*). Thus the binding is apparently "irreversible," a result suggesting that the osmium is either covalently bound or very tightly enmeshed in the secondary structure.

Since Os VI • 2pyridine is known to form stable adducts with *cis*-diol groups (*1*) (indeed, this was the rationale behind trying the reaction), the yeast Os-tRNA^{fMet} crystals were dissolved and tested to see whether or not the terminal 3′OH was free to accept methionine. Yeast Os-tRNA^{fMet} accepted 1.45 nmole of methionine per unit of absorbance at 260 nm (A_{260} unit), whereas tRNA^{fMet} obtained from parent crystals accepted 1.55 nmole of methionine per A_{260} unit. Since the difference in acceptance between the derivative and parent is less than the differences normally encountered between various batches of crystalline tRNA^{fMet}, it is clear that under typical assay conditions (*2*) the Os-tRNA^{fMet} could be enzymatically charged to the same extent as the parent molecule. In view of the firmness with which osmium is bound, we conclude that the 3′OH terminus is not the primary site of osmium attachment. Although a Michaelis constant has not yet been determined for the yeast Os-tRNA^{fMet} adduct, rapid and full enzymatic acylation of Os-tRNA^{fMet} suggests that the site of osmium attach-