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STUDY OF HIBERNATION-INDUCED ALTERATIONS OF
ERYTHROCYTE LIPIDS IN THE 13-LINED GROUND
SQUIRREL, SPERMOPHILUS TRIDECEMPLINEATUS

by

James Clarence Veltman

A Thesis Submitted to the Faculty of the Graduate
School of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of

Master of Science

May

1979

Dedication

With All My Love

To my Mary Jo

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VITAE

The author, James Clarence Veltman, was born on February 16, 1951, the oldest son of Clarence and Diane Veltman of Chicago, Illinois.

His elementary education was received at Holy Rosary Grade School in Chicago, where he graduated in June, 1965. His secondary education took place at Mendel Catholic High School in Chicago. He received his diploma in June, 1969.

In the following September, he attended Loyola University of Chicago, where he majored in Biology. He received a Bachelor of Science degree in June, 1974.

He enrolled in the Masters program at Loyola University and was the recipient of a teaching assistantship from September, 1974 - June, 1977.

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LIST OF ABBREVIATIONS

- I. Fatty Acids: Fatty Acids are listed in the abbreviated format. In it, the first number is the carbon length; the second number is the number of double bonds (Lehninger, 1976).
- II. Phospholipids: DPG = diphosphatidyl glycerol
(cardiolipin)
LPC = lysophosphatidyl choline
(lysolecithin)
LPE = lysophosphatidyl ethanolamine
PA = phosphatidic acid
PC = phosphatidyl choline (lecithin)
PE = phosphatidyl ethanolamine
PI = phosphatidyl inositol
PS = phosphatidyl serine
Sph = spingomyelin
- III. Anti-oxidants: BHT = butylated hydroxytoluene
- IV. Anti-coagulants: EDTA = Ethylene diamine tetra-acetic acid

ABSTRACT

Erythrocyte membranes of warm-acclimated, cold-acclimated or hibernating 13-lined ground squirrel were assayed for their content of fatty acids and phospholipids.

During cold-acclimation, fatty acid composition changed significantly with respect to that of warm-acclimated controls. Concentrations of palmitic acid (16:0), oleic acid (18:1), and linolenic acid (18:3) increased, while those of docosahexaenoic acid (22:6) and docosapentaenoic acid (22:5) diminished. Hibernation potentiated these changes and caused the disappearance of arachidonic acid (20:4).

Cold-acclimation produced no significant alterations of membranous phospholipid content. However, during hibernation, concentrations of phosphatidyl ethanolamine (PE), phosphatidyl choline (PC) and diphosphatidyl glycerol (DPG) diminished, while those of lysophosphatidyl ethanolamine (LPE), lysophosphatidyl choline (LPC) and phosphatidic acid (PA) increased.

These changes may insure an adequate stability and permeability of the erythrocyte membranes during torpor, which in turn may enhance the survival of the species.

INTRODUCTION

Over the last two decades, hibernation has acquired many definitions. Among homeotherms, hibernators are unique in their ability to maintain the essential functions of life with insignificant tissue damage, while enduring prolonged depression of body temperature (Swan et al., 1977). Such depression of body temperature occurs on a seasonal basis and actually reflects an endogenous circannual rhythm (Prosser, 1972; Pengelley, 1974). A number of environmental parameters affect this rhythm by influencing the complex thermoregulatory patterns which occur during the heterothermic period. Among such factors are light (Pengelley, 1974), food deprivation (Pengelley, 1968), and temperature (Pengelley and Asmundson, 1969).

Depending upon prior acclimation history, the last steps of entering hibernation involve dramatic internal changes (Hoffman, 1964; Hudson, 1973; Folk, 1974). As body temperatures cool, the cardiac rate becomes inhibited to a level not governed by cold alone (Hoffman, 1964; Lyman, 1965; Hudson, 1974) while progressive differential vasoconstriction reduces the blood flow to various organs (Folk, 1974; Hudson, 1974).

The thirteen-lined ground squirrel, Spermophilus tridecemlineatus, is a typical hibernator (Lyman, 1961; Pengelley, 1974). As its body temperature declines and approaches low ambient temperatures, the ground squirrel's cardiac rate diminishes from 400 to 2 beats/min (Spurrier & Dawe, 1973). During entrance into hibernation, distinctive hematological and serological alterations also occur (Spurrier & Dawe, 1973). These include a reduced hematocrit, an increased mean corpuscular volume, an increased resistance to hemolysis and a 40-50% folding over of the erythrocytes. Two distinct hemoglobin fractions have been described (Spurrier & Dawe, 1973), as well as a decreased level of 2, 3- diphosphoglycerate and a 55% reduction of 5' - ATP within erythrocytes (Larkin, 1973).

Many of these hematological changes which occur within ground squirrels, including the morphological alterations, may be a consequence of hypoxia and acidosis during torpor (Larkin, 1973). The site where these changes may occur in these erythrocytes is most likely the membrane (Livne et al., 1972; Swan et al., 1977), possibly due to alterations in its lipid composition (Aloia et al., 1974; Swan et al., 1977). For example, increased unsaturation of the lipid-acyl groups of the membrane has been found to result in a greater osmotic stability in heat-exposed hamsters (Livne et al., 1972). Such changes preserve red cellular membrane flexibility, permeability and internal fluidity during hibernation. Moreover,

the preservation of membrane fluidity may prevent any discontinuity of activation energy and thus modulate membrane-bound enzymes during hibernation of the ground squirrel (Aloia et al., 1974). An excellent review of the effects which lipids exert upon membrane-bound enzymes has been published recently (Sanderman, 1978).

Changes occur in the phospholipid core as well as the fatty acyl groups of various tissues of hibernating animals (Platner et al., 1972). If hibernating animals resemble cold-acclimated poikilotherms (Hoffman, 1964; Bligh, 1973), then tissues of hibernators may show greater levels of polyunsaturated fatty acids, as do those within poikilotherms (Kemp & Smith, 1970; Caldwell & Vernberg, 1970).

If such trends are present in the erythrocytes of hibernating ground squirrels, they could contribute to the survival ability of the species. A structurally modified erythrocyte would be of functional value not only during hibernation, but also provide ample oxygenation to key organs during arousal. It is the purpose of the present investigation to determine whether such changes occur in the erythrocytes of the hibernating thirteen-lined ground squirrel, Spermophilus tridecemlineatus.

REVIEW OF LITERATURE

I. Definition of Hibernation

Hibernation is a naturally recurring state of torpor which occurs on a circannual basis among a select group of homeotherms (Pengelley, 1974). Mammals experiencing hibernation exhibit decreased body temperatures which approach low ambient levels. Upon arousal, such animals are able to re-warm themselves spontaneously without external assistance (South, 1972). Body cooling occurs with little metabolic resistance and can be endured for prolonged periods of time without any permanent damage of tissues (Swan et al., 1977).

In contrast, animals experiencing hypothermia must have their thermogenic abilities overwhelmed. Further, their lowered body temperatures can be endured only on a short term basis (Hoffman, 1964; Lyman, 1965).

At least two schools of thought exist to explain mammalian hibernation in the evolutionary sense. One school contends that mammalian hibernation developed from primitive thermoregulators. This group contends that hibernation is analogous to the dormancy experienced by poikilotherms (Hoffman, 1964; Bligh, 1973), in that comparable strategies of biochemical restructuring have been developed to cope with the state of torpor (Somero, 1972). The second school views

hibernation as a special adaptation of homeothermy, which results from resistant adaptation to lowered temperatures (Willis, 1972; Bligh, 1973).

A number of external stimuli, described as "zeitgebers", occur on a circannual basis. These, in turn, entrain endogenous responses within hibernators. Among such "zeitgebers" are changes of temperature, photoperiod (Pengelley, 1974) and food supply (Bligh, 1973). Presently it appears that no single external factor can act independently of the others (Pengelley, 1972; 1974). Among the circannual fluctuations which they elicit are changes of body temperature (Galster & Morrison, 1975), weight (Pengelley, 1969), and fat (Mrosovsky, 1976). As the degree of external environmental factors lessen in severity, it appears that the flexibility of endogenous rhythms increases (Pengelley, 1972).

Interestingly, this same flexibility may result from the waxing and waning of a specific "trigger" molecule, whose presence completes a series of biochemical reactions leading to hibernation (Dawe & Spurrier, 1974; Pivorun, 1977; Dawe, 1978). Indeed, the preparative stages preceding hibernation, as well as hibernation itself, may result from the rapid accumulation of these molecules (Dawe & Spurrier, 1972). Subsequent to hibernation, arousal and active states are characterized by a decreased concentration of uncomplexed trigger molecules (Dawe & Spurrier, 1972).

II. Cardiovascular Changes during Hibernation

The cardiovascular system, like all body systems, shows dramatic changes during all phases of hibernation (Lyman, 1965; Hudson, 1973). During entry into hibernation, there is a marked inhibition of cardiac rate, as well as a reduction of respiration and oxygen consumption (Lyman, 1965). Increasing parasympathetic activity causes the cardiac rate to decline, as evidenced by a progressively increased diastolic run off time (Hudson, 1973). The stroke volume is minimally altered, so that any ventricular force generated is only slightly different than during active periods (Armour et al., 1974).

This decline in heart rate during hibernation is accompanied by a marked reduction in systolic and diastolic pressures (Lyman, 1965). Mean blood pressures remain near the active range, reflecting a dramatic increase of peripheral resistance (Lyman, 1965). This stepped-up resistance is caused in part by the cold and its vasoconstricting effects, as well as by autonomic vascular control (Lyman, 1965). Indeed blood flow to areas of the splanchnic bed, the bladder and the kidneys, becomes limited severely (Hudson, 1973).

During hibernation, changes in the serological and hematological composition appear to parallel the progressive decline of blood flow in ground squirrels as well as other

hibernators. Serological fluctuations during hibernation show large increases of serum lipid in the 13-lined ground squirrel (Galster & Morrison, 1966). Moreover, these fluctuations in the 13-lined ground squirrel may be analagous to those in hibernating hedgehogs, whose serum levels of cholesterol rose over 50% along with a decline of free fatty acids by the same percentage (Kontinnen et al., 1964). In addition, there is in hibernating ground squirrel, the gradual appearance of several different particles in the serum (Spurrier & Dawe, 1973). Judging from their ability to adhere to the erythrocyte surface, some of these particles appear to be lipoidal in nature. Their presence, as well as increased serum lipids, tend to increase blood viscosity, which in turn reduces blood flow (Spurrier & Dawe, 1973).

With the onset of hibernation, erythrocytes of the hibernating ground squirrel undergo adaptive compensation in response to reduced circulation (Nansel & Knoche, 1972; Spurrier and Dawe, 1973). In hibernating, 13-lined ground squirrels, hematocrits become lowered as mean corpuscular volume increases (Spurrier & Dawe, 1973). The population of erythrocytes in these animals exhibits a 40-50% "folding over" which may serve to increase blood flow through constricted vessels (Spurrier & Dawe, 1973). Further the rate of red cell aging in ground squirrels becomes reduced like that of the marmot, presumably to offset the effect of lowered

erythropoiesis during hibernation (Brock, 1960; Szilagyi et al., 1974). This is reflected by the diminished levels of 2, 3-diphosphoglycerate in hibernating ground squirrels, which results in decreased oxygen affinity to hemoglobin (Larkin, 1973). Similar changes were noted in other hibernating animals, e.g. marmots and hamsters (Temple et al., 1975; Harkness et al., 1974). The combined effect of these alterations presumably enhances the functional and metabolic status of erythrocytes within hibernators (Larkin et al., 1972).

III. Alterations of Lipid Composition

Several hypotheses have been advanced to explain the morphological changes which occur subsequent to biochemical modification of erythrocytes during hibernation. Of these, the hypothesis linking lipid alternations with the altered morphology seems most plausible (Spurrier & Dawe, 1973).

Studies of temperature effects on lipid deposition and composition are not new (Fawcett & Lyman, 1954; Hudson, 1973). Investigations into the chemical nature of deposited white and brown adipose tissue have become the springboard for further studies of lipid patterns in both hibernating and cold-acclimated mammals (Folk, 1974). Studies of the unsaturation index of hibernating hamsters have yielded data indicating significant changes in unsaturation of depot fats with hibernation (Fawcett & Lyman, 1954). Similar findings were obtained in hamsters after prolonged acclimation to the cold (Hudson, 1973). Interestingly, these patterns are analagous

to lipid changes seen in tissues of cold-acclimated poikilotherms, which showed large increases of polyunsaturated fatty acids (Caldwell et al., 1970; Kemp et al., 1970).

With the advances made in gas-liquid chromatography, which have made lipid identification and quantitation more exact, later probes into lipid composition have substantiated this theory. Cold-acclimation of hamsters produced increases in larger polyunsaturated fatty acids (Chaffee et al., 1968). However, studies on hibernation-induced alterations of cardiac and hepatic membrane lipid of mitochondria did show a different pattern (Platner et al., 1976; Swan et al., 1977). These investigations revealed a trend toward shorter fatty acid chain lengths as well as larger concentrations of monounsaturated fatty acids (Platner et al., 1976).

Refined methods of thin-layer chromatography have shown several dissimilarities between hibernating mammals and poikilotherms. Studies of tissues of cold-acclimated poikilotherms have demonstrated increased percentages of DPG and PE (Caldwell et al., 1970). However, hibernation-induced alterations of cardiac tissue membranes have been found to result in PE, DPG and PC, along with increased concentrations of PA, LPE and LPC (Aloia et al., 1974).

The significance of these changes is that they maintain membrane fluidity (Aloia et al., 1974; Swan et al., 1977). Indeed investigations of membrane function during hibernation,

as reflected in continued enzyme activity, show that fluidity is crucial (Raison et al., 1971; Cannon et al., 1975).

Membrane stability is crucial for the ground squirrel erythrocyte, particularly in maintaining its role of oxygen transport both during hibernation and especially in arousal. Lipid alterations involving greater incorporation of oleic acid (18:1) and linolenic acid (18:3) have been shown to stabilize the erythrocyte membrane in warm-acclimated hamsters (Livne et al., 1972). Furthermore, transport function of such erythrocyte membranes did remain viable, though reduced, thus keeping the ionic milieu of the cells near the active state (Willis et al., 1972). This result corresponds with circadian rhythm studies of ion transport function in membranes, which determined that membrane lipid was the modulating factor for ion transport (Njus et al., 1974).

This investigation will attempt to determine which alterations occur in erythrocyte lipids of cold-acclimated as well as hibernating, ground squirrels. An attempt will be made to correlate these changes with membrane function.

MATERIALS AND METHODS

During the early fall of 1975, thirteen-lined ground squirrels, Spermophilus tridecemlineatus, were trapped in Northern Illinois and subsequently maintained in the Hibernation Laboratory of Drs. A. Dawe, S. Jones and Miss W. Spurrier at the Loyola University Medical Center, Hines, Illinois. In mid-November, these animals entered hibernation, which lasted until mid-March.

Groups of animals were maintained at two different temperature conditions. A warm control group was kept at $22^{\circ} + 3^{\circ}$ C. under an 8 hour photoperiod. A cold-acclimated group was maintained at $5^{\circ} + 3^{\circ}$ C. in total darkness. Those animals which were hibernating were designated as such, while active animals were designated as cold room controls. Bedding remained unchanged to avoid premature arousals of hibernating squirrels. Food, consisting of Purina Rat and Purina Lab Chows supplemented with vegetables and sunflower seeds, and water were supplied ad libitum at staggered time intervals to all groups of animals.

During January and February, 1976, animals from each group were sacrificed by decapitation. Blood samples were collected immediately into 3.0 ml of cold 10 mM EDTA - 10,000 units Heparin in 0.9% saline solution. Blood samples were

centrifuged for 10 minutes at 1500 x g in an I.E.C. refrigerated centrifuge. Plasma and buffy coats were subsequently removed by aspiration (Dodge & Phillips, 1967). Pelleted red cells were next washed in 0.9% cold saline and centrifuged at 1500 x g for 10 minutes. This procedure was repeated twice. After the last wash, the pellet of red cells was resuspended in 10 mM EDTA - 10,000 units Heparin in 0.9% saline on a 1:1 basis (v/v), flushed with N₂ and sealed in a 1.0 ml freezing capsule (Cooke Pro-Vial). Samples were quickly frozen in liquid Nitrogen and stored at -50° C. in a Revco freezer until assayed. Such samples have been reported to be stable from lipid auto-oxidation for up to 2 years (Nelson, 1972; Van Deenan & De Gier, 1974).

To retard auto-oxidation of the extracted lipids, 50 mg of BHT per liter was added to all solvents (Dodge and Phillips, 1967). A final solvent to sample ratio of 50:1 was used in each stage of the lipid extraction to insure complete extraction of the red cell lipids (Nelson, 1972). A volume of 6.0 ml of the red blood cell suspension was added slowly to 100 ml of cold methanol and allowed to mix for 10 minutes. This was followed by the addition of 200 ml of cold chloroform and mixed for 15 minutes. Afterwards this mixture was filtered through a glass funnel plugged with glass wool. Both the funnel and the glass wool had been pre-washed with chloroform. The filtrate was placed into a 500 ml extraction flask while

the extraction procedure was repeated with the residue (Nelson, 1972).

Combined filtrates were purified according to a procedure of Williams and Merilees (1970). In this procedure, Sephadex G-25 coarse was added directly to the filtrate on a 1:1 basis (w/w) of Sephadex G-25 to the amount of water in the extract. The filtrate was then dried on a rotary evaporator in vacuo at 36° C. A volume of 20 ml of chloroform per gram of Sephadex was added to resuspend the filtrate, which was then redried in the rotary evaporator. A second addition of chloroform resuspended the filtrate, which was then poured into a 1.0 cm diameter filter tube. Washing the Sephadex with 100-200 ml chloroform removed most of the lipid (Williams & Merilees, 1970). The purified lipid was evaporated to near dryness in the rotary evaporator and subsequently re-dissolved in 10 ml Benzene-Methanol (4:1 v/v) (Broekhuysse, 1969). These extracts were stable up to 1 year when stored at -25° C. (Kates, 1972).

Total lipid determinations were carried out by adding 50 ml of the extract to tared vials, evaporating the solvent overnight over KOH pellets, and reweighing the vial on a Mettler analytical balance (Kates, 1972).

Analysis of the fatty acid composition required 1.0 ml of the stored extract. The solvent was removed by heating the sample to 25° C. under nitrogen (Wessels et al., 1973),

then mixing with 0.5 ml of hexane. Fatty acid methyl esters were prepared using 2.0 ml BF_3 - Methanol. The mixture was then heated at 100°C . for 15 Minutes (Morrison & Smith, 1964; Supelco Bulletin #721B, 1975). Methyl esters were analyzed on a Perkin-Elmer 810 Gas Chromatograph (F.I.D.) using 6' long, 1/8" (O.D.) stainless steel columns packed with 10% SP-2330 on 100/120 chromosorb W/AW (Supelco Bulletin #746, 1975). Assays were performed at 175°C . with a N_2 carrier gas flow of 40 cc/min. Qualitative determinations were performed using standards (Chemical Research Services, Addison, Illinois). Quantitative determinations were a result of comparing peak areas (Supelco Bulletin #770, 1977), and were expressed as g/100g \pm S.E.M. (Wessels et al., 1973).

Phospholipids were assayed on thin-layer glass plates covered with Supelcosil-42A (Supelco Redi-Coats, Supelco Inc., Bellefonte, Pennsylvania). Plates were initially activated in a 110°C . drying oven for 20 minutes, then cooled for 3 minutes. Approximately 500 to 1000 μg of sample, assayed in duplicate, were applied to the plates in the lower right hand corner, about 25 mm from each edge of the plate. Two dimensional phospholipid separation took place using chloroform/methanol/ NH_4OH (65/25/5, v/v/v) in the first dimension, and chloroform/acetone/methanol/acetic acid/water (3/4/1/1/0.5, v/v/v/v/v) in the second dimension (Supelco Bulletin 713, 1971). A time of 6 minutes was allowed for the plate to dry between developments.

Spots were developed using a specific phosphate stain (Kates, 1972) as well as a char reagent (0.6% potassium dichromate in 50% sulfuric acid). After the spots were visualized and photographed, they were aspirated into tubes and digested with a 2:1 mixture of 85% sulfuric acid: 70% perchloric acid until they became clear and colorless (Nelson, 1972).

Quantitative determinations were made by analyzing the phosphorus content of the digested phospholipids. To the digested lipids were added 8.0 ml of H₂O, 1.0 ml of 10% fresh ascorbate, and 1.0 ml of 2.5% ammonium molybdate. This mixture was heated in boiling water for 5 minutes, cooled, centrifuged, and read at 820 n.m. against a reagent blank corresponding to the size of the sample (Rouser et al., 1966; Kates, 1972). Results were expressed as percentage of the total \pm S.E.M.

All data were analyzed statistically using the student's unpaired "t" test (Zar, 1974).

RESULTS

The fatty acid composition of erythrocytes exhibited marked changes during cold-acclimation and hibernation. Table 1 shows that the percent concentration of oleic acid (18:1) and linolenic acid (18:3) significantly increased in red cells of cold room controls. Conversely, the concentration of palmitoleic acid (16:1) significantly decreased in RBC's of cold room controls, while that of docosapentaenoic acid (22:5) and docosahexaenoic acid (22:6) also decreased, but not significantly.

These trends were enhanced during hibernation with additional changes occurring (Table 2). Levels of palmitic acid (16:0) showed a statistically significant two-fold increase whereas those of 20:4 were significantly decreased. Additionally, fatty acids with chain lengths longer than 20:0 were no longer detectable in hibernators as compared to warm room controls.

Conversely, the percent concentration of stearic acid (18:0) and linoleic acid (18:2) appeared not altered by hibernation (Table 2), in a fashion similar to that caused by cold exposure (Table 1).

In regard to RBC phospholipid percent concentration, two significant changes were observed (Table 3).

Table 1. EFFECTS OF COLD-ACCLIMATION ON FATTY ACID
COMPOSITION OF ERYTHROCYTES.

FATTY ACID COMPOSITION ^a

| | Warm Room Control ^b | Cold Room Control | P _c |
|------|--------------------------------|-------------------|----------------|
| | n _d = 4 | n = 5 | |
| 16:0 | 13.14 ± 1.55 | 9.40 ± 5.58 | N.S. |
| 16:1 | 41.64 ± 4.95 | 18.71 ± 4.21 | <0.01 |
| 18:0 | 17.25 ± 1.43 | 26.68 ± 4.10 | <0.02 |
| 18:1 | 10.04 ± 1.02 | 21.05 ± 3.64 | <0.002 |
| 18:2 | 9.13 ± 1.14 | 10.65 ± 1.76 | N.S. |
| 18:3 | 2.70 ± 0.48 | 7.55 ± 1.95 | <0.01 |
| 20:4 | 2.39 ± 0.46 | 2.98 ± 0.88 | N.S. |
| 22:4 | 0.69 ± 0.62 | 2.31 ± 1.06 | N.S. |
| 22:5 | 0.67 ± 0.37 | 0.03 ± 0.02 | N.S. |
| 22:6 | 2.72 ± 0.84 | 0.32 ± 0.19 | <0.05 |

a: fatty acid composition in g/100g

b: ± S.E.M.

c: P = probability according to unpaired T-test;

N.S. = Not Significant

d: n = number of animals

Table 2. EFFECTS OF HIBERNATION ON FATTY ACID COMPOSITION OF ERYTHROCYTES.

FATTY ACID COMPOSITION ^a

| | Warm Room Control ^b | Hibernators | P _c |
|------|--------------------------------|--------------|----------------|
| | n _d = 4 | n = 10 | |
| 16:0 | 13.14 ± 1.55 | 29.91 ± 2.83 | <0.01 |
| 16:1 | 41.64 ± 4.95 | 20.53 ± 0.27 | <0.001 |
| 18:0 | 17.25 ± 1.43 | 16.80 ± 0.80 | N.S. |
| 18:1 | 10.04 ± 1.02 | 15.52 ± 0.72 | <0.001 |
| 18:2 | 9.13 ± 1.14 | 10.55 ± 0.75 | N.S. |
| 18:3 | 2.70 ± 0.48 | 9.38 ± 0.95 | <0.001 |
| 20:4 | 2.39 ± 0.46 | 0.30 ± 0.56 | <0.001 |
| 22:4 | 0.69 ± 0.62 | 0.0 ± 0 | N.S. |
| 22:5 | 0.67 ± 0.37 | 0.0 ± 0 | <0.05 |
| 22:6 | 2.72 ± 0.84 | 0.0 ± 0 | <0.002 |

a: fatty acid composition in g/100g

b: ± S.E.M.

c: P = probability according to unpaired T-text;

N.S. = Not Significant

d: n = number of animals

Diphosphatidylglycerol (DPG) concentration was significantly decreased while that of sphingomyelin (Sph) was significantly increased by cold exposure. The remaining phospholipid levels were not altered by cold-acclimation.

During hibernation, however, phospholipid levels in erythrocytes were dramatically altered (Table 4). Percentages of major diacyl phospholipids significantly decreased while monoacyl lysoglycerophospholipids appeared significantly increased. Levels of phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) were reduced 33% and 50%, respectively, whereas those of lysophosphatidyl choline (LPC) and lysophosphatidyl ethanolamine (LPE) showed significant increases of six to ten-fold, respectively. DPG levels were significantly depressed by hibernation similarly to that caused by cold-acclimation. As opposed to the lack of effect caused by cold-acclimation on phosphatidic acid (PA), a precursor of many phospholipids, hibernation produced a significant six-fold increase in this agent.

Table 3. EFFECTS OF COLD-ACCLIMATION ON PHOSPHOLIPID
COMPOSITION OF ERYTHROCYTES.

ERYTHROCYTE PHOSOPHOLIPIDS ^a

| | Warm Room Control | Cold Room Control | P _c |
|-----|--------------------|-------------------|----------------|
| | n = 4 _b | n = 5 | |
| PE | 19.15 ± 1.58 | 21.78 ± 1.14 | N.S. |
| PC | 30.91 ± 1.58 | 24.74 ± 3.14 | N.S. |
| Sph | 21.90 ± 1.498 | 31.56 ± 1.388 | <0.01 |
| PI | 4.32 ± 0.538 | 4.64 ± 1.350 | N.S. |
| PS | 14.07 ± 1.603 | 10.72 ± 1.210 | N.S. |
| LPC | 3.17 ± 0.188 | 3.3 ± 0.629 | N.S. |
| LPE | 0.35 ± 0.184 | 0.0 ± 0.0 | N.S. |
| DPG | 5.575 ± 1.131 | 0.12 ± 0.119 | <0.001 |
| PA | 0.275 ± 0.274 | 0.0 ± 0.0 | N.S. |

a: % ± S.E.M. recovery range: 95.0 - 99.1%

b: n = number of animals

c: P = Probability; unpaired T-test N.S. = Not Significant

Table 4. EFFECTS OF HIBERNATION ON ERYTHROCYTE PHOSPHOLIPID COMPOSITION.

ERYTHROCYTE PHOSPHOLIPIDS ^a

| | Warm Room Controls | Hibernators | P _c |
|-----|--------------------|---------------|----------------|
| | n = 4 _b | n = 9 | |
| PE | 19.15 ± 1.58 | 12.26 ± 1.36 | <0.005 |
| PC | 30.91 ± 1.58 | 13.93 ± 1.236 | <0.001 |
| Sph | 21.90 ± 1.498 | 21.75 ± 1.379 | N.S. |
| PI | 4.32 ± 0.538 | 3.71 ± 0.249 | N.S. |
| PS | 14.07 ± 1.603 | 11.42 ± 0.659 | N.S. |
| LPC | 3.17 ± 0.188 | 17.66 ± 1.312 | <0.001 |
| LPE | 0.35 ± 0.184 | 13.98 ± 1.531 | <0.001 |
| DPG | 5.575 ± 1.131 | 2.26 ± 0.539 | <0.01 |
| PA | 0.275 ± 0.274 | 4.12 ± 0.948 | <0.02 |

a: % ± S.E.M. recovery range: 95.0 - 106.5%

b: n - number of animals

c: P = Probability; N.S. = Not Significant;

unpaired T-test

DISCUSSION

During hibernation, a number of morphological and biochemical changes occur within cellular membranes. Such alterations appear to be compensatory adaptations which permit survival not only of the altered cells, but also of the species itself (Willis, 1972; Spurrier & Dawe, 1973).

The results of this study indicate that red cell fatty acids of ground squirrels are characterized by shorter chain lengths and greater unsaturation during cold-acclimation (Table 1). These changes may occur at the expense of membrane fatty acids whose lengths exceed 20 carbons. Hibernation produces even more drastic changes on the composition of red cell fatty acids (Table 2). Similar trends have been reported for cardiac, cortical and hepatic membranes of hibernating ground squirrels (Swan et al., 1977), as well as for erythrocyte ghosts of cold acclimated hamsters (Chaffee et al., 1968). Serum and brown fat of ground squirrels display opposite patterns of fatty acid composition during hibernation (Platner et al., 1972). Since fatty acids are not synthesized in the mature red cell, the significance of these serum changes may lie in the turnover rates of fatty acids between red cells and serum (Nelson, 1972; Van Deenan and De Gier, 1974).

Erythrocytes of hibernators are confronted with special adaptative challenges as a result of lowered cardiac rates and massive vasoconstriction. To circumvent these problems, red cell pliability increases, allowing for an easier circulation through constricted blood vessels (Spurrier & Dawe, 1973).

The present data suggest that significantly increased levels of oleic acid (18:1) and linolenic acid (18:3) may contribute to the improved red cell flexibility seen at low temperatures (Table 2). Similar changes have been shown to stabilize erythrocyte membranes of warm-acclimated hamsters (Livne, et al., 1972). These modifications could be responsible in part, for the greater resistance of hibernating ground squirrel erythrocytes to hemolysis (Spurrier & Dawe, 1973). Such changes may afford protection against the increasing extracellular accumulation of cations as well as the infiltration of urea into the cell (Willis, 1972; Hudson, 1973; Galster & Morrison, 1975).

In this study the phospholipid composition of red cells showed little change during cold-acclimation (Table 3). Comparable results have been reported for cold-acclimated hamsters (Cannon et al., 1974, 1975).

However, the levels of PE, PC and DPG decreased significantly during hibernation, while those of LPC, LPE and PA increased significantly (Table 4). These changes are similar

to those reported for cardiac phospholipids of hibernating, gold mantled ground squirrels (Aloia et al., 1974).

A model which may be helpful in explaining the changes in red cells of hibernating ground squirrels (Table 4) is that proposed by Aloia et al. (1974) and Goldman (1975). According to this model, increased levels of lysoglycerophosphatides may represent reactants in an incomplete transacylation reaction within the membrane (Nelson, 1972; Aloia et al., 1974; Van Deenan & De Gier, 1974). Complex mechanisms such as the lecithin-cholesterol acyl transfer reactions, remove cholesterol from the red cell membranes. The cholesterol then becomes esterified to a form to which the membrane is impermeable (Chapman, 1975). High concentrations of urea accelerate this esterification by dissolving hydrophobic bonds within the matrices of the membranes (Van Deenan & De Gier, 1974). Since serum cholesterol and tissue urea levels are known to increase in hibernators (Kontinnen et al., 1964; Galster & Morrison, 1975), similar changes in hibernating ground squirrels may be consistent with the proposed model. Furthermore, the increased unsaturation of phospholipid fatty acids during hibernation (Table 2) might alter the solubilizing properties which phospholipids exhibit toward non-polar substrates, such as cholesterol (Van Deenan & De Gier, 1974; Sanderman, 1978). These changes during hibernation may then exclude cholesterol, which imposes steric hindrances to the acyl chains by condensing the phospholipid core, which would

dampen fluid mobility (Papahadjapolous, 1973).

The combined effects of these various alterations during hibernation are to increase membrane fluidity. (Aloia et al., 1974; Goldman, 1975; Cannon et al., 1975; Swan, 1977). Together they prevent a lipid phase transition into a gel-crystalline state, which could cause conformational changes of the membrane-bound enzyme proteins. The absence of such changes allows circumvention of the natural homeo-thermic barrier to lower body temperatures (i.e. 23-25° C.), and permits a continual energy of activation of membrane-bound enzymes (Raison et al., 1971; Swan et al., 1977). Since only a 5% increase of linoleic acid (18:2) and linolenic acid (18:3) can prevent phase changes in hepatic mitochondria of hibernating ground squirrels (Raison & Lyons, 1971), it may be possible that similar changes also occur in the red cell membranes.

Examples of lipid dependent enzymes in the red cell membrane include Mg^{2+} - dependent ATPase, Na^+ , K^+ - ATPase (Van Deenan & De Gier, 1974), and acetylcholine esterase (Sanderman, 1978). Of these, Na^+ , K^+ - ATPase is suggested to have continual activity at lower temperatures due to the fluidity of the lipid-altered membrane (Aloia et al., 1974). Moreover, it is the lysoglycerophosphatides which appear to be responsible for this enzyme activity (Aloia et al., 1974; Keith et al., 1975). Hence, the increased unsaturation of

the fatty acids (Table 2) coupled with elevated levels of lysophosphatides (Table 4) can contribute to the increasing disorder of the membrane; this in turn maintains fluidity during hibernation (Raison et al., 1971; Keith et al., 1975).

Since the activity of Na^+ , K^+ -ATPase is an acceptable index of transport function (Willis, 1972), continual enzyme activity means that transport may be slowed but not abolished (Goldman, 1975). Indeed, red cells of hibernating ground squirrels lost K^+ at a much slower rate than other tissues (Willis, 1972), indicating a reduced Na^+ efflux pump.

Moreover, continued Na^+ , K^+ -ATPase activity could have an effect on the membrane lipids. It would allow energy to be utilized in the transacylation of LPC into PC, thereby maintaining a steady state that would regulate solubilization within the membrane (Papahadjapoulos, 1973; Sanderman, 1978). Then, upon arousal, stepped up Na^+ , K^+ -ATPase action would easily transform the lysophosphatides back to active conditions (Keith et al., 1975).

The accumulation of PA in the hibernating erythrocytes suggests a marked reduction in the synthesis of new phospholipids (Table 4). This may be due either to cold lability of the membrane-bound enzyme, phosphatidic acid phosphorylase, required for conversion of PA into other phospholipids, or insufficient energy reserves required for interconversion (Larkin, 1973). Similar explanations have

been advanced to explain the changes observed in cardiac tissue of hibernating ground squirrels (Aloia et al., 1974). Other enzyme systems in the erythrocyte have yet to be investigated.

In summary, the data presented in this thesis are consistent with previous findings which have suggested that hibernation-induced alterations of membrane lipids enhance the survival value of the red cells by rendering their membranes more flexible and stable. Such alterations may enhance the expulsion of cholesterol from the membrane, as well as to prevent its return. Further, they increase membrane disorder, thereby preventing a gel-crystalline state that would severely impair membrane-bound enzymic activity. Additional studies are needed to further extend these observations.

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

April 23, 1979
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