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Paula S. Fritsch
Winona State University

Dave Dapkus
Winona State University

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1994 Winchell Award Winner

EFFECTS OF SELECTION FOR HALOTHANE RESISTANCE ON LIPID CONCENTRATION AND COMPOSITION IN *DROSOPHILIA MELANOGASTER*[†]

PAULA S. FRITSCH[‡] AND DAVE DAPKUS

ABSTRACT

Drosophila melanogaster is increasingly used as a model system for anesthesia studies. Lipids may play a role in anesthetic resistance by sequestering the hydrophobic anesthetics from nervous tissue. Alternatively, changes in membrane lipid composition (phospholipids and/or cholesterols) may contribute to resistance through alteration of neuronal membranes. This project studied the relationship of lipids to anesthetic resistance in a strain of *Drosophila melanogaster* with a high level of halothane resistance produced by 13 generations of mass selection. The estimated dose of anesthetic needed to produce anesthesia in one-half of the flies tested (ED_{50}) for this strain was 2.3 times that of the unselected control. Total lipids were extracted and the concentration of total lipids was determined. The average percent of total lipids ± standard error found in *D. melanogaster* was 8.73% ± 0.08 for the resistant strain and 7.57% ± 0.03 for the control, a relative increase of 15.3%. After determining that the total lipid content was greater in the resistant population ($p = 0.0004$), the fatty acids of the phospholipids were analyzed. The purpose of this study was to determine if alterations in fatty acids were associated with the increase in anesthetic resistance, as predicted by the fluidization hypothesis. Gas chromatography was used to analyze the percentage of saturated fatty acids in phospholipids. Both populations had the same mean normalized percent of saturated fatty acids. This result did not support the hypothesis that resistance was due to altered phospholipid fatty acids.

INTRODUCTION

Although anesthetics are commonly used, their mechanism of action has yet to be determined (1). Current thinking is that inhalation anesthetics probably act on the plasma membrane of neurons (2). This membrane consists of a lipid bilayer with integral and peripheral proteins (3). It is unknown whether anesthetic molecules interact with lipid or protein components (2).

Drosophila melanogaster is a commonly used model for anesthetic studies. Rasmuson (4) was the first to study altered anesthetic responses in *D. melanogaster*. In the course of a bristle selection experiment, Rasmuson detected and analyzed a strain particularly sensitive to ether. Gamo et al (5) later studied a strain derived from a single ether-resistant female. They determined that resistance could be intensified by selection and that the strain exhibited multiple resistance to ether, chloroform, and halothane. Further analysis revealed changes in the relative amounts of some phospholipid fatty acids (6). Recently, Krishnan and Nash (7) conducted experiments on four different strains, produced by chemical mutagenesis, that were resistant to halothane. They concluded that D. melanogaster undergoes a typical anesthetic response which could be altered by mutation and subjected to genetic analysis. Finally, Tinklenberg et al (1) pursued the possible role of a membrane protein in anesthetic resistance. They found altered anesthetic resistance in a strain having the "shaker" mutation. The shaker mutation is known to have altered potassium channels (8).

This research focused on the role of lipids in anesthetic resistance. Our hypothesis was that a change in lipid concentration and/or composition was responsible for the altered halothane resistance observed in the selected, resistant strain. We used a strain of *D. melanoguster* highly resistant to halothane. This strain had been produced by artificial selection. Our goals were to 1) determine if there was a difference in the total lipid concentration between the control and resistant flies and 2) analyze the fatty acid composition of phospholipids by gas-liquid chromatography (GC). We chose to focus our efforts on the phospholipids because they are the primary lipids of neuronal membranes. Our work on fatty acid composition was designed to test one of the prevailing lipid-based theories of anesthetic action, the fluidization theory. This theory asserts that anesthetics act to fluidize the neuron's membrane and thus temporarily block impulse transmission (9). We

[†] Contribution from the Department of Biology, Winona State University, Winona, Minnesota 55987.

[‡] Corresponding author.

reasoned that alteration of phospholipid fatty acid saturation might make the neuronal membrane more resistant to fluidization.

Materials and Methods

D. melanogaster strains: The anesthetic resistant *D. melanogaster* used were produced by artificial selection. Nearly 200 wild flies were collected from Quarry Hill Nature Center in Rochester, Minnesota to form a base population. This initial population was then divided into two groups. The first group was maintained as an unselected control population, Quarry Hill control (QHC). The second group was selected over 13 generations for halothane resistance. The selection was performed by placing batches of one to two thousand flies in an inebriometer (10). The flies were then exposed to halothane. Flies that fell through the column first had lesser resistance and the last flies to succumb had greater resistance.

The 100 most resistant males and 100 most resistant females from each generation were selected to serve as the parents for the next generation. Initially, the halothane concentration used was 0.5%. As the selection process proceeded through the generations, greater halothane concentrations, up to 3.0%, were required to collect the most resistant flies. The most recent generation formed the resistant population, Quarry Hill resistant (QHR). The ED_{50} of QHR was about 2.3 times that of QHC. Both populations were maintained side by side on standard corn meal-agar-molasses medium at room temperature (about 20 °C).

Lipid Concentration: Lipid concentrations were determined using a gravimetric technique (11). Lipids were extracted from samples of QHR and QHC using a modification of the technique of Folch et al (12). Fly samples and lipid samples were weighed to 100 µg. Samples of about 500 mixed sex flies weighing 340.6 to 517.6 mg were analyzed. Pairs of samples, one QHR and one QHC were taken from bottles set up at the same time and maintained side by side. Weighed samples of flies were stored under nitrogen at -20 °C until analyzed. Flies were ground in a Waring Blender with 10 mL of 2:1 chloroform:methanol (v/v) for 3 min. The blender was rinsed twice by blending additional 10 mL and 5 mL aliquots of 2:1 chloroform:methanol for 1 min. The three extracts were then pooled and mixed for 5 min. This mixture was then filtered through Whatman #1 filter paper into a 50-mL glass, tapered bottom, centrifuge tube. The filter was re-extracted with 10 ml chloroform into the same tube. Seven mL of Folch's multiple salt solution (12) were added to the tube and the contents were mixed. Tubes were centrifuged for 10 min at 1,130 x g. The upper aqueous layer was removed and the lower organic layer was transferred to a pre-weighed Erlenmeyer flask. The sample was dried under vacuum in a desiccator containing anhydrous Ca_2SO_4 to a constant weight (12 to 24 hr). The weight of the lipids extracted was determined by reweighing the flask. The concentration of lipids in the fly sample was determined by dividing the weight of extracted lipids by the live weight of flies analyzed. The concentration was expressed as a percent of live weight.

Lipid Composition: Lipids were extracted as above except from 20 male flies. Solvent was removed under vacuum in a Rotavapor 115Ex. Lipids were re-dissolved in 2 mL chloroform, transferred to a screw-top culture tube, sealed under nitrogen and stored at -20 °C.

Thin layer chromatography (TLC) was used to separate the four major lipid classes: phospholipids (PL), free fatty acids (FA), triglycerides (TG), and cholesterol esters (CE). Chloroform was removed from the stored extracts under a stream of nitrogen. The lipid residue was re-dissolved in 200 ml chloroform. This sample was streaked on a Whatman TLC plate (20 cm x 20 cm silica gel G, 250 micron). Plates were placed in glass tanks with a developing solvent consisting of 80:20:1 Skelly F (Petroleum ether 30 - 60 °C; Mallinckrodt Co., Paris, Kentucky): diethyl ether: glacial acetic acid (v:v:v). After developing, the plates were allowed to dry and were sprayed with a solution of 0.2% 2',7'-dichlorofluorescein in ethanol. Bands were visualized under ultraviolet light. The plates developed with the following order of relative distances from the origin to the solvent front: PL > FA > TG > CE. The PL band was identified by its relative position and scraped from the plate into a screw top culture tube containing 2 mL of 20% HCl:MeOH (w:v). The tubes were then sealed under nitrogen, mixed with a vortex mixer, and placed in a 100 °C heating block for 1.5 hr. This resulted in hydrolysis of the PL and trans-esterification of the fatty acids to fatty acid methyl esters. One mL distilled water and 2 mL Skelly F were added to the cooled tubes. Tubes were capped under nitrogen, mixed on a vortex mixer and the liquids allowed to separate. The upper, organic layer, containing the methyl esters of the fatty acids (ME), was transferred to a clean screw cap culture tube, sealed under nitrogen and stored at -20 °C until GC analysis.

The methyl esters were analyzed using a Hewlett-Packard 5840A GC. A 30 m open tubular-wall, coated column with an inner diameter of 530 µm. The stationary portion was a free fatty acid phase (FFAP), the carrier gas was helium and a flame ionization detector was used. The oven was temperature programmed from 145 °C to 190 °C at a rate of 1.5 °C min^{-1} with an initial time of 5 min. The injector and detector temperatures were maintained at 250 °C.

Phospholipid methyl esters (PLME) were removed from storage, solvent was removed under nitrogen,

Table 2. Analysis of Variance Table for Lipid Concentration.

Source	DF	SS	F	p
Day (A)	3	0.0507	1.88	0.3080
Halothane resistance (B)	1	2.7028	301.01	0.0004
A*B	3	0.0269		
Total	7	2.7805		
Grand Average	1	531.54		

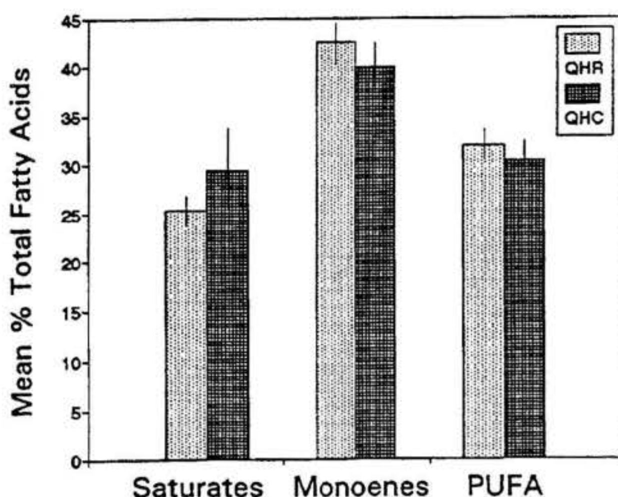

Fig. 2. Mean normalized percentage of phospholipid fatty acids in three saturation classes for each population. Data were determined by GC analysis.

Table 3. Analysis of Variance Table for Normalized Percentage of Saturated Fatty Acids

Source	DF	SS	F	p
Day (A)	3	30.44	0.15	0.9216
Halothane resistance (B)	1	34.21	0.51	0.5251
A*B	3	199.55		
Total	7	264.20		
Grand Average	1	6003.10		

logical deduction from this theory was the expectation that a more rigid membrane is more resistant to the effects of anesthetics and thereby allows impulse transmission to continue at a greater halothane concentration. Membranes can be made more rigid by increasing the concentration of saturated fatty acids.

When a lipid bilayer, such as a neuronal membrane, is composed of phospholipids with saturated fatty acids, the fatty acids of the two layers, at appropriate temperatures, fit together in a paracrystalline array such that lateral translation of the phospholipids is minimized and the membrane is more rigid. The presence of unsaturated fatty acids in the membrane disrupts the paracrystalline structure and thereby allows more lateral movement and membrane fluidity (3). The QHR population was expected to have an increased percentage of phospholipid saturated fatty acids leading to a more rigid, but anesthetic-resistant membrane. However, no increase in the percentage of saturated fatty acids in the resistant population was observed.

Gamo et al, found decreased PUFAs in the Eth-29 strain (6). These results were consistent with the fluidization hypothesis. The Eth-29 strain was originally derived from a female which showed increased resistance to ether anesthesia. Resistance in this strain was intensified by 29 generations of selection for resistance to death by over-etherization. The resultant strain was multiply resistant to ether, chloroform, and halothane (6). So, although the origin was different from QHR, the history of selection was somewhat similar and the resulting strain showed a change in phospholipid fatty acid saturation. Because so little is known about the mechanism or mechanisms of anesthesia, it seems possible that these two populations became anesthetic resistant by different mechanisms even though the method of selection for resistance was similar. Thus, a difference in mechanisms could account for the apparently conflicting results.

Another possible explanation for our results could relate to the anesthetics partitioning into the lipids. In theory, halothane should partition into lipids and lipid containing structures. This would include adipose tissue, myelin sheath tissue, and nervous tissue membranes. Therefore, the only halothane which is effective for anesthesia would be that which reaches the nervous tissue membranes. A 15.3% increase in total fly lipids should reduce the amount of halothane available to partition into the nervous tissue membranes, if all other factors are held constant. Selection of a fat fly population could explain what occurred because fatter flies are expected to be less susceptible to anesthesia.

Future studies of anesthetic resistance in *D. melanogaster* could expand on this study. Some areas of interest are: 1) analysis of a portion of the organism that is rich in neuronal tissue, 2) analysis of the sterols, and 3) study of the role of proteins.

Because anesthesia involves the central nervous system (7), local changes in fatty acid saturation might be undetectable in the whole animal lipid extract. The sensitivity of the test might be improved by testing an

area rich in neuronal tissue, such as the head. Lipid composition studies done on the QHR and QHC populations using just heads might yield different results.

This study considered only one mechanism by which membrane rigidity might be altered. Membranes are also made more rigid by increasing the sterol concentration in the membrane (3). It is possible that the increased lipid concentration in the QHR population is due to an increased sterol concentration. Further experiments could quantitatively compare the cholesterol concentration in the QHR and QHC populations. Based on the fluidization theory, one would expect to observe an increased percentage of sterols in the resistant population as compared to the control.

Finally, lipid theories of anesthesia are just one subset of possible mechanisms of alterations of anesthetic resistance. Considerable work has also been done on the role of proteins in producing anesthesia. It is entirely possible that the lipid and protein components of the neuronal membrane could be working in tandem to produce anesthesia (9).

In conclusion, an increase in lipid concentration in an anesthetic resistant population was detected, indicating a possible role of lipids in anesthetic resistance. Based on the fluidization hypothesis, an analysis of phospholipid fatty acid saturation was conducted. This test, however, failed to detect any difference between the populations. Further analysis of the QHR population could include determination of the cholesterol concentration because of the rigidifying effects of sterols on the membrane. Also, an analysis of protein differences might reveal a role for proteins in QHR's increased resistance.

Acknowledgement

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